BEHAVIOURAL AND ECOLOGICAL CONSEQUENCES OF URBAN LIFE IN BIRDS

EDITED BY: Caroline Isaksson, Amanda D. Rodewald and Diego Gil
PUBLISHED IN: Frontiers in Ecology and Evolution

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-497-6
DOI 10.3389/978-2-88945-497-6

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BEHAVIOURAL AND ECOLOGICAL CONSEQUENCES OF URBAN LIFE IN BIRDS

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Urban jackdaw. Image: Amparo Herrera-Dueñas.

Urbanization is next to global warming the largest threat to biodiversity. Indeed, it is becoming increasingly evident that many bird species get locally extinct as a result of urban development. However, many bird species benefit from urbanization, especially through the abundance of human-provided resources, and increase in abundance and densities. These birds are intriguing to study in relation to its resilience and adaption to urban environments, but also in relation to its susceptibility and the potential costs of urban life. This Research Topic consisting of 30 articles (one review, two meta-analyzes and 27 original data papers) provides insights into species and population responses to urbanization through diverse lenses, including biogeography, community ecology, behaviour, life history evolution, and physiology.

Citation: Isaksson, C., Rodewald, A. D., Gil, D., eds. (2018). Behavioural and Ecological Consequences of Urban Life in Birds. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88945-497-6

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frontiers in Ecology and Evolution

EDITORIAL
published: 26 April 2018
doi: 10.3389/fevo.2018.00050

Editorial: Behavioural and Ecological Consequences of Urban Life in Birds

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Keywords: biodiversity, environmental stress, pollution, species interactions, urbanization

Editorial on the research topic

Behavioural and Ecological Consequences of Urban Life in Birds

INTRODUCTION

The footprint of urban areas continues to expand across the globe, with urban land cover expected to triple between 2000 and 2030 (United Nations, 2014). This dramatic transformation from natural or less-intensively used land to impervious surfaces and buildings has altered species composition and distributions, homogenized communities, and even prompted local extinctions (Marzluff and Ewing, 2001; McKinney, 2002; Shochat et al., 2010). Because the most intense urban development is projected to occur within biodiversity hotspots (Elmqvist et al., 2013), urbanization stands to be one of the greatest threats to species persistence in the future. Yet nearly 20% of the roughly 10,000 described bird species can still be found in cities (Aronson et al., 2014). Thus, understanding the enabling factors that allow species to persist within urbanized landscapes is as important as identifying the drivers of species loss.

The present issue on "Behavioural and Ecological Consequences of Urban Life in Birds" showcases 30 articles that provide insights into species responses to urbanization through diverse lenses, including biogeography, community ecology, life history evolution, and physiology. Behavioral responses are a central theme across articles, in part because behavior often defines initial responses to urbanization and provide clues about how certain species cope with urban habitats, human presence, and novel resources (e.g., food and nest boxes) and conditions (e.g., pollution, noise, artificial light). As Topic Editors we aimed both to cover a wide range of topics and approaches and to highlight research around the world, especially those studies conducted outside of Europe and North America. We believe that it is important to cover research in the Southern Hemisphere, as this comparatively less studied area contains the highest levels of avian diversity in the world (Thomas et al., 2008). In total, 19 countries from 6 continents are represented in the articles within this issue, which collectively report on more than 700 bird species. The top-three species for urban/rural comparisons were, not surprisingly, the great tit (*Parus major*), the house sparrow (*Passer domesticus*) and the blackbird (*Turdus merula*). This pattern is consistent with the suggestion in the contributing paper by Ibáñez-Álamo et al., who show that urbanization not only affects the birds but also how we, researchers, study them. The more urban a species is the more frequently it is studied.

Here we review and synthesize the key findings of this issue. The articles provide a nice overview of the general advances in the field of urban ecology, especially in relation to birds. In addition, these studies tackle many still unanswered questions at both macro- and micro-ecological levels, along with phenotypic and evolutionary responses to environmental change that urgently need to be addressed to maintain avian diversity on an urbanizing planet.

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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *21 March 2018*
Accepted: *10 April 2018*
Published: *26 April 2018*

Citation:
Isaksson C, Rodewald AD and Gil D (2018) Editorial: Behavioural and Ecological Consequences of Urban Life in Birds. Front. Ecol. Evol. 6:50. doi: 10.3389/fevo.2018.00050

Fear and Boldness

Millions of people around the world enjoy birds through bird-feeding or bird-watching, but the reality is that the experience is not always mutually pleasant from the perspective of birds. Whereas, humans often enjoy the interaction and entice birds to come closer, birds must evaluate the risk at every encounter.

A common expectation is that urban birds should be less fearful of humans than rural counterparts. Several contributed papers address the potential fear experienced by birds by using flight initiation distance (FID) as a proxy for "fear" toward humans i.e., the shorter FID, the closer humans can approach and the more fearless is the bird. Indeed, two meta-analyses of 32 (Samia et al.) and 42 European species (Symonds et al.), respectively, report that the more urbanized a species is (i.e., longer time since colonization of cities), the shorter the FID and, presumably, the less fear is experienced by the birds. A field-intensive study of burrowing owls (*Athene cunicularia)* by Carrete and Tella also found that breeding pairs were more fearless toward humans in urban compared to rural habitats.

But what drives those patterns? Symonds et al. explicitly tested the extent to which encephalization (brain complexity and size) affected tolerance level of urban birds, but found no significant association between brain size and FID in this set of 42 species. Interestingly, the authors of the two meta-analyses suggest contrasting conclusions to the shorter FIDs of urban birds—*released selection* via reduced predation (Samia et al.) or *enhanced selection* of birds with low responsiveness toward humans (Symonds et al.). Sprau and Dingemanse applied a statistical approach to distinguish plastic responses from patterns of non-random distributions of behavioral variation in FID and aggression in the great tit. The striking result was that the two axes of behavior were non-randomly distributed, such that fearless (bold) birds occurred more frequently in areas with more cars but fewer humans, while fearful (shy) individuals were predominantly found in areas with fewer cars and more humans (Sprau and Dingemanse). Potential explanations for this pattern include non-random settlement, habitat- and type-specific survival, or irreversible plasticity in response to long-term exposure to urban environmental stress. The unexpected finding of shorter FID with fewer humans might be explained by vehicle noise, as Petrelli et al. found that noisy urban environments facilitated closer human approaches, and thus shorter FIDs, than more quiet habitats.

Other contributed papers explored behavior (often these assays are linked to so-called personalities or behavioral syndromes) using other approaches. Two studies examined coping responses of urban and rural great tits when handled, a widely used technique to score aggression. Senar et al. found that urban tits used a more pro-active coping strategy via more distress calls (fear screams) and higher pecking rates (aggression), whereas Charmantier et al. detected no such differences in great tits from Montepellier. Instead, the urban great tits from Montpellier were more explorative to a novel environment (Charmantier et al.), which is consistent with the exploratory behavior that Carrete and Tella report for urban burrowing owls. Great tit aggression was also assayed in the study by Sprau and Dingemanse using staged trials with a caged mount and playback of song. As for FID (see above)—boldness increased with traffic-load.

Behavioral and Fitness Consequences of Human-Provided Resources

The novelty, availability, and predictability of resources, such as bird-feeders and nest boxes, are among the most striking ways that urban landscapes differ from non-urban areas. Yet the extent to which urban-dwelling species respond to and rely upon human-provided resources varies widely. The so-called "urban exploiters" depend on the anthropogenic resources and are often abundant within cities, whereas the "urban adapters" are more opportunistic (McKinney, 2002).

While the extent to which anthropogenic resources positively or negatively affect birds varies among species, systems, and geographies, several papers in this issue provide new insights on this respect. Anthropogenic resources, such as birdseeds and invasive fruits, may create more predictable and homogenous environments that reduce selective pressures compared to natural environments. Rodewald and Arcese corroborate this hypothesis by demonstrating reduced variability in reproductive contributions within and among-female Northern cardinals (*Cardinalis cardinalis*) in urban than rural environments, despite comparable variation in body condition. In this way, urban females were relatively homogeneous in terms of performance, whereas rural females spanned a wider range of high to low performers. While bird-feeding is associated with many positive outcomes for birds, Reynolds et al. remind that community responses may differ between continents and hemispheres; for example, gregarious non-native species tend to dominate at feeding tables in the Southern hemisphere. Indeed, Galbraith et al. report that 10 of the 11 species recorded using urban bird feeders in New Zealand were non-native, including the two dominating species of the house sparrow *P. domesticus* and the spotted dove *Streptopelia chinensis*. This improved benefit for invading species may explain the patterns of abundance and spreading that characterize these species.

Other articles highlight the tradeoffs between quantity and quality of urban-associated resources, particularly when urban resources are less diverse and of lower nutritional value. Isaksson et al. revealed differences in the nutritional fatty acid physiology of four common passerine species between the urban and rural habitats that were not attributed to specific foods. Rather, the fatty acid profile of urban tits (Paridae) and sparrows (Passeridae) suggest that the urban diet of these two families could affect the birds through two different pathways, inflammation and oxidative stress, respectively.

Of course, food is not the only urban resource, and many birds are attracted to nest boxes, especially when natural holes are limited. The presence of nest boxes can allow a species to colonize areas that otherwise would have been inhospitable and may boost reproductive success of certain species. At the same time, nest boxes may increase risk of predation if they are more readily located than natural cavities. Consistent with this idea, a meta-analysis conducted by Vincze et al. showed that artificial nests (both hole and cup nests) in cities were more likely to be depredated than natural nests, but surprisingly, *vice versa* in less urbanized areas. Although differences might be attributed to shifts in predator composition, abundance, or behavior between rural and urban landscapes, no ecological drivers were identified.

Apart from predation, another ecological factor that can influence breeding success in an artificial nest box is weather. Despite the careful attention given to the design and construction of nest boxes, it was surprising to learn from Duckworth et al. that nest boxes are typically less insulated than natural nest cavities and, consequently, are associated with lower survival of nestlings in inclement weather.

Physiological and Behavioral Effects to Novel Abiotic Stressors

Urbanization is commonly associated with pollution, whether due to emissions in air, artificial light at night (ALAN) or noise (e.g., Salmón et al., 2018). Indeed, Bailly et al. demonstrate that although levels of nitrogen gas (NO_2) were consistently higher in urban than in rural areas, most non-essential metals were undetectable in the blood of great tits (*P. major*) living in urban and rural environments.

Passive exposure (e.g., oral intake and inhalation from ambient environment) is not the only form of pollution that can affect birds. In fact, some urban birds actively seek out toxic substances, such as cigarette butts, to include in their nest. Previously, it has been shown that this reduces ectoparasite load of nestlings (Suárez-Rodríguez et al., 2012). Here the contributed paper by Suárez-Rodriguez et al. show genotoxic effects of cigarette butts on the incubating and caring parent of house finch (*Carpodacus mexicanus*) and house sparrow (*P. domesticus*) from Mexico City that led to an interesting trade-off between parasite repellence and DNA-damage. A number of papers in this issue investigate other markers of cellular stress and damage, specifically oxidative damages to protein and lipids, telomere length and gene expression of inflammatory genes between urban and rural passerines (Herrera-Duenas et al.; Isaksson et al.; Biard et al.; Capilla-Lasheras et al.). Results provided mixed evidence across species; sparrows (Passeridae) for instance, show overall more damage in relation to urbanization compared to tits (Paridae) (see also Salmón et al., 2018). For all these correlative studies the exact causal driver of the stress and damage experienced in urban birds could not be identified. In contrast, ALAN was experimentally manipulated by Welbers et al., who exposed breeding great tits to either white, green, red LED light or left dark as a control and investigated its effect on daily energy expenditure (DEE). The DEE of great tits exposed to white and green light during chick feeding was significantly lower than those exposed to the control treatment—a pattern that the authors regarded as an indirect consequence of the positive association between lights and insect abundance. This is an intriguing positive effect of an urban pollution source that may lead to balance out some of the more negative effects.

There is a growing literature of studies showing that noise pollution is an important factor affecting acoustic communication in birds, and that many bird species show modifications in song amplitude, frequency and temporal characteristics that increase the chances of song reaching receivers (Gil and Brumm, 2014). Two articles in this issue deal with acoustic communication in urban environments. The first of them, by Sewall and Davies, identifies differences between urban and rural populations in the brain expression of an early gene (FOS), a proxy for recent neural activity, indicating different neural responsiveness to stimuli that could underpin differences in behavior in the song sparrow (*Melospiza melodia*). Another study, by Sierro et al., shows that, in the particular noise environment of an airport, European blackbirds modify the composition of their song, but not their overall frequency characteristics, reducing the part of the song which has the lowest amplitude. In addition, birds near the airport advance the timing of their dawn chorus at the period of the season when overcraft activity overlaps most with their singing. In contrast, this advance disappears later in the season, when the natural timing of song is much earlier than the airport activity.

Reproduction and Life History

Understanding the ecological and evolutionary consequences of urbanization requires, in part, that we identify how urban-associated factors impact fitness across phenotypes, populations, and species. Several articles reported that cities reduced breeding success via a range of pathways including clutch size, fledgling success and chick quality (Biard et al.; Capilla-Lasheras et al.; Charmantier et al.; Corsini et al.), though others found equivalent breeding performance and condition among rural and urban populations (Rodewald and Arcese).

Although reduced productivity is often attributed to predation, this pattern may also reflect a life-history strategy characterized by urban birds adopting a slower pace of life compared to rural individuals (Sepp et al., 2018). In this special issue, Charmantier et al. provide counterevidence to a slow life in the city by showing that urban great tits have behavioral phenotypes linked to a fast-pace of life, although they produce smaller clutches. Thus, urban great tits in this study seemed to be constrained during reproduction rather than these differences being the result of a proper life-history strategy. One constraint might be related to lower overall access of natural high quality foods or a mismatch between timing of breeding and available food.

Phenological shifts can also influence reproduction in urban populations. Due to the higher temperatures in the cities (i.e., heat island effect), cities often show phenological advances in spring with earlier bud burst and insect emergence. If birds are not able to respond to this environmental change the consequences for breeding can be detrimental (Visser and Both, 2005). Here, however, a study on great tits showed that urban birds advance their breeding which is in line with the presumed earlier phenology of a city (Charmantier et al.). In contrast, the breeding performance of the black sparrowhawk (*Accipiter melanoleucus*), but not the timing of breeding *per se*, showed different seasonal patterns in urban and rural areas, declining across the season in cities but improving in rural habitats (Rose et al.). The black sparrowhawks have relatively recently colonized the urban habitats of Cape Town, and thus the seemingly selective advantages of earlier breeding in the city may have not arisen yet. In the contributed paper by Fudickar et al. the underlying mechanism of advancing the timing of breeding is investigated in male dark-eyed juncos (*Junco hyemalis*). By comparing sedentary urban birds with migratory individuals, they show that the earlier timing of breeding in the urban population is facilitated by earlier increase in upstream baseline activity of the hypothalamic-pituitary-gonadal (HPG) axis and an earlier release of gonodal suppression affecting testosterone production (Fudickar et al.).

Biodiversity and Conservation in Urban Habitats

Urbanization stands out as one of the most important threats to biodiversity on our planet. Several articles in the special issue examined this threat explicitly, and most contributors at least alluded to the conservation implications of their findings. Focusing on the Seattle Metropolitan area in the US, Shryock et al. showed that relationships between avian species richness and vegetative productivity, as measured by the Normalized Difference Vegetation Index (NDVI), are mediated by urban development. Specifically, they found that species richness declined with NDVI within areas undergoing active development, but less so in areas of established housing development and forested reserves. Urbanization also may affect the functional diversity of avian communities in ways that might have ecosystem-level consequences. Oliveira Hagen et al. compared avian functional diversity of 25 urban areas using 27 traits from more than 500 species. Interestingly, they found that avian functional diversity is higher in cities than in semi-natural habitats, a pattern attributed to higher functional habitat diversity in cities compared to single habitats within more natural landscapes. Both studies suggest that city planners can moderate the effect of urbanization on the avian community by maintaining or restoring diverse and heterogeneous native vegetation. Habitat management also may facilitate species movements and dispersal, which can be constrained in urban systems even for highly mobile organisms, as shown by Evans et al.

The altered habitat structure and the simplified avifauna composition of urban habitats change the life conditions of many species and the interspecific interactions among them, something that can strongly benefit some species whereas other species will suffer. A species that has benefited from these modifications is the North American brown-headed cowbird (*Molothrus ater*). This is a brood parasitic species that has increased in population densities with deforestation. Two contributed papers raise the concern of the increased abundance of this species for other urban-dwelling birds. In a first study by Stiles et al. it is shown that cowbirds together with humans and feral dogs have a direct negative effect on several native bird species. The second paper by Ladin et al. uses a modeling approach to test how the negative trend of cowbirds on the population growth of the forest umbrella species, the wood trush (*Hylocichla mustelina*) could be averted. Their models suggest that removing cowbirds along with reforestation could stop the decline of the wood thrush.

CONCLUSION

This special issue highlights a few overarching themes among studies of urban birds, and the large number of articles reflect the great interest and timeliness of the topic. Many of the contributed studies involve a comparison of two single populations - one rural and one urban. Though comparative studies are certainly useful in identifying possible mechanisms and patterns, unreplicated designs provide limited inference about mechanisms and the variability in outcomes, as well as the underlying drivers of the effects and variabilities. Identifying drivers is challenging given that cities differ from rural habitats in a many respects, just as cities differ from each other. Although experiments are typically difficult to conduct, we would like to stress the need to envisage manipulations whenever possible to do so.

Taken together, the articles in this special issue highlight the wide variety of responses—both positive and negative—to urbanization and the challenge that biologists face in trying to generalize. The differential resilience of species in the face of urbanization changes community composition and affects interspecific relationships, thus fuelling new feedback mechanisms that add complexity to the final picture. Thus, the field of urban avian ecology have many challenges ahead and intriguing new venues for future research.

AUTHOR CONTRIBUTIONS

All three authors have made substantial work with the topic issue and with the present editorial piece.

FUNDING

DG was funded while editing this special issue by a research grant from the Ministerio de Economía y Competitividad (CGL2014-55577R).

ACKNOWLEDGMENTS

We wish to thank all the contributing authors for their efforts to make this issue a success. We also wish to thank the team working for Frontiers in Ecology & Evolution for their patience and their support.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 26 June 2017
doi: 10.3389/fevo.2017.00066

Rural-Urban Differences in Escape Behavior of European Birds across a Latitudinal Gradient

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *17 November 2016*
Accepted: *02 June 2017*
Published: *26 June 2017*

Citation:
Samia DSM, Blumstein DT, Díaz M, Grim T, Ibáñez-Álamo JD, Jokimäki J, Tätte K, Markó G, Tryjanowski P and Møller AP (2017) Rural-Urban Differences in Escape Behavior of European Birds across a Latitudinal Gradient. Front. Ecol. Evol. 5:66. doi: 10.3389/fevo.2017.00066

Behavioral adjustment is a key factor that facilitates species' coexistence with humans in a rapidly urbanizing world. Because urban animals often experience reduced predation risk compared to their rural counterparts, and because escape behavior is energetically costly, we expect that urban environments will select for increased tolerance to humans. Many studies have supported this expectation by demonstrating that urban birds have reduced flight initiation distance (FID = predator-prey distance when escape by the prey begins) than rural birds. Here, we advanced this approach and, for the first time, assessed how 32 species of birds, found in 92 paired urban-rural populations, along a 3,900 km latitudinal gradient across Europe, changed their predation risk assessment and escape strategy as a function of living in urban areas. We found that urban birds took longer than rural birds to be alerted to human approaches, and urban birds tolerated closer human approach than rural birds. While both rural and urban populations took longer to become aware of an approaching human as latitude increased, this behavioral change with latitude is more intense in urban birds (for a given unit of latitude, urban birds increased their pre-detection distance more than rural birds). We also found that as mean alert distance was shorter, urban birds escaped more quickly from approaching humans, but there was no such a relationship in rural populations. Although, both rural and urban populations tended to escape more quickly as latitude increased, urban birds delayed their escape more at low latitudes when compared with rural birds. These results suggest that urban birds in Europe live under lower predation risk than their rural counterparts. Furthermore, the patterns found in our study indicate that birds prioritize the reduction of on-going monitoring costs when predation risk is low. We conclude that splitting escape variables into constituent components may provide additional and complementary information on the underlying causes of escape. This new approach is essential for understanding, predicting, and managing wildlife in a rapidly urbanizing world.

Keywords: alert distance, antipredator behavior, buffer distance, flight initiation distance, phi index, pre-detection distance, rural-urban difference, urbanization

INTRODUCTION

Urbanization is one of the main drivers of the current global biodiversity crisis (Foley et al., 2005; Grimm et al., 2008; McDonald, 2008). More than half of the world's humans live in cities and this proportion is expected to increase over the next decades (United Nations, 2014). In addition, the urban areas are increasing at an even greater rate than the urban population (United Nations, 2014). Given a rapidly urbanizing world, we need to better understand the traits that permit species to coexist with humans, which are often perceived as predators by animals (Frid and Dill, 2002). In this sense, behavioral plasticity is one of the key elements that permits species to coexist with humans (Sol et al., 2013). For instance, because escape from non-threatening humans is costly (Ydenberg and Dill, 1986; Cooper and Frederick, 2007; Samia et al., 2016), and because humans in cities rarely hunt or otherwise intentionally kill animals (Berger, 2007), urban prey are expected to respond to humans by reducing costly anti-predator behavior. In addition, humans in urban areas often displace predators (Møller, 2012), creating human shields where prey are relatively safe (Berger, 2007; Ibáñez-Álamo et al., 2012; Møller, 2012). If animals in urban areas experience reduced risk of predation by their native predators, this should select for reduced anti-predator responses.

In line with these expectations, a growing body of literature has shown that animals generally have relaxed anti-predator behavior in urban environments (e.g., Møller and Ibáñez-Álamo, 2012). In particular, individuals in urban populations generally tolerate more human disturbance by reducing their flight initiation distances (FID = predator-prey distance when escape of the prey begins; **Figure 1A**) compared to rural populations (Samia et al., 2015c). Despite this apparently ubiquitous pattern, the drivers of the rural-urban difference in human tolerance have just begun to be explored. Recent studies indicate an important role of a species' body size on urbanization with larger birds being more tolerant (Samia et al., 2015c) and having increased survivorship in cities (Brown and Graham, 2015). Brain size also plays a key role: successful urban invaders and exploiters are characterized by having larger brains (Sol et al., 2005, 2013; Maklakov et al., 2011) and several studies have shown a negative correlation between brain mass and FID (Møller and Erritzøe, 2014; Samia et al., 2015a; but see Carrete and Tella, 2011). Studies also suggest that big-brained species are more innovative and flexible in their ability to deal with the novel challenges imposed by urban environments, such as the chronic disturbance caused by human presence. Because predation risk is expected to decrease with group size (Hamilton, 1971; Pulliam, 1973; Alexander, 1974), birds in larger flocks might tolerate more human disturbance than birds alone or in smaller flocks (Dill and Ydenberg, 1987; Reimers et al., 2006), although current evidence does not show a substantial effect of flock size on tolerance to humans of urban birds (Møller, 2015; Samia et al., 2015c). Some studies also suggest that increased tolerance to humans is a function of disturbance frequency (Levey et al., 2009; Engelhardt and Weladji, 2011; McGiffin et al., 2013), which suggests that either the individual habituation process (i.e., phenotypic plasticity; Blumstein, 2016), differential sorting according to individual's personalities (Carrete and Tella, 2013), or strong selection against fearful individuals (Carrete et al., 2016) may be involved in generating tolerance.

Among many recognized correlates of human tolerance, latitude has recently been identified as a potentially important correlate of tolerance in rural and urban environments. Latitude is used as a proxy for predation risk since predator density tends to decrease with increasing latitude (Laurila et al., 2008; Schemske et al., 2009). In a study of 159 species of European birds, FID decreased as latitude increased, in parallel with a clinal reduction in raptor abundance (raptors are key avian predators; Díaz et al., 2013). Other interspecific studies of FID across latitudinal gradients—or among continents—have recently revealed novel patterns in escape behavior (Møller et al., 2014, 2016; Samia et al., 2015b). However, these large scale studies have not considered a main predictor of FID, the prey's alert distance (AD; **Figure 1A**), which is the predator-prey distance when an individual prey becomes aware of and begins to monitor the predator (Cooper and Blumstein, 2015). Along with body mass, AD explains most of variation in FID in taxa studied to date (Samia et al., 2013; Cooper et al., 2015). A strong relationship between FID and AD implies that they both reflect the general level of fear that individuals experience while both scanning for predators and fleeing following a predator's approach. Variation in the strength of this relationship will however reflect different escape tactics in response to varying levels of predation risk (Cooper and Blumstein, 2014).

While a robust body of evidence has shown that FID is generally smaller in urban than rural places (review in Samia et al., 2015c), latitudinal studies of rural-urban differences in FID that account for variation in AD should permit us, for the first time, to assess how birds modify their predation risk assessment and escape strategy (in addition to modifying their FID) when living in cities. These anti-predatory decisions can be inferred from two variables. First, the prey's pre-detection distance (**Figure 1A**), which is a measure of how long a prey takes to become aware of an approaching human [i.e., the

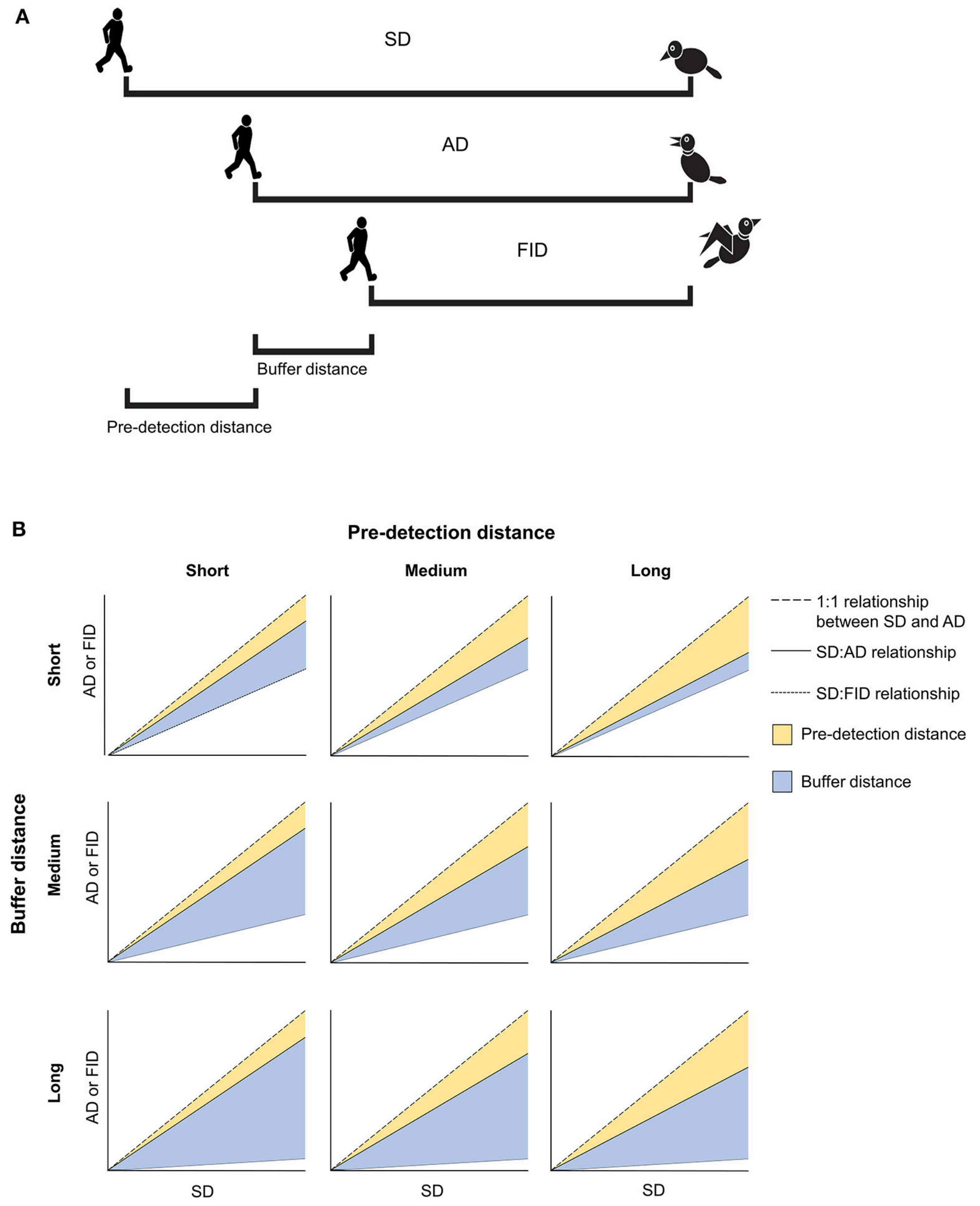

FIGURE 1 | (A) Illustration of the five anti-predator behaviors considered in the present study. Starting distance (SD) is measured as the distance at which an approaching human begins to approach a prey. Alert distance (AD) is measured as the distance at which prey responds by orienting toward the approaching human. Flight initiation distance (FID) is measured as the human-prey distance when the prey begins to flee. Pre-detection distance is a measure of how long a prey takes to become aware of an approaching human (i.e., the difference between SD and AD). Buffer distance is an estimate of tolerance calculated as the distance at which a prey fled after being alerted by an approaching human (i.e., the difference between AD and FID). **(B)** Some hypothetical relationships between SD, AD, and FID as a function of short, medium, or long pre-detection distances and short, medium, or long buffer distances.

distance between starting distance (SD) and AD; where SD is the distance to the bird when approach begins]. Second, the prey's buffer distance (**Figure 1A**), which is a more rigorous estimate of tolerance estimated as the distance at which a prey fled "after being alerted by an approaching human" (i.e., the distance between AD and FID; Cooper and Blumstein, 2015). Using this approach, we can explore whether the previously identified reduction in urban birds' FID is traded-off with an increase in other costly anti-predatory behaviors. For instance, a shorter FID associated with a larger AD might imply increased vigilance costs since the prey's attention cannot simultaneously be focused on other fitness-enhancing activities, such as foraging. By contrast, a shorter FID associated with a shorter AD would result in a shorter buffer distance, implying that birds escape sooner after detecting an approaching human. If this reduction in tolerance is associated with a longer pre-detection distance, it may illustrate a strategy by which birds reduce their monitoring costs. **Figure 1B** illustrates these and other hypothesized scenarios.

We studied the difference in escape decisions of urban and adjacent rural populations of 32 bird species along a large latitudinal gradient across Europe (3,900 km). We investigated whether rural and urban birds differ in their awareness and tolerance along this gradient by estimating their pre-detection distance and buffer distance. We also studied the factors potentially related to rural-urban differences in these anti-predatory indicators by exploring seven potential correlates of pre-detection distance and buffer distance: latitude, species' body mass, species' brain mass, number of humans living in the city, rural-urban difference in mean SD (hereafter, ΔSD), rural-urban difference in mean AD (hereafter, ΔAD), and rural-urban difference in mean flock size (hereafter, Δflock size). Our hypotheses concerning the effect of these predictor variables are summarized in **Table 1**.

MATERIALS AND METHODS

Field Data Collection

We collected data from April to September 2015 (breeding season) using a standard protocol (Blumstein, 2006). Data were collected in urban and adjacent rural areas of 10 cities from eight European countries: Czech Republic, Denmark, Estonia, Finland, France, Hungary, Poland, and Spain (data set available in Supplementary Material 1). Observers used binoculars to identify birds that were foraging or engaged in "relaxed behavior" (i.e., roosting or preening). Highly vigilant or obviously alarmed individuals, or individuals near their nests were not approached. Subsequently, each individual bird was approached in a straight line by a human walking at a constant speed (0.5 m/s). The distance at which the experimenter started to approach the focal bird was recorded as SD, while the distance at which the bird first oriented toward the approaching human and stopped its previous activity was recorded as AD, and the distance at which the animal began to flee was recorded as FID. The number of birds within a radius of 10 m around the focal individual was recorded as flock size. We avoided sampling the same individual twice by moving to another site immediately after samples were taken. If the same general area was visited, only individuals of different species, sex or age than those sampled before were tested. A modest degree of re-sampling subjects, however, has been shown to not influence the results of studies like this (Runyan and Blumstein, 2004).

We used a paired study design with one urban and one rural area in each study location. The distance between each pair of urban and rural site varied between 1 and 20 km. All urban study sites included both urban centers, characterized by areas with multi-story buildings, as well as suburban areas, characterized by areas with single-family houses. Rural areas were dominated by open farmland with scattered houses. Definitions of urban (at least 50% of built-up area, building density >10 buildings/ha and, a residential human density >10 humans/ha) and rural habitats (5–20% built-up areas, a building density <2.5 buildings/ha, and residential human density between 1 and 10 humans/ha) follow Marzluff et al. (2001). The benefit of this paired study design is that neighboring study sites will share most potentially confounding environmental characteristics (e.g., weather, altitude, soil), yet philopatry and assortative mating may decrease, or even prevent, movements between urban and rural study sites (Møller, 2009, 2015).

Additional Data

Information about latitude and human population size of the cities were extracted from Wikipedia (https://www.wikipedia.org). Body mass information was extracted from Cramp and Perrins (1977–1994). Brain mass information was extracted from Møller and Erritzøe (2014). As explained above, mean flock size was estimated by field observations of individual birds.

Calculating the Phi Index

The relationship between SD, AD, and FID is constrained by an envelope. This means that AD can only assume values $\leq$ SD (according to methodology applied to study escape behavior, an individual is never experimentally approached if already alerted by an approaching observer; see Section Field Data Collection) and FID can only assume values $\leq$ AD (a prey individual cannot escape from a predator before it has detected it). The phi index (Φ) is a goodness-of-fit metric that was originally developed to provide estimates, unbiased by this envelope relationship, of how close FID is to AD, which corresponds to our definition of buffer distance (Samia and Blumstein, 2014). Formally,

$$\Phi = 1 - \frac{\sum_{i=0}^{n} \frac{(\mathbf{e}_i - \mathbf{o}_i)}{\mathbf{e}_i}}{n}$$

where, e_i is the AD, o_i is the FID, and n is the sample size. Nevertheless, Φ can also be used to estimate how close AD (in this case, o_i) is to SD (e_i) and then estimate the pre-detection distance. Importantly, Φ can be used as an effect size measure, which provides the magnitude of the effect of SD on AD (hereafter, pre-detection-Φ) and of AD on FID (hereafter, buffer-Φ; Samia and Blumstein, 2014). Φ is a standardized metric ranging from 0 to 1. Pre-detection-Φ values closer to 1 imply that birds were aware of approaching humans as soon as the human started to approach the focal bird, while buffer-Φ values closer to 1 imply that birds escape as soon as they were alerted to an approaching human.

Statistical Methods

We fitted a phylogenetically informed mixed-effect model using the *rma.mv* function of the *metafor* R package (Viechtbauer, 2010). Because there were multiple observations for each species, and because a shared evolutionary history makes data statistically dependent (Garamszegi, 2014), we incorporated species identity as a random factor while controlling for the phylogenetic dependence among species using a recent phylogenetic hypothesis (Jetz et al., 2012; Supplementary Figure 1).

Our dependent variables were the rural-urban difference in Φ estimated for a species from a single city (e.g., Φ of the rural population of *Passer domesticus* from around Madrid minus the Φ of the urban population of *P. domesticus* from the city of Madrid); hereafter termed Δpre-detection-Φ and Δbuffer-Φ. The use of these dependent variables emphasizes our aim of studying rural-urban differences in these antipredator behaviors.

TABLE 1 | Hypotheses concerning predictors.

Predictor	Response variable	Rationale	Expectation
ΔSD	Pre-detection distance	Predation risk decreases as soon as prey detect an approaching predator.	Rural-urban difference should increase with ΔAD: we expect that urban birds show a weaker positive relationship between buffer distance and AD because of a reduced perceived risk of predation in urban environments.
ΔAD	Buffer distance	FID often show a strong positive relationship with AD, very likely because of increased attentional costs of monitoring an approaching predator, or because perceived risk increases as the duration of a predator's approach increases.	Rural-urban difference should increase with ΔAD: we expect that urban birds show a weaker positive relationship between buffer distance and AD because of a reduced perceived risk of predation in urban environments.
Δflock size	Pre-detection distance	Vigilance increases with group size because of many eyes effect.	Rural-urban difference should increase with Δflock size: we expect that urban birds live in smaller flocks because of reduced predation risk in the cities and then take longer to be alerted about an approaching human.
	Buffer distance	*Per capita* predation risk tends to decrease with group size because individual risk is diluted.	Rural-urban difference should increase with Δflock size: we expect that urban birds live in smaller flocks because of reduced predation risk in the cities and also tolerate closer human approaches after detection because of the reduction in perceived predation risk.
Latitude	Pre-detection distance	Predation risk decreases with latitude because of reduced predator density.	Rural-urban difference should decrease as latitude increases since the difference in predation pressure between rural and urban environments is smaller at high latitudes.
	Buffer distance	Predation risk decreases with latitude because of reduced predator density.	Rural-urban difference should decrease as latitude increases since the difference in predation pressure between rural and urban environments is smaller at high latitudes.
Body mass	Pre-detection distance	FID and AD usually increase with body mass for a variety of hypothetical causes: increased conspicuousness of large prey, decreased agility to escape larger animals, preferred prey item because of increased energetic intake to the predator, increased risk taking by smaller animals because their metabolic rates make acquiring sufficient energy more immediate.	Rural-urban difference should increase as body mass increases.
	Buffer distance	FID and AD usually increase with body mass for a variety of hypothetical reasons: increased conspicuousness of large prey, larger animal's decreased agility to escape, large prey are preferred prey items, increased risk taking by smaller animals because their energetic requirements are more immediate given relatively high metabolic rates.	Rural-urban difference should increase as body mass increases.
Brain mass	Pre-detection distance	Large-brained species are better able to adapt to novel environments (e.g., cities), assess predation risk and increase net benefits before fleeing from an approaching threat.	Rural-urban difference should increase as brain mass increases.
	Buffer distance	Large-brained species are better able to adapt to novel environments (e.g., cities), assess predation risk and increase net benefits before fleeing from an approaching threat.	Rural-urban difference should increase as brain mass increases.
Human population size	Pre-detection distance	For animals that showed increased tolerance to humans, evidence suggests that the degree of tolerance increases with frequency of non-lethal human disturbances, likely because of a habituation process.	Rural-urban difference should increase with a city's human population size.
	Buffer distance	For animals that showed increased tolerance to humans, evidence suggests that the degree of tolerance increases with frequency of non-lethal human disturbances, likely because of a habituation process.	Rural-urban difference should increase with a city's human population size.

SD, starting distance; AD, alert distance; FID, flight initiation distance; Δ, symbol indicates the rural-urban difference of a given variable.

We only included populations with at least eight observations per urban site and at least eight observations per rural site (i.e., at least 16 observations per species' population) in the analyses to avoid estimates based on very small sample sizes.

We tested the effect of the predictors on Δpre-detection-Φ and Δbuffer-Φ using models that were weighted by sample size to account for differences in sampling effort among populations (Garamszegi and Møller, 2011; Garamszegi, 2014). The inverse sample size of a given species' population (= contrast) was used as a proxy for variance in the *rma.mv* function (Garamszegi, 2014). Continuous variables were $\log_{10}$-transformed before analyses to ensure normality of residuals.

We performed stepwise backward model selection based on corrected Akaike Information Criteria (AIC_c), using a threshold AIC_c value of 2. We present both full and minimum adequate models. We assessed the importance of each predictor based on their effect sizes calculated as partial correlation coefficients (Nakagawa and Cuthill, 2007). We followed criteria listed by Cohen (1997) for small ($r = 0.10$, explaining 1% of the variance), intermediate ($r = 0.3$, explaining 9% of the variance) or large effect sizes ($r = 0.5$, explaining 25% of the variance). As expected, brain mass was strongly positively correlated with body mass ($r = 0.9$), but the remaining predictor variables had low multicollinearity (all variance inflation factors, VIFs, <1.21). Multiple regression is the best approach to control for undesirable confounding effects among correlated covariates (such as body and brain masses), yielding unbiased coefficient estimates (Freckleton, 2002). For this reason, we retained body mass and brain mass in the same model to control for their confounded effect. Moreover, we found no relationship between body mass and latitude (model's $P = 0.36$; effect size $r = -0.07$), or body mass and an interaction between latitude and site (rural × urban; model's $P = 0.94$; effect size $r = -0.005$), suggesting that any effect of latitude on the escape variables was not confounded by a potential latitudinal increase in avian body mass. All analyses were conducted with R version 3.2.2 (R Core Team, 2014).

RESULTS

Our final data set contained 5,987 observations of 32 bird species, resulting in 92 paired comparisons of rural and urban populations (Supplementary Material 1). Paired *t*-tests showed that, on average, SD, AD, FID, and buffer-Φ were smaller in urban than in matched rural populations, all with large effect sizes (**Figure 2**). Although the SD used to approach urban birds was much smaller than the SD for rural birds (with a large effect size, $r = 0.64$), urban birds took longer than rural birds to be alerted to an approaching human, as evidenced by a smaller pre-detection-Φ in urban populations (the rural-urban difference had a large effect size; **Figure 2**). Flock size did not differ between rural and urban populations (with a very small effect size; **Figure 3**), suggesting that urban birds did not take longer to detect humans because of fewer eyes to detect predators.

The minimum adequate model of Δpre-detection-Φ retained only one variable, latitude, which had a large effect size (**Table 2**). We found that the rural-urban difference in pre-detection distance increased with latitude (**Table 2**, **Figure 4A**). Specifically, we found that although both rural and urban populations tended to take longer to be alerted to an approaching human as latitude increased (i.e., both have smaller pre-detection-Φ values as latitude increased), urban birds took even longer than rural birds as latitude increased (**Figures 4A,B**).

The minimum adequate model of Δbuffer-Φ retained two variables: ΔAD (intermediate to large effect size) and latitude (small to intermediate effect size; **Table 3**). We found that the rural-urban differences in buffer distance increased with the difference in their mean AD (**Table 3**, **Figure 5A**). When we excluded an outlier with high leverage from analysis, we found that rural birds did not have a significant relationship between mean AD and buffer-Φ (**Figure 5B**), while urban birds had a negative relationship between these variables ($P = 0.019$; **Figure 5C**). The latter relationship was marginally significant when the outlier was kept in the analysis ($P = 0.053$; Supplementary Figure 2). We also found that rural-urban differences in buffer distance decreased as latitude increased (**Table 3**, **Figure 6A**). Although both rural and urban populations tended to escape sooner once alerted, as latitude increased (i.e., both have larger Φ-values as latitude increased), urban birds tended to delay their escape more at low latitudes when compared with rural birds (**Figure 6B**).

Finally, regression between buffer-Φ and pre-detection-Φ by site revealed an interesting pattern (**Figure 7**). In rural environments, populations that were alerted soon after a human began approaching them responded by delaying or anticipating flight, whereas all but one population that took longer to become alerted to humans escaped soon afterwards (**Figure 7A**). By contrast, in urban environments, the significant negative relationship between buffer-Φ and pre-detection-Φ (small to intermediate effect size, $r = -0.26$; intercept = 0.783, $b = -0.203$, $P = 0.012$) suggests a trade-off between these escape decisions (**Figure 7B**).

DISCUSSION

When compared with rural birds, urban European birds have shorter alert distances (AD), flight initiation distances (FID), and delay escape more (as reflected by smaller buffer-Φ values). Despite the fact that the starting distance (SD) used to approach individual birds was much smaller in urban sites, urban birds took longer to be alerted by an approaching human (as reflected by a smaller pre-detection-Φ values), making our conclusions even more robust. Despite the fact that the observer is partly determining SD, it is also a feature of habitats and species associated with specific habitats. In urban habitats, birds may observe an approaching human or predator later because of high building density or because of urban noise, and therefore urban birds may have less time to make their escape decisions. Importantly, since AD and FID were also substantially reduced in urban habitats (both with large effect sizes), the large reduction in pre-detection distance and buffer distances in urban locations were not an artifact caused by Φ, a metric which calculates the relative, not the absolute, difference between these behavioral estimates (Samia and Blumstein, 2014).

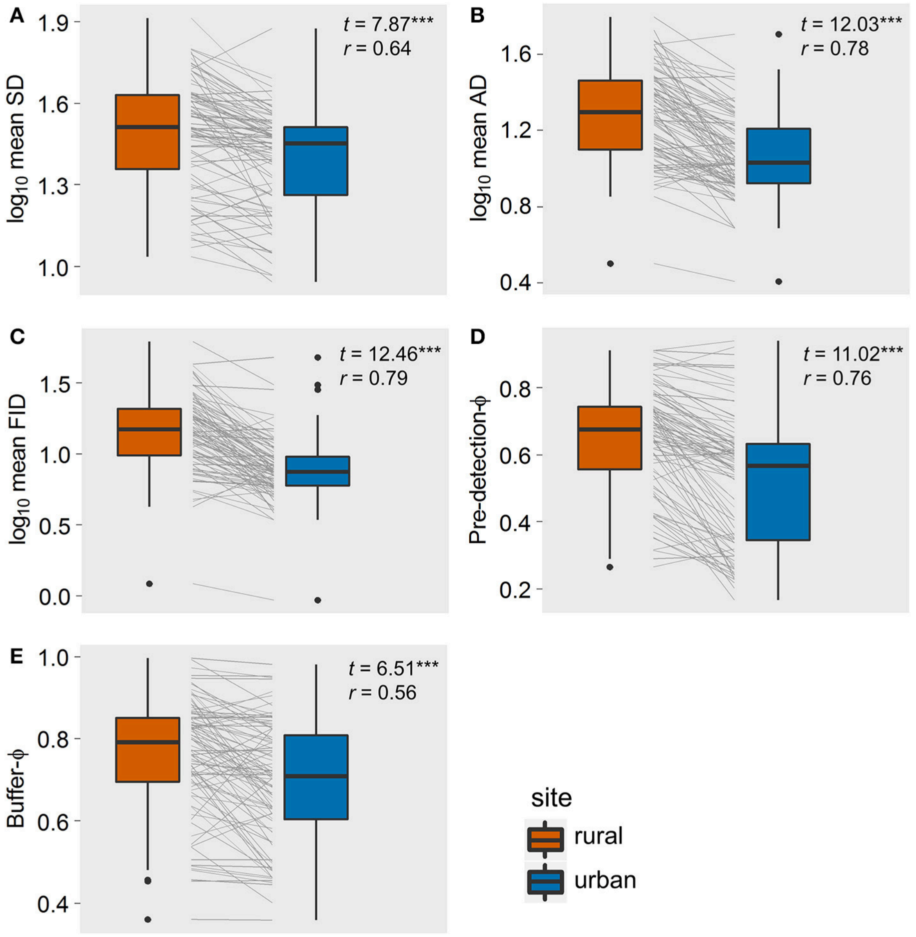

FIGURE 2 | The rural-urban differences of five components of escape behavior of the 92 bird populations studied in Europe: **(A)** starting distance, SD; **(B)** alert distance, AD, **(C)** flight initiation distance, FID; **(D)** pre-detection-Φ (lower values mean that birds took longer to be alerted to an approaching human); **(E)** buffer-Φ (lower values mean that birds tolerate more human approach after detection). Box-plots show median, quartiles, 5- and 95-percentiles and extreme values. Each population's trajectory is illustrated with a gray line. *t*-values and effect sizes calculated as partial correlation coefficients are shown. All *t*-values were highly significant (***$P < 0.001$).

The great reduction in anti-predatory responses in urban environments suggests that urban birds in Europe live under lower predation risk than their rural counterparts, a causal hypothesis supported by previous studies (e.g., Anderies et al., 2007; Møller and Ibáñez-Álamo, 2012; Díaz et al., 2013). Economic escape theory postulates that prey should counterbalance predation risk and the costs of fleeing (which includes the loss of foraging opportunities) when deciding to escape from a potential predator (Ydenberg and Dill, 1986; Cooper and Frederick, 2007). By reducing awareness and delaying flight after detecting a potential threat, urban birds are expected to be able to allocate more of their limited energy to other key activities, such as foraging and reproduction (Ydenberg and Dill, 1986; Cooper and Frederick, 2007). Indeed, urban birds often have longer reproductive periods and more breeding bouts when compared with their rural counterparts, although climatic and ecological factors are also involved in this process (Deviche and Davies, 2013; Møller et al., 2015).

Besides the difference in predation risk, there are at least two other possible drivers of these rural-urban differences in anti-predatory responses. First, it is possible that urban birds live in "heat islands" and this climatic effect, rather than variation in predation risk, drives the patterns in the life history decisions reported here. We find this possible, but not likely because of the effect sizes we identified and because of the relationship between predation and FID that we (and others) reported elsewhere (Stankowich and Blumstein, 2005; Díaz et al., 2013; Cooper and Blumstein, 2015; Samia et al., 2015c). Second, variation in flock size, which could influence risk perception, could also explain these patterns. Differences in flock size between rural and urban populations could contribute to differential awareness and risk taking by birds that results from the effects of many eyes scanning for predators (Pulliam, 1973). Individuals in larger flocks could also tolerate more human disturbance because of a reduction in *per capita* risk of predation (Alexander, 1974). And, if tolerance of non-lethal human disturbance is socially

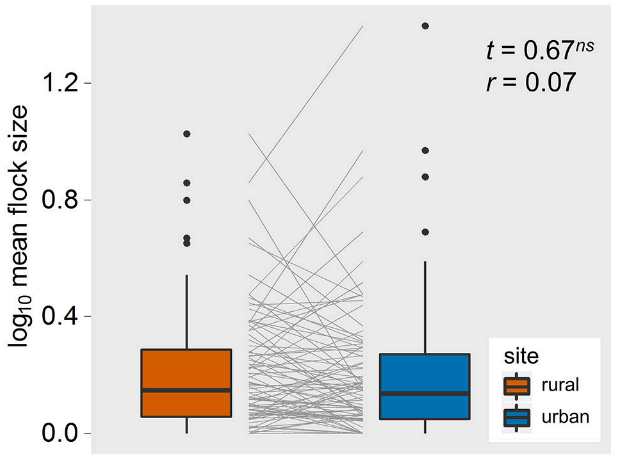

FIGURE 3 | The rural-urban differences in flock size of the 92 bird populations studied in Europe. Box-plots show median, quartiles, 5- and 95-percentiles and extreme values. Each species' trajectory is illustrated with a gray line. *t*-values and effect sizes calculated as partial correlation coefficients are shown. ns, non-significant.

TABLE 2 | Full and minimum adequate models explaining the rural-urban differences in pre-detection distance (Δpre-detection-Φ) of European birds.

Predictor	Estimate	*SE*	*Z*	*p*	Effect size
FULL MODEL (AIC$_c$ = −93.84)					
(Intercept)	−0.137	0.206	−0.662	0.508	
ΔSD	0.142	0.145	0.980	0.327	0.11
Latitude	0.004	0.002	2.106	**0.035**	0.23
Body mass	0.076	0.076	0.998	0.318	0.11
Brain mass	−0.108	0.121	−0.893	0.372	−0.10
Human population	−0.013	0.019	−0.693	0.489	−0.08
ΔFlock size	0.010	0.090	0.108	0.914	0.01
MINIMUM MODEL (AIC$_c$ = −113.32)					
(Intercept)	−0.078	0.094	−0.821	0.412	
Latitude	0.004	0.002	2.162	**0.031**	0.23

Effect sizes are partial correlation coefficients. P-values in bold indicate statistical significance ($P < 0.05$).

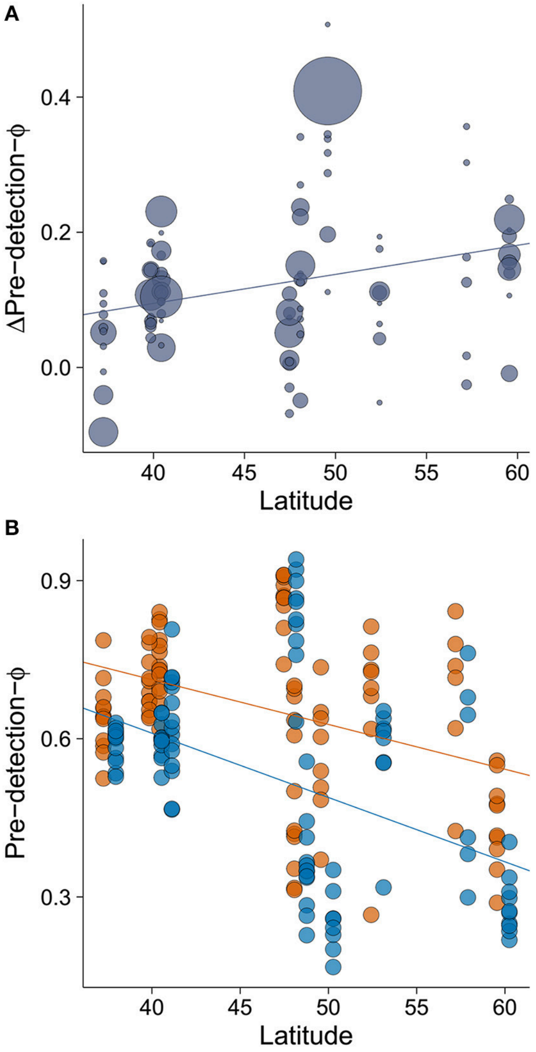

FIGURE 4 | The relationship between **(A)** latitude and rural-urban difference in pre-detection-Φ (Δpre-detection-Φ) and **(B)** the relationship between latitude and pre-detection-Φ by site. Lower values of pre-detection-Φ mean that birds took longer to be alerted to an approaching human. The size of the circles in plot A is proportional to sample size for a given population (min = 19, max = 358). In plot **(B)**, orange points and orange line represent rural populations whereas blue points and blue line represent urban populations. Circle size was fixed but points representing urban birds have been slightly displaced to the right (0.7° of latitude) to improve data visualization. Lines illustrate linear regressions.

transmitted, one might expect the effect of social transmission to be enhanced in larger groups (Griffin, 2004). However, the rural and urban bird populations we studied in Europe did not differ in their flock sizes. Although, a recent study found that wild birds increased tolerance to humans as group size increased (Samia et al., 2015a), flock size was not important in explaining rural-urban differences in tolerance in a meta-analysis of 180 bird species distributed around the globe (Samia et al., 2015c), nor was it important in a meta-analysis of all estimates of FID in birds (Møller, 2015). Nevertheless, the similarly-sized flocks in rural and urban bird populations in our study suggests alternative explanations for rural-urban differences in these anti-predatory responses.

To understand the changes in escape strategies of rural and urban birds we need an integrative view of the patterns that arise from the main drivers of pre-detection distance and buffer distance. As latitude increases, pre-detection distances increase and buffer distances decrease in both rural and urban birds. If we assume that predation risk decreases with increasing latitude (Schemske et al., 2009; Díaz et al., 2013), this implies that as risk decreases, birds take longer to become aware of approaching humans while they take flight more rapidly following detection. This pattern suggests that birds prioritize the reduction of monitoring costs when facing lower predation pressure, which occurs at higher latitudes and in urban areas.

TABLE 3 | Full and minimum adequate models explaining the rural-urban differences in buffer distance (Δbuffer-Φ) of European birds.

Predictor	Estimate	*SE*	*Z*	*p*	Effect size
FULL MODEL (AIC$_c$ = −110.72)					
(Intercept)	0.222	0.184	1.209	0.227	
ΔAD	0.317	0.080	3.966	**<0.001**	0.43
Latitude	−0.005	0.002	−2.490	**0.013**	−0.27
Body mass	−0.0004	0.07	−0.006	0.996	<0.01
Brain mass	0.072	0.107	0.675	0.499	0.07
Human population	0.0003	0.017	0.018	0.986	<0.01
ΔFlock size	0.028	0.082	0.343	0.731	0.04
MINIMUM MODEL (AIC$_c$ = −126.69)					
(Intercept)	0.222	0.085	2.603	0.009	
ΔAD	0.332	0.078	4.246	**<0.001**	0.45
Latitude	−0.005	0.002	−2.407	**0.016**	−0.26

Effect sizes are partial correlation coefficients. P-values in bold indicate statistical significance ($P < 0.05$).

Because attention is a limited entity (Dukas, 1998, 2004), prey that allocate attention so that they can detect predators must reduce or divert their attention from other fitness-enhancing activities, such as foraging or social activities. For this reason, ongoing monitoring is costly because of lost opportunities. In fact, a recent meta-analysis showed that factors associated with opportunity costs had the largest effect size on escape decisions in lizards (Samia et al., 2016). This raises the question whether it is reasonable to expect selection on animals to reduce monitoring costs in areas with reduced predation risk. Future studies that report a negative relationship between latitude (or other environmental correlates of predation risk) and vigilance behavior in birds would be consistent with the causal hypothesis of reduction of attentional costs.

While larger pre-detection distances at higher latitudes is consistent with the reduced predation risk hypothesis, the more immediate escape of both rural and urban birds at higher latitudes might sound counterintuitive. However, this observation can be explained by the spatial constraints associated with escape. Escape variables are constrained so that SD ≥ AD ≥ FID, with FID always being > 0 for prey which flee an approaching threat at any given moment (Cooper et al., 2015). The larger a prey's pre-detection distance, the closer is AD to the minimal safe distance (Blumstein, 2003; Cooper and Blumstein, 2015). In economic escape theory, the minimal safe distance (or Zone I sensu Blumstein, 2003) represents a distance at which prey flee immediately because the risk of being depredated is maximal given its very close distance to the predator. Thus, it is not expected that prey tradeoff risks against costs, but rather flee immediately (Blumstein, 2003; Cooper and Blumstein, 2015). Therefore, because birds seemingly increase their pre-detection distance to avoid ongoing monitoring costs at high latitudes, their AD shifts closer to their minimal safe distance, resulting in even more immediate escape at higher latitudes. Although the existence of a minimal safe distance has empirical support (e.g., Cooper, 2005, 2008), future studies identifying the factors that determine it, and whether the minimal

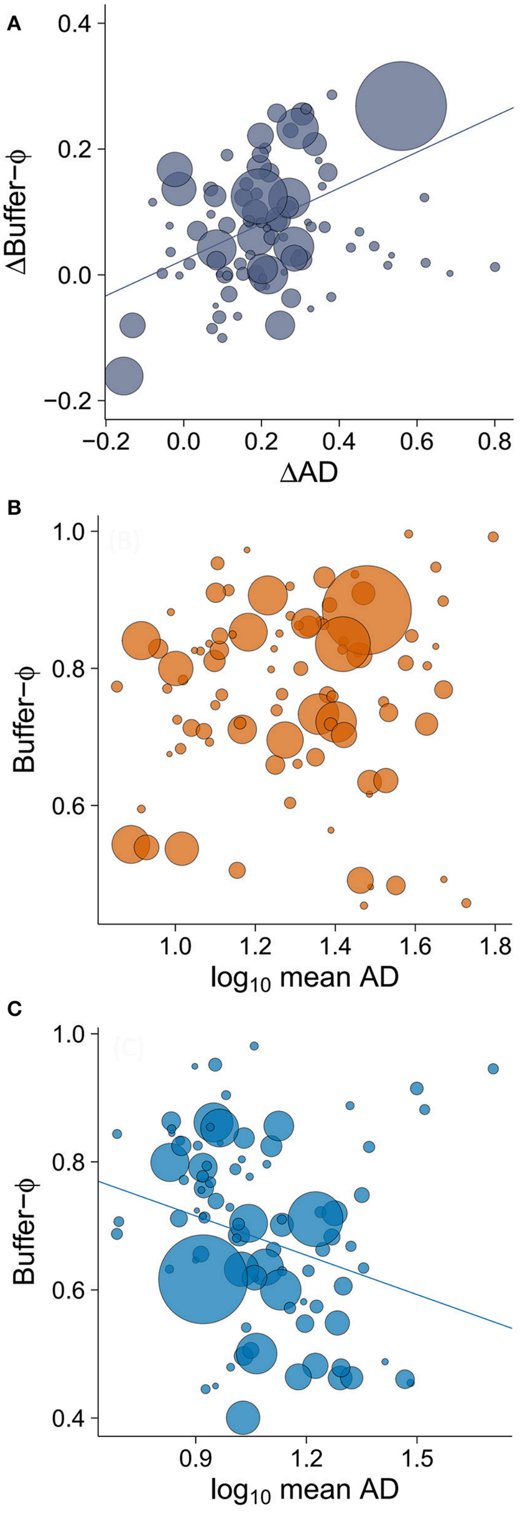

FIGURE 5 | The relationship between **(A)** rural-urban difference in mean alert distance (ΔAD) and rural-urban difference in buffer-Φ (Δbuffer-Φ), and the relationship between mean AD and buffer-Φ in **(B)** rural and **(C)** urban sites. Lower values of buffer-Φ mean that birds tolerate more human approach after detection. The size of the circles is proportional to sample size for a given population (plot A: min = 19, max = 358; plot B: min = 8, max = 122; plot C, min = 8, max = 275). Lines illustrate linear regressions.

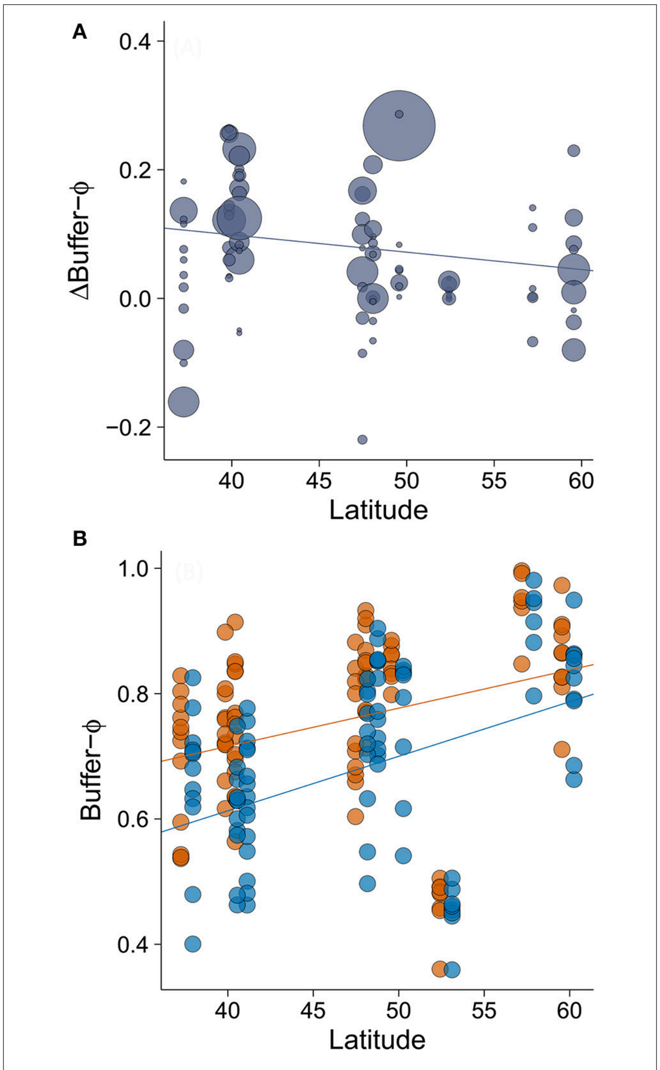

FIGURE 6 | The relationship between **(A)** latitude and rural-urban difference in buffer-Φ (Δbuffer-Φ) and **(B)** the relationship between latitude and buffer Φ by site. Lower values of buffer-Φ mean that birds tolerate more human approach after detection. Circle size in plot A is proportional to the sample size for a given population (min = 19, max = 358). In plot B, orange points and orange line represent rural populations whereas blue points and blue line represent urban populations. Circle size was fixed and points representing urban birds have been slightly displaced to right (0.7 degrees of latitude) to improve data visualization. Lines illustrate linear regressions.

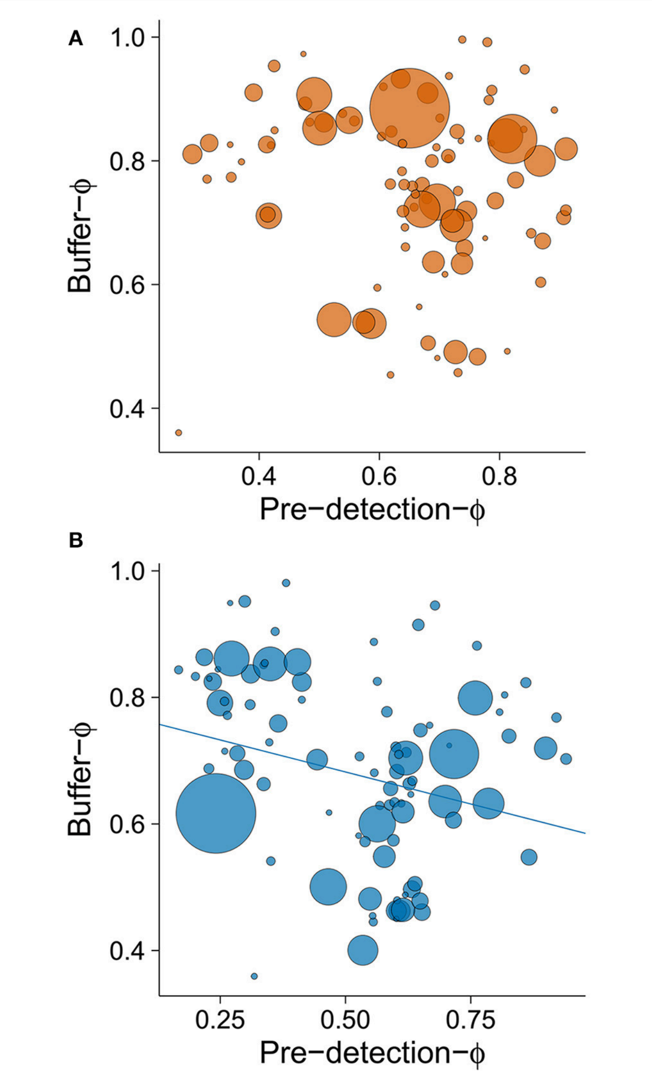

FIGURE 7 | The relationship between pre-detection-Φ and the buffer-Φ for **(A)** rural and **(B)** urban populations. Lower values of pre-detection-Φ mean that birds took longer to be alerted to an approaching human, whereas lower values of buffer-Φ mean that birds tolerate more human approach after detection. Circle size is proportional to sample size for a given population (plot A: min = 8, max = 122; plot B: min = 8, max = 275). Line illustrates linear regression.

safe distance changes over time and context, will help us to understand the processes leading to the success of urban populations.

This causal hypothesis of the relationship between buffer distance and latitude is supported by two other findings. First, the observation of different relationships between AD and buffer distance that varied across sites. In rural areas, with larger average AD, which were further from bird's minimal safe distances, there was no relationship between mean AD and buffer distance. By contrast, this relationship was negative in urban areas where mean AD was shorter and thus closer to the bird's minimal safe distances. Second, the observation of the inverse relationship of pre-detection distance and buffer distance that varied with latitude. This relationship suggests that the minimal safe distance is a potential threshold for escape in birds. The rural-urban difference in buffer distance is larger in Southern Europe, the region where the difference in pre-detection distance is smaller. Conversely, the rural-urban difference in buffer distance is smaller in Northern Europe, where the difference in pre-detection distance is larger.

Although the observed latitudinal variation in behavior is consistent with the predation risk hypothesis (when not distinguishing rural from urban populations), the observed "rural-urban difference" in these behaviors with latitude is challenging to explain, particularly since some expect patterns were not observed (see **Table 1**).

Contrary to our expectation, rural-urban differences in pre-detection distance increased at higher latitudes, with urban birds taking even longer to be alerted of approaching humans in higher latitudes. There are at least two hypotheses that may explain these patterns. First, in Northern Europe, rural villages are small and birds may effectively avoid people because they have a reduced tolerance of people (e.g., because they are less exposed, they are less likely to habituate to humans) and because of a weaker human shield effect in these areas (Berger, 2007; Ibáñez-Álamo et al., 2012; Møller, 2012). Thus, the difference between urban centers and rural areas may be exacerbated given the size of rural villages. Second, the differences between Northern and Southern Europe could result by their different types of predator communities. Predator assemblages in Northern towns are less diverse and abundant than assemblages in the Southern towns. For example, no stray cats and red foxes live in Northern European towns (e.g., Jokimäki et al., 2005), despite both of them being common in Southern European towns. In addition, many raptor species (e.g., *Falco peregrinus* and *F. tinnunculus*) are urbanized in the South but have not become urbanized in the North. These differences are partly related to much longer history of urbanization in Southern than in Northern Europe. Thus, urban birds in the North may live in even safer environments and this could enhance the pre-detection differences between rural and urban areas. However, it is also possible that both human presence/disturbance (Carrete and Tella, 2010) as well as differences in the predator assemblages might cause differences in avian anti-predatory behavior.

Brain size was not a main driver of rural-urban differences in escape decisions in this set of European birds. Brain size was a main driver of escape decisions of North American and Australian birds when interspecific differences were investigated (Samia et al., 2015a). At the species level, differences in brain size are often associated with variation in cognitive abilities and/or behavioral flexibility (Sol et al., 2005, 2013; Maklakov et al., 2011). However, as we found, a species' brain size may not have a substantial effect on the rural-urban changes in avian escape decisions within species. Despite that, the effect of brain size on rural-urban difference in escape decisions of European birds might differ from that in North American and Australian birds due to a longer history of close interactions between birds and humans in the Old World, dating back more than 30,000 years. For example, Møller et al. (2014) showed that the relationship between FID and population trends differed between Europe and Australia on one hand and North America on the other. Whether this difference is based on cognitive, and hence brain size, differences among continents still remains to be determined. Future studies that explicitly analyze the relationship between rural-urban differences in escape decisions and brain size on continents that vary in their history of exposure to human activity are warranted. Such differences in behavior may have consequences for life history, which are already known to differ between the Old and the New World with reduced nest predation and increased fecundity and iteroparity in the Old World (Martin and Clobert, 1996; Ghalambor and Martin, 2001). The present study indicates that effects of urbanization, acting through selection on life history decisions, may be more profound than previously realized.

We conclude by noting that, while the study of FID has played a crucial role in the development of risk assessment theory and has been widely used to study the effects of urbanization, we have shown that other components of escape behavior can respond differently (both to urbanization and latitude) and provide a complementary perspective to the study of antipredator responses. This new approach is essential if we are to understand, predict and manage wildlife in a rapidly urbanizing world.

ETHICS STATEMENT

Data were collected on public and private land after acquiring any required permits. By design, experimental approaches were designed to create only a brief disturbance and we are not aware of any lasting harm caused by the experimental approaches. In addition, and to reduce the likelihood of any negative effects, endangered species were not targeted, and we only targeted birds away from their nests. In fact, the disturbance produced to birds by our methodology did not differ from standard "background" disturbance caused by any persons walking outdoors.

AUTHORS CONTRIBUTIONS

Conceived the study: DS and AM. Collected data: All authors, except DS and DTB. Analyzed data: DS. Discussed the results: All authors. Wrote the first draft of the manuscript: DS, DTB, and AM. All authors edited the manuscript.

FUNDING

DS was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2015/06734-1). DTB was supported by NSF. TG was supported by the Human Frontier Science Program award (RGY83/2012). JI was funded by a postdoctoral contract (TAHUB-104) from the program "Andalucía Talent Hub" (co-funded by the European's Union Seventh Framework Program Marie Skłodowska-Curie actions—COFUND—and the regional Government of Andalucía). KT was supported by the Estonian Research Council (institutional research funding IUT number 34-8). GM was supported by National Research, Development, and Innovation Office (NKFIH, K-115970).

ACKNOWLEDGMENTS

We thank Tomás Pérez-Contreras for his help during the field work and R. Viitanen for designing the **Figure 1A**.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00066/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 04 October 2016
doi: 10.3389/fevo.2016.00117

Time Since Urbanization but Not Encephalisation Is Associated with Increased Tolerance of Human Proximity in Birds

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The examination of links between a high degree of encephalisation (i.e., a large brain mass relative to body size) and the capacity of wildlife to inhabit anthropogenic habitats has formed the basis of several recent studies, although typically they have not uncovered any relationship. It, however, remains unclear whether encephalisation is directly related to a species' capacity to develop tolerance to human proximity (i.e., a reduction in response to approaching humans). It is also unknown whether such a relationship is related to the size of specific areas of the brain. Using published data on flight-initiation distance (FID), the distance at which animals flee from an approaching human, we estimate the degree of tolerance of human proximity for 42 bird species by comparing FIDs in urban and rural areas, with relatively high and low exposure to humans, respectively. We used a phylogenetic, comparative approach to analyse the relationship of degree of tolerance, and of FID in urban and rural populations more directly, to relative sizes of whole brains (42 species) and brain components (25 species) for the species, and examine the effect of the year that the bird species was first recorded in an urban area (year of urbanization). We demonstrate an interaction between bird habitat and year of urbanization on FIDs. Urban populations of species that have a longer history of inhabiting urban areas have lower FIDs (i.e., birds that were urbanized earlier are more tolerant), which may suggest local selection for birds with reduced responsiveness to humans in urban areas. The pattern is not seen in rural populations of the same species, providing additional evidence that it is greater exposure to humans that has resulted in this tolerance. While we found that forebrain mass and optic lobe mass are influential positive predictors of FID there was no indication that degree of tolerance itself was related to any brain size metric and hence no support for the idea that urban populations of species with larger brains are better able to habituate to human presence. This suggests that processes other than encephalisation explain the high degree of tolerance evident in urban-dwelling birds.

Keywords: brain size, flight initiation distance, habituation, optic lobe, phylogenetic generalized least squares regression, urbanization

Edited by:
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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *20 July 2016*
Accepted: *20 September 2016*
Published: *04 October 2016*

Citation:
Symonds MRE, Weston MA, van Dongen WFD, Lill A, Robinson RW and Guay P-J (2016) Time Since Urbanization but Not Encephalisation Is Associated with Increased Tolerance of Human Proximity in Birds. Front. Ecol. Evol. 4:117. doi: 10.3389/fevo.2016.00117

INTRODUCTION

Some species of birds have colonized urban areas from ancestral, rural populations. The "cognitive buffer" hypothesis suggests that larger-brained animals (such as birds) are better able to adapt to novel environmental conditions, such as those created by urbanization (Sol et al., 2005a). In addition, larger brains may be associated with more proactive (or "bold") personality types in which animals may be quicker to explore novel environments (Kotrschal et al., 2014). In theory, birds with larger brains may be able to more accurately judge risk when presented with evolutionary novel stimuli, or be more able to learn (habituate or sensitize) to adjust responses appropriately, based on their previous experience (Guay et al., 2013c). Despite these predictions, comparative studies have found no evidence that bird species with relatively larger brains are more likely to colonize urban habitats (Kark et al., 2007; Evans et al., 2011; Møller and Erritzøe, 2015), nor that larger brain size in birds is linked to the time at which a species started living in urban habitats (Møller and Erritzøe, 2015). However, these studies focus on urbanization *per se,* rather than the individual behaviors that may be associated with adaptation to urban environments. Of these, the capacity to discriminate benign, but common, potential threats (e.g., humans) from more dangerous ones is thought to be especially adaptive in urban environments because escape responses can be costly (Ydenberg and Dill, 1986). One way of measuring such discrimination, risk perception, and responsiveness of animals is through the flight-initiation distance (FID), the distance between a threatening stimulus and an animal when an escape response is initiated (Weston et al., 2012). Many species exhibit reduced FIDs to humans in areas where humans are more common (Kitchen et al., 2010; Weston et al., 2012; McGiffin et al., 2013; Gravolin et al., 2014; van Dongen et al., 2015b; Vines and Lill, 2015). These species can be said to have developed "tolerance" of human proximity—defined as when animals permit closer approaches by humans without overtly responding or fleeing (Blumstein, in press).

This tolerance is often inferred to be caused by habituation (the reduction of responsiveness with increasing exposure to a stimulus) most commonly the result of within-animal learning (Møller, 2010; Weston et al., 2012). But, in fact, other "habituation-like processes" may act to reduce responsiveness in animals in areas where humans are more common (Blumstein, in press). For example, local selection involving bolder individuals settling or remaining in busier areas (van Dongen et al., 2015b) may act across generations to reduce responsiveness over time. This is especially the case where species invade urban areas from adjoining non-urban areas. Under these circumstances species with a longer histories of urbanization are expected to have the greatest reduction in responsiveness because these selection processes will act across generations. Indeed, among birds, the earlier the year in which a species colonizes urban areas the shorter it's FID, suggesting evolution of avian responses to humans (Møller, 2008, 2010; but see Gendall et al., 2015).

If the degree of tolerance to humans in urban populations is also the result of learning and habituation then the cognitive buffer hypothesis would predict that species with larger brains should display greater degree of tolerance to humans in urban habitats. A key challenge for this theory, however, is how to measure cognitive capacity. Relative whole brain size is often used as a surrogate for a species' cognitive ability and is available for a broad range of birds (Madden, 2001; Sol et al., 2005a; Healy and Rowe, 2007; Guay and Iwaniuk, 2008; Sol, 2009). More recently, and because of recognition of functional specialization of different parts of the brain (Healy and Rowe, 2007), brain components have been used (e.g., Symonds et al., 2014). In birds the detection of, and response to threats is likely to be associated with brain components involved in vision and perception (the optic lobe), cognition and assessment of risk (the forebrain), and physiological and motor responses (the brain stem and the cerebellum) (Paulin, 1993; Burish et al., 2004; Feenders et al., 2008). We therefore predict that the size of these individual brain components influences responses to perceived threats. For example, the forebrain is involved in cognition and learning, processes which underpin behavioral traits associated with habituation. Therefore, species with larger forebrains should show the greatest decrease in FID in urban environments. By contrast, species that are more likely to respond quickly to predators (i.e., greater FIDs overall) are likely to have greater perception abilities and a capacity to respond quickly (hence larger cerebellums, optic lobes, and brain stems). However, analyses of whole brain and brain components, and their link to FID, have yielded conflicting results. Møller and Erritzøe (2014) found that bird species with relatively larger brains overall had reduced FIDs, but that species with relatively larger cerebellums did indeed have longer FIDs. By contrast, Guay et al. (2013c) and Symonds et al. (2014) found no such patterns. The relationships of FID with brain size generally, and with specific brain components in particular, therefore remain unclear.

Despite the apparent lack of a relationship between relative whole brain size and the propensity to inhabit urban environments, there is greater intraspecific variation in brain sizes of species which have also colonized urban areas compared to those that have not, and such species exhibit greater behavioral plasticity (e.g., FIDs, Møller, 2010; Carrete and Tella, 2011; Møller and Erritzøe, 2015). Whilst Carrete and Tella (2011) considered relative whole brain size and FIDs (evoked by cars) in birds in rural vs. urban areas, to date no study has examined the influence of brain size components on the reduction in FID associated with living in urban habitats.

This study uses both whole brain and brain component size to examine whether brain size influences birds' tolerance of humans (as evoked by the commonest stimulus evident in urban areas, people). To investigate this, we examine FIDs of urban and rural populations of avian species, and used these values to generate a "degree of tolerance" exhibited by each species. We tested two predictions: (1) that species with longer histories of urbanization would exhibit a greater degree of tolerance (i.e., a bigger difference between FID in urban vs. rural populations) indicative of a local selection effect, and (2) that brain size and brain component sizes would also influence the extent of the degree of tolerance, due to larger brained species being more likely to habituate to a greater extent.

We also examined the way that the absolute values of FID in urban and rural populations of avian species differ in relation to brain and brain component sizes. If the cognitive buffer theory applies, we predict an interaction between habitat and brain size in predicting FID, such that (1) species with larger overall brain sizes will have smaller FIDs and (2) in species with larger brains there would be a greater difference in FID between rural and urban individuals (see **Figure 1**). This would provide evidence of larger-brained birds being able to habituate (decrease their FIDs) to humans to a greater extent in urban areas (Guay et al., 2013a).

MATERIALS AND METHODS

Data Collection

Data on FID, Starting Distance (SD, the distance at which an investigator approach started), and year of urbanization (first known year of breeding in an urban environment, as derived from observations and historical accounts) were sourced from Garamszegi et al. (2007) and Møller (2008). Data on FID and SD (in France and Denmark) from both the aforementioned sources were combined, as these measurements are repeatable between observers (Guay et al., 2013b; van Dongen et al., 2015a). We derived separate estimates of FID and SD for urban and rural populations of 42 species.

Data on brain and brain component masses were sourced from Mlíkovský (1989a,b,c, 1990) and Portmann (1947). Where brain volumes were reported, brain mass was calculated using the mean density of brain tissue (1.036 g/ml), as reported elsewhere (Iwaniuk and Nelson, 2002). The brain regions were forebrain, cerebellum, optic lobe (comprising the optic tectum and underlying structures, such as the inferior colliculus), and brain stem. Body masses were obtained from Dunning (2008). Although we were able to extract whole brain masses for all 42 species in our analysis, data on individual brain components was restricted to a subset of 25 species. Consequently, analyses examining the role of individual brain components on FID are restricted to this smaller data set.

FID, SD, body mass and brain masses/brain component masses were log transformed prior to analysis to better conform with assumptions of normality. We used the difference in FID values between rural and urban populations to generate a measure of "degree of tolerance" for each species (Glover et al., 2015). However, such a direct comparison of FID is complicated by the differences in SD between the two populations. To control for this, mean FIDs were then plotted against mean SDs for both rural and urban populations (SD is positively related to FID; Weston et al., 2012). The residual FID (ResFID) was then calculated for each species in both habitat types (i.e., $\text{ResFID}_{\text{urban}}$

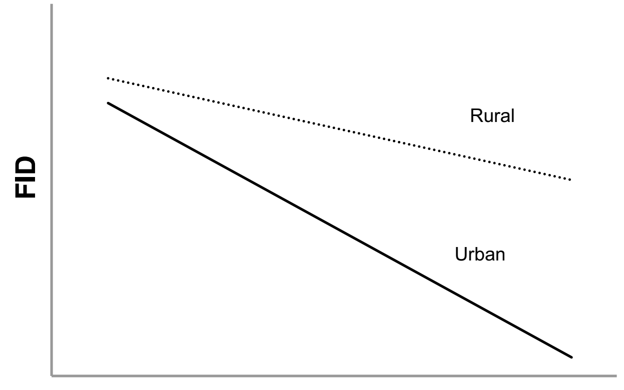

FIGURE 1 | Predicted interspecific relationships between relative brain size and FID in rural (dotted line) and urban birds (solid line) if larger brained birds are better able to adjust to human presence. The pattern demonstrates an interaction between habitat and brain size in predicting FID.

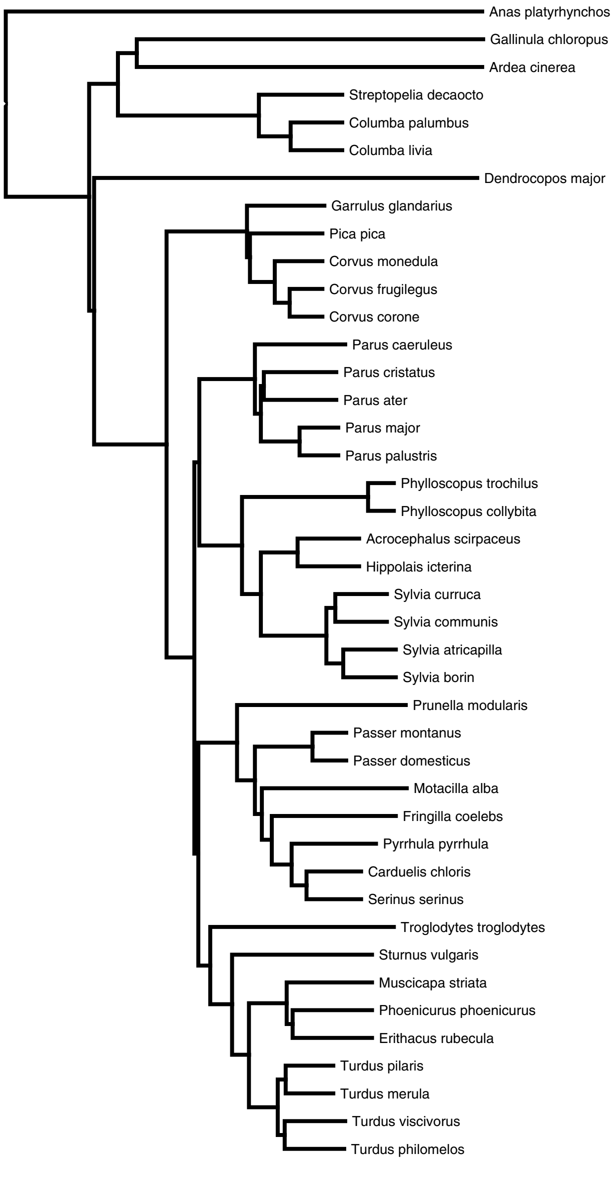

FIGURE 2 | Consensus phylogeny for the 42 bird species in the analysis.

and $\text{ResFID}_{\text{rural}}$). A mean SD for each species (pooling both habitats) was then used to calculate predicted FID in each habitat, based on the coefficients of the correlation between FID and SD (predicted FID = 1.1958∗MeanSD + ResFID). These values represented predicted FIDs for each species in each environment at a standardized starting distance. Predicted FIDs were then back transformed and used to calculate degree of tolerance, using the following formula:

$$Degree\ of\ Tolerance = \frac{predictedFID_{rural} - predictedFID_{urban}}{predictedFID_{rural}} \times 100$$

Therefore, higher values indicate a greater degree of tolerance in urban habitats.

Comparative Analysis

As with earlier analyses of brain components and FID (Guay et al., 2013c; Symonds et al., 2014), we employed a phylogenetic comparative approach when analyzing our data, to control for potential non-independence of data due to shared ancestry among species. In this study, we obtained the phylogeny used as the basis for analysis from the "Global Phylogeny of Birds" website—www.birdtree.org (Jetz et al., 2012). Specifically, we downloaded a set of 2000 trees for our subsets of species from the pseudo-posterior distribution of trees using the Hackett et al. (2008) backbone. We then used this tree set to calculate a 50%-majority-rule consensus tree (see **Figure 2**) using the Mesquite package (Maddison and Maddison, 2010). We conducted our comparative analysis in two different ways. The first approach used degree of tolerance for each species as the response variable. The second approach employed the absolute FID data from two different populations (urban and rural) for each species as the response variable. In the second case we treated the populations as two separate "species" in our analysis. Consequently, we split each species tip in the phylogeny into two, separated by minimal branch length (length = 0.001) from their ancestral node.

We constructed phylogenetic generalized least squares (PGLS) models with brain size (or brain component sizes), body mass, and year of urbanization as predictors of degree of tolerance, and with SD and habitat as additional predictors in the analysis of FID. PGLS analysis was carried out using the *caper* package in R (Orme et al., 2012).

Because of the high degree of correlation ($r > 0.8$) with body mass, we obtained measures of relative brain size (or relative brain component sizes) by calculating the residuals of the PGLS regression of brain size on body size, i.e., the observed value minus the predicted value from the PGLS regression of the log-transformed trait against log body mass. In the second analysis, to evaluate whether brain size influences the way that rural and urban populations differ in their FID responses, the interaction term between habitat and brain size was also evaluated in models. Likewise, an interaction between year of urbanization and habitat (urban/rural) was also included to assess the prediction that only urban populations should show a response in FID in relation to year of urbanization.

We used an information theoretic approach to analyse the explanatory power of our predictor variables in explaining FID. We conducted the analyses with two different data sets: using either the whole brain mass (42 species) or the 4 individual brain component masses (25 species) in combination with the other predictor variables. All PGLS model combinations

TABLE 2 | Parameter estimates from models with ΔAICc < 2, explaining variation in degree of tolerance among birds.

Predictor variable	Coefficient	*SE*	*t*
(A) 42 SPECIES DATASET			
Model 1			
Intercept	**972.5**	**276.1**	**3.522**
Year of Urbanization	**–0.482**	**0.141**	**–3.425**
Model 2			
Intercept	**788.9**	**321.4**	**2.454**
Body Mass	10.78	9.73	1.109
Year of Urbanization	**–0.398**	**0.160**	**–2.489**
(B) 25 SPECIES DATASET			
Model 1			
Intercept	**1498.5**	**425.2**	**3.524**
Year of Urbanization	**–0.758**	**0.219**	**–3.470**
Model 2			
Intercept	**1317.6**	**461.5**	**2.855**
Body Mass	14.13	14.05	1.006
Year of Urbanization	**–0.679**	**0.232**	**–2.924**

Influential parameters (i.e., whose confidence intervals do not include zero) are shown in bold.

TABLE 1 | The best-ranked PGLS regression models (models with ΔAICc < 2) predicting degree of tolerance across bird species.

Model structure	*k*	AICc	ΔAICc	w_i	R^2
(A) WITH WHOLE BRAIN MASS AS PREDICTOR (42 SPECIES)					
Year of Urbanization	2	421.2	0.00	0.436	0.23
Year of Urbanization + Body mass	3	422.2	1.02	0.262	0.25
(B) WITH INDIVIDUAL BRAIN COMPONENTS AS PREDICTORS (25 SPECIES)					
Year of Urbanization	2	262.1	0.00	0.196	0.34
Year of Urbanization + Body mass	3	263.6	1.47	0.094	0.37

Models are ranked using AICc, with ΔAICc and Akaike weight (w_i) for each model shown. Also shown is the number of parameters in the model (k) and measure of model fit (R^2).

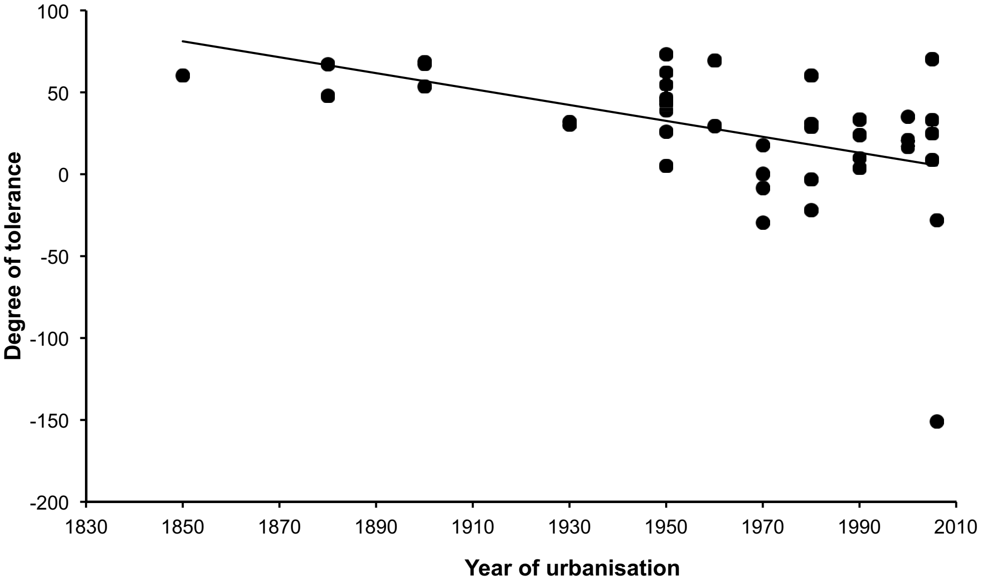

FIGURE 3 | Relationship between degree of tolerance (see text) and year of urbanization in 42 bird species. Species that have a longer history of urbanization tend to show higher degrees of tolerance to humans (more positive values).

TABLE 3 | The best-ranked PGLS regression models (models with ΔAICc < 2) predicting FID across bird species (including rural and urban populations of those species).

Model structure	k	AICc	ΔAICc	w_i	R^2
(C) WITH WHOLE BRAIN MASS AS PREDICTOR (42 Species)					
Habitat + Body mass + SD + Year of Urbanization + Year of Urbanization:Habitat	6	−109.7	0.00	0.713	0.81
(D) WITH INDIVIDUAL BRAIN COMPONENTS AS PREDICTORS (25 SPECIES)					
Habitat + Body mass + SD + Year of Urbanization + Year of Urbanization:Habitat + Forebrain	7	−60.5	0.00	0.312	0.86
Habitat + Body mass + SD + Year of Urbanization + Year of Urbanization:Habitat+ Optic Lobe	7	−60.1	0.37	0.259	0.86
Habitat + Body mass + SD + Year of Urbanization + Year of Urbanization:Habitat	6	−58.7	1.75	0.130	0.84

Models are ranked using AICc, with ΔAICc and Akaike weight (w_i) for each model shown. Also shown is the number of parameters in the model (k) and measure of model fit (R^2).

of the predictor variables were compared using Akaike's Information Criterion controlled for small sample size (AICc, Burnham and Anderson, 2002; Symonds and Moussalli, 2011). Models with the lowest AICc score are held to be the best approximating models explaining the response variable. We calculated the relative support for models by examining the ΔAICc score (the difference in AICc score between the best approximating model and each other model in the candidate set), and also the Akaike weight (w_i)—the probability that the model is the best model in the set). We compiled the list of top models (those models with ΔAIC < 2) with associated parameter estimates for each model. Influential effects were those where the 95% confidence interval around the parameter estimate did not cross zero (Burnham and Anderson, 2002). The evaluation of all models was achieved using the MuMIn package in R (Bartoń, 2014). In the analyses using brain components, because of the high co-linearity between these component masses, we did not include any models that involved combinations of components, just those using individual components, and their interaction terms with habitat.

For visual representation of relationships between FID, habitat, and year of urbanization or brain size, we calculated FID values for each species as the residuals from the PGLS model predicting log FID with log body mass and log SD as predictors.

RESULTS

There was no effect of whole brain mass or individual brain component masses on degree of tolerance to human proximity (**Table 1**). The only clear predictor of degree of tolerance across species was year of urbanization, which was strongly negatively related to the degree of tolerance (**Table 2**). Species which have inhabited urban areas longer are more tolerant to human presence (**Figure 3**). In both analyses of degree of tolerance, the top model returned was the model with year of urbanization as the sole predictor.

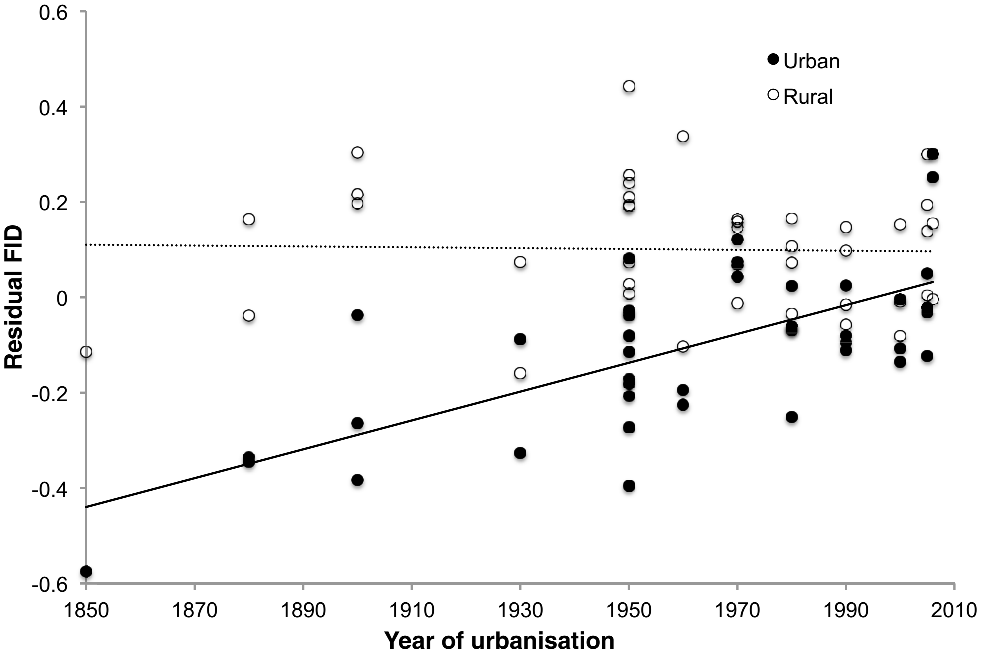

FIGURE 4 | Relationship between FID and year of urbanization in rural and urban populations of 42 bird species. Urban populations of species that have a longer history of urbanization tend to show greater reductions of FID, but there is no such relationship in rural populations of the same species. FID values are the residuals from the regression of absolute log FID against log body mass and log starting distance.

In the separate analysis of absolute FID values, all best approximating models featured SD, body mass, and habitat as strong predictors of FID (**Table 3**), the former two with positive effects, and the latter showing the clear effect that birds in urban populations have shorter FIDs than birds in rural populations (**Table 4**). In all top models, year of urbanization features as a predictor, although pooled across all species/populations, its individual effect has a parameter estimate whose confidence intervals include zero. However, there is a clear interaction between habitat and year of urbanization. Urban populations of birds have a positive association between FID and year of urbanization, with more recently urbanized bird populations showing longer FIDs. No association between year of urbanization and FID is evident for rural birds (**Figure 4**).

Analysis using whole brain, brain stem or cerebellum masses revealed no direct effect of these variables on FID in the best models (**Table 3**). However, both forebrain and optic lobe mass appeared as positive predictors of FID in the best models, although their inclusion in models only explains an extra 2% of variance, indicating that their individual effects are weak (**Table 4**). In no case did an interaction term between brain size and habitat appear in the top models, indicating no evidence that larger-brained bird species show a greater discrepancy in FID between urban and rural populations. **Figure 5** demonstrates the relationship between forebrain and optic lobe masses and FID, demonstrating the similar nature of the relationship for both rural and urban populations.

DISCUSSION

Urbanization is associated with reduced responsiveness to, i.e., increased tolerance of, humans (Møller, 2010; this study). As has also been previously reported, the longer a species has been exposed to urbanization the more tolerance to humans is evident (Møller, 2008, 2010; but see Gendall et al., 2015). This pattern of increase in tolerance over time suggests selection is acting whereby more responsive individuals die or do not breed, or less responsive individuals have some other fitness benefits in urban settings, although it may possibly also involve historical founder effects if bolder birds initially instigated colonization of urban areas (Møller, 2010; Weston et al., 2012; van Dongen et al., 2015b). Human behavior, especially the occurrence of hunting in rural areas, may differ between habitats and influence FID (Magige et al., 2009; Sreekar et al., 2015). Alternatively, site selection by birds may involve more responsive individuals moving away from, or not settling in, urban areas (van Dongen et al., 2015b). The relationship between time since urbanization and tolerance, and more specifically the fact that rural birds have apparently not altered their responsiveness through time while urban birds have exhibited a decrease in responsiveness, strengthens the idea that it is specifically urbanization, and not some other variable, that has driven the increased degree of tolerance in species that have longer histories of urbanization. The linear decrease in FIDs through time for urban birds suggests that tolerance appears to occur across generations, and defies the somewhat commonly held view that it is solely learning within individuals (habituation) that is the principle mechanism

TABLE 4 | Parameter estimates from models with ΔAICc < 2, explaining variation in FID among birds.

Predictor variable	Coefficient	*SE*	*t*
(A) 42 SPECIES DATASET			
Intercept	−0.012	1.061	−0.012
SD	**0.522**	**0.098**	**5.340**
Body Mass	**0.229**	**0.037**	**6.119**
Year of Urbanization	−0.00004	0.0005	−0.083
Habitat (Urban)	**−7.036**	**1.213**	**−5.800**
Year of Urbanization:Habitat	**0.003**	**0.0006**	**5.612**
(B) 25 SPECIES DATASET			
Model 1			
Intercept	0.224	1.295	0.173
SD	**0.650**	**0.137**	**4.750**
Body Mass	**0.193**	**0.034**	**5.652**
Forebrain Mass	**0.273**	**0.093**	**2.925**
Year of Urbanization	−0.0002	0.0006	−0.323
Habitat (Urban)	**−7.787**	**1.696**	**−4.592**
Year of Urbanization:Habitat	**0.004**	**0.0009**	**4.440**
Model 2			
Intercept	0.424	1.311	0.323
SD	**0.676**	**0.136**	**4.991**
Body Mass	**0.186**	**0.034**	**5.486**
Optic Lobe Mass	**0.555**	**0.194**	**2.858**
Year of Urbanization	−0.0003	0.0007	−0.504
Habitat (Urban)	**−7.777**	**1.702**	**−4.568**
Year of Urbanization:Habitat	**0.004**	**0.0009**	**4.420**
Model 3			
Intercept	−0.121	1.311	−0.092
SD	**0.653**	**0.141**	**4.635**
Body Mass	**0.217**	**0.047**	**4.660**
Year of Urbanization	−0.00007	0.0006	−0.113
Habitat (Urban)	**−7.786**	**1.523**	**−5.106**
Year of Urbanization:Habitat	**0.004**	**0.0008**	**4.938**

Influential parameters (i.e., whose confidence intervals do not include zero) are shown in bold.

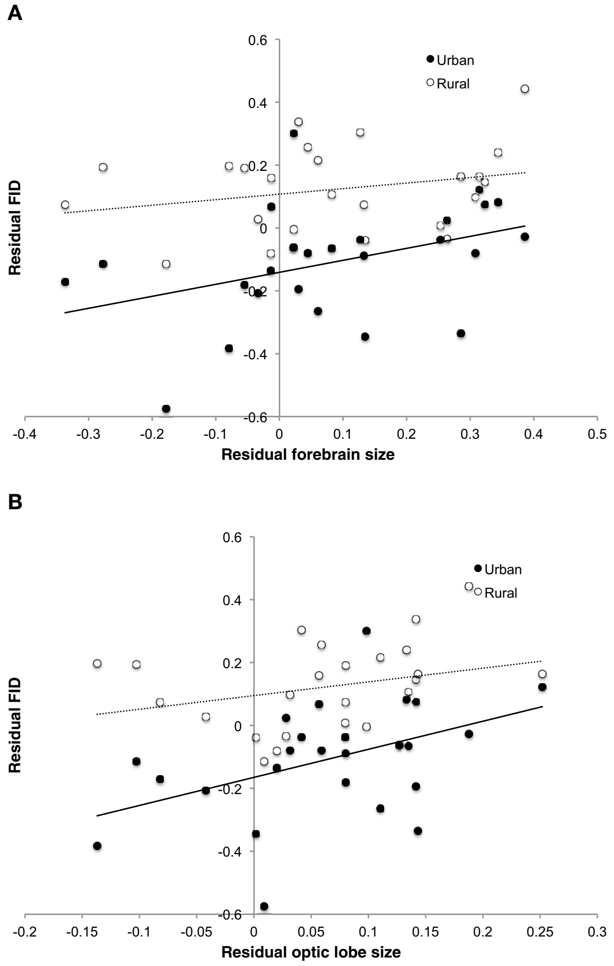

FIGURE 5 | Relationship between FID and (A) forebrain size and (B) optic lobe size in both rural and urban populations of 25 bird species. While rural populations, and species with greater brain component masses, have longer FIDs, there is no difference between populations in the relationship of brain component sizes to FID. FID values are the residuals from the regression of absolute log FID against log body mass and log starting distance. Brain size values are the residuals of the log transformed trait mass against log body mass.

through which tolerance of humans increases (see Weston et al., 2012).

Encephalisation had no apparent effect on tolerance, either as measured through degree of tolerance or through the interaction between habitat and brain size in predicting FID. These results suggest that habituation, if it also does explain to some extent the increased tolerance in urban birds, is not being mediated by brain size. Three potential influences may explain the lack of an effect. First, our measures of brain size and FID did not adequately characterize intraspecific variation in these traits. Brain sizes vary within populations of birds and whilst mean brain size may be equal, the distribution of brain sizes (standard deviation and skewness) can differ amongst populations and may influence tolerance of humans intraspecifically (Møller and Erritzøe, 2015). Given the high neural density in avian brains, small differences in size may represent substantial differences in cognitive abilities (Olkowicz et al., 2016). Therefore, whilst a relationship between brain size and tolerance may not be detected among species, it may exist within species between individuals (Blumstein, in press). Second, fear is not the sole driver of brain architecture or size. Urban founder effects and selective pressures, including, among other influences, the nutritional requirements associated with growing and maintaining larger brains, are also likely to influence relative bird brain size in urban vs. rural habitats (Møller and Erritzøe, 2015). Finally, studies of whole brain size (and less commonly brain component size) and FID in birds indicate contrasting results linking escape behavior to the size of brain structures (Guay et al., 2013c; Møller and Erritzøe, 2014; Symonds et al., 2014). Hence any effects of encephalisation on the reduction in FID may be difficult to discern, and may reflect the inherent difficulties and generalizations of relating complex behaviors with specific areas of the brain (Healy and Rowe, 2007).

Interestingly, decreased fear of humans has co-evolved with *decreased* brain size in domestic birds (Desforges and Wood-Gush, 1975; Ebinger and Löhmer, 1985, 1987), perhaps mediated by a relatively stronger selection for atrophy of the optical processing areas of the brain as compared to the overall brain mass (Ebinger and Löhmer, 1987) and/or a decreased eye size in domestic birds (Ebinger et al., 1989).

The finest level of neural anatomy available to us across species (brain components) uncovered a generally positive relationship between forebrain mass, optic lobe mass, and FID. Larger forebrains in birds have been linked to behavioral flexibility, such as foraging innovations (Lefebvre et al., 1998; Nicolakakis and Lefebvre, 2000), leading us to expect that larger forebrains could be linked with moderation of FIDs where humans are common and benign. However, contrary to our predictions we found that whilst larger forebrains were associated with *longer* FIDs among species in general, they were not associated with the degree of decrease in FIDs in urban populations. Perhaps larger forebrains underpin threat perception at greater distances, and this overrides any effects of enhanced risk assessment and moderated response. Forebrain size is subject to ecological constraints and is implicated in major life history differences between birds (e.g., longer-migrating species have smaller forebrains; Winkler et al., 2004; Sol et al., 2005b), and thus is subject to a variety of selective pressures which vary among species, and which we did not include in these analyses. The role of forebrain size, if any, in moderation of escape behaviors in birds warrants further investigation.

FID is positively correlated with eye size (Møller and Erritzøe, 2010), and the optic lobe in birds increases in size in relation to eye size (Brooke et al., 1999). It has been suggested, based on flight speeds, that larger eyes (and therefore optic lobes) enable detection of stimuli at greater distances (Brooke et al., 1999), which is consistent with the longer FIDs we found here, but again there is no evidence that optic lobe size plays a role in determining the extent to which species reduce their FID in urban environments. Whether the acuity of vision varies between species at the starting distances and stimulus (humans) used in this study remains unclear.

Our results therefore indicate an evolutionary response in FID to urban-living in birds, such that tolerance has increased over historical time, as is shown by the finding that bird species with longer histories of urbanization exhibit a greater degree of tolerance. However, although we find some evidence that brain component (forebrain, optic lobe) sizes are linked with FID response in general among the species studied, there is no evidence that increased encephalisation influences the extent to which tolerance of humans has developed in urban populations of birds. Comparisons of fine-scale brain structures of birds inhabiting environments with different prevailing human regimes may uncover subtler differences.

AUTHOR CONTRIBUTIONS

Conceived study: MS, PG, MW, WvD, AL. Collected data: PG, WvD, MW. Analysed data: MS, WvD, PG, MW. Contributed funding and materials RR. Wrote the paper: MS, PG, MW, WvD, AL.

FUNDING

This research was supported by funding from Melbourne Water Corporation and a Victoria University Fellowship and a Faculty of Health Engineering and Science Collaborative Research Grant Scheme to PG.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00117

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 31 May 2017
doi: 10.3389/fevo.2017.00054

Behavioral Correlations Associated with Fear of Humans Differ between Rural and Urban Burrowing Owls

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Behavioral studies are fundamental to understanding how animal populations face global change. Although much research has centered upon the idea that individuals can adaptively modify their behaviors to cope with environmental changes, recent evidence supports the existence of individual differences in suites of correlated behaviors. However, little is known about how selection can change these behavioral structures in populations subject to different environmental constraints. The colonization of urban environments by birds has been related to their inter-individual variability in their fear of humans, measured as their flight initiation distance to an approaching human, such that urban life would select for fearless individuals. This behavior has been demonstrated to be heritable and highly consistent throughout the adult lifespan of burrowing owls (*Athene cunicularia*). Here, we experimentally assessed, in field conditions, whether urban life involves changes in other behaviors such as exploration and antipredatory response through their correlation with fear of humans. Breeding urban birds were more fearless toward humans and were quicker to explore a new food resource and defend their nests from predators than their rural counterparts. However, while fear of humans positively correlated with exploration and antipredatory response in the rural population, it only correlated with exploration in the urban one. Predator release in urban environments could relax—and even counterselect—antipredator behaviors, thus dismantling the behavioral correlation existent in natural populations. Altogether, our results suggest that rural and urban animals may differ in some behavioral aspects, may be as a consequence of the selection processes acting during the colonization of urban areas as well as the different ecological environments encountered by individuals.

Keywords: antipredator behavior, exploration behavior, flight initiation distance, neophily, personalities, risk-taking

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *13 February 2017*
Accepted: *10 May 2017*
Published: *31 May 2017*

Citation:
Carrete M and Tella JL (2017) Behavioral Correlations Associated with Fear of Humans Differ between Rural and Urban Burrowing Owls. Front. Ecol. Evol. 5:54. doi: 10.3389/fevo.2017.00054

INTRODUCTION

Behavioral studies are fundamental to our understanding of how animals respond to environmental changes (Sih et al., 2011). Over the past four decades, much research has centered upon the proposition that individuals can alter their behavioral phenotype to cope adaptively with environmental conditions that change within their lifetime (Piersma and Drent, 2003; Réale and Dingemanse, 2010). Indeed, a meta-analysis of more than 3,000 rates of recent phenotypic change suggested that most of the phenotypic changes associated with rapid human-induced environmental changes involve phenotypic plasticity rather than immediate genetic evolution (Hendry et al., 2008). Despite this flexible nature, however, there is also a tendency for individuals to behave consistently through time, and there is a growing body of evidence indicating that behavioral variation among individuals within populations sometimes exceeds the variation expressed by individuals over time or across contexts. Such stable interindividual variation is referred to as "animal personality" (Dall et al., 2004), "temperament" (Réale et al., 2007), and "coping style" (Koolhaas et al., 1999), and several reviews have emphasized its potential evolutionary causes and functions under current global change (e.g., McDougall et al., 2006; Réale et al., 2007; Smith and Blumstein, 2008; Sih et al., 2011).

Urbanization is one of the most prevailing and lasting forms of rapid human-induced habitat change occurring worldwide, and is causing the loss of biodiversity through local extinction processes (McKinney, 2006; Sol et al., 2014). However, the response of species are greatly variable, and although most of them are unable to occupy these new habitats, others persist or even reach higher densities in urban than in rural areas (Stracey and Robinson, 2012; Sol et al., 2013; Rodriguez-Martínez et al., 2014; Tella et al., 2014). Thus, a growing number of studies have explored ecological and life history traits that allow some species to thrive in urban environments (Bonier et al., 2007; Sol et al., 2014). Recently, it has been proposed that the ability of certain species to colonize urban habitats is related to their interindividual variability in the fear of humans, measured as the distance between an approaching human and a focal animal at which the latter flees (i.e., flight initiation distance; Carrete and Tella, 2011). Although differences in fear of humans among populations subject to different degrees of human disturbance have been traditionally interpreted as indicative of habituation (e.g., Blumstein et al., 2003; Martínez-Abrain et al., 2008; Rodríguez-Prieto et al., 2009), recent work demonstrated that this behavior is highly repeatable within an individual's adulthood (Carrete and Tella, 2010, 2013) and heritable (Møller, 2014; Carrete et al., 2016), leaving a small margin for behavioral flexibility (Vincze et al., 2016). Thus, urban invasion seems to be the result of tame individuals from species with high interindividual variability in their fear of humans crossing the disturbance frontier, supporting the disturbance-induced habitat selection hypothesis (Carrete and Tella, 2010).

Recent studies have shown that individuals within a population can be defined as bold or aggressive across a wide range of situations (i.e., territorial, feeding, parental, or antipredator behavior), while others are consistently nonaggressive or shy. The existence of these suites of correlated behaviors (Sih et al., 2004) suggests that behaviors should be evaluated together rather than as isolated units due to their potential consequences on individual fitness and to understand potential trade-offs or conflicts. The idea that urban areas (and humanized environments in a more broad sense) can act to select individuals with tolerant behaviors toward people (Arroyo et al., 2017) opens the question of whether they can also induce changes in other behaviors because of genetic correlations (constraint hypothesis) or through pressures acting in a similar direction (adaptive hypothesis; Bell, 2005, 2007; Dingemanse et al., 2007). In this sense, previous studies have correlated flight initiation distance with exploration or aggression toward conspecifics (Garamszegi et al., 2009; Evans et al., 2010), suggesting that other behaviors in addition to fear of humans could also change when birds enter urban areas.

Here, we test whether urbanization influences the distribution and diversity of individual behaviors, selecting for populations with particular behavioral structures. For this purpose, we focused on the burrowing owl, a bird species largely studied as a model of recent urban colonization (Carrete and Tella, 2010, 2011, 2013; Rodriguez-Martínez et al., 2014; Rebolo-Ifrán et al., 2015; Carrete et al., 2016). We experimentally show that rural and urban individuals differ in their fear of humans and in its association with antipredatory and exploratory behaviors. This suggests that behavioral correlations in this species are the result of selective pressures acting on the different behaviors and supporting their adaptive nature.

MATERIALS AND METHODS

Study Species, Area and Population Monitoring

The burrowing owl (*Athene cunicularia*) is a small owl found across American open landscapes, showing diurnal activity and nesting in burrows excavated by themselves or by mammals (del Hoyo et al., 1999). Pairs are territorial and highly conspicuous in the daytime during the breeding season (from October to early February in the study area), and are easily located usually within 50m of their nests. Sexual differences in coloration and plumage patterns (del Hoyo et al., 1999) allow experienced observers to sex breeding adults at a distance using binoculars (Carrete and Tella, 2010).

During the 2009–2010 breeding season, we GPS-located 192 active nests of burrowing owls in a 3,500 km^2 area, comprising the city of Bahía Blanca (Argentina) and its surrounding rural areas. Urban birds excavated their own nests in private and public gardens and in spaces among houses in urbanized residential areas, usually within 10–100 m of inhabited buildings, but also on curbs of streets and even on large avenues in the city. Rural birds, however, breed in the surrounding large expanses of natural grasslands and pastures devoted to wide-ranging livestock and low-intensive cereal crops, where owls excavate their own nests but can also occupy burrows of fossorial mammals (Carrete and Tella, 2011). There, human presence and activities are extremely low (Carrete and Tella, 2011). There is not a clear gradient neither habitat barriers (rivers, mountains, forests, etc.) between urban and rural habitats, as the city is immediately surrounded by large and flat rural expanses.

Territories were regularly visited to monitor breeding success, capture birds, and perform behavioral tests. Breeding birds were captured using bow nets and ribbon carpets placed at the entrance of their nests, and marked by using a plastic ring with an individual alphanumeric code readable at a distance.

Behavioral Experiments

From November 2009 to January 2010, we visited all territories to establish the experimental conditions needed to characterize behavioral traits in different ecological situations, namely: (1) presence of a new food source (exploration), (2) presence of predators (aggression toward predators) and (3) fear of humans (risk taking). To homogenize the underlying state of the individuals as much as possible, we only performed behavioral tests in those territories where breeders where rearing chicks, excluding those with fledglings or unsuccessful nests (e.g., nests where chicks were predated). Behavioral tests were performed sequentially (exploration, antipredatory behavior toward a terrestrial predator, and antipredatory behavior toward an aerial predator) to facilitate comparison between individuals, except for the fear of humans, which was measured throughout the study period. We are not aware of any lasting harm (e.g., nest failure, territory abandonment, individual injury) caused by the experimental approaches performed in this study.

Risk Taking

Fear of humans is indicative of the risk that individuals are willing to take in our presence, and has been shown to be key to understanding avian urban invasion (Carrete and Tella, 2011). We measured it as the distance at which a bird flees when approached by a human, using the standard procedure of walking toward undisturbed focal individuals (perched on the ground or on small poles close to their nests, **Figure 1A**), following a direct trajectory at a constant speed of 0.5 m/s, with no obstacles between the bird and the observer. Distances at which birds fled were measured using a laser telemeter (Leica Geovid, range: 10–1,300 m) or counting paces for distances of less than 10 m (Carrete and Tella, 2010, 2011, 2013). FIDs were measured during the day, when owls were easily located at a distance, given the bare ground and short vegetation surrounding their nests. Due to the high within-individual repeatability of FID in urban and rural owls, both within ($r = 0.84$–0.92; Carrete and Tella, 2010) and across breeding seasons covering the lifespan of individuals ($r = 0.90$–0.96; Carrete and Tella, 2013), we only used one measure per individual for analysis (average values when more than one measure was obtained from a single individual).

Exploration/Avoidance

We tested differences in an individual's behavior when facing a novel food source by presenting birds with an unfamiliar food item placed in an unfamiliar object close (1 m) to the entrance of their nests. The novel food used was a white laboratory mouse kept within a small cage in a metallic trap (**Figure 1B**). Thus, the exploration/avoidance experiment coincided with the capturing sessions. Owls can prey on house mice *Mus musculus* and other rondentines, which are mainly brownish, so we inferred that birds had not previously exploited such a novel (white mouse) but potential food source. Moreover, the non-camouflaged cage in which the mice were presented also changes the way mice are usually encountered. Therefore, we assumed that the responses of individuals would reflect how they cope (exploration or avoidance) with altered foraging opportunities. For each bird, we measured time (in minutes) to approach the trap (i.e., perching close ≤ 1 m- to the trap with hunting attitude) as a measure of its willingness to explore the new food. As trapability can be affected by aspects other than the interest of the animal in the mouse, we did not take into account the final result of the capture session (captured or not). Observations were recorded from a vehicle using binoculars (10 × 40) and telescopes (20–60×) at a minimum distance of twice the FID of the most shy individual of the pair to avoid interfering in the activity of the birds. The experiment was completed after a variable time depending on an individual's behaviors.

Antipredatory Behavior

After the exploration/avoidance experiment, we quantified the antipredatory behavior of birds toward a terrestrial (Pampa Fox *Pseudalopex gymnocercus*) and an aerial (peregrine falcon *Falco peregrinus*) predator, both of which are native to the study area. Predator models (**Figures 1C,D**) were sequentially exposed to the same nests (firstly the fox, and secondly the falcon) during the period in which breeders were rearing their offspring, with a lag of ca. 10 days between them.

During the experiments, we placed a polyester reproduction of the predator close (1 m) to the entrance of the nests for 15 min to minimize disturbance. We measured the aggressive reaction toward the predator as the time (in minutes) to approach it (i.e., when the individual perched close to the predator, performing displays, and was ready to attack). As in the previous experiments, observations were recorded from a vehicle using binoculars and telescopes at a distance to avoid interfering with the activity of the birds.

We estimated the repeatability of antipredatory behavior using a Bayesian Markov chain Monte Carlo technique implemented in the MCMCglmm package in R (Hadfield, 2010), modeling the latency to approach a predator (log-transformed to reach normality) as dependent variable, including predator species as a fixed effect and individual as a random term. Models were run with priors for the random variances set to 1, and a degree of belief $n = 2$. We used a "cengaussian" distribution as latencies were right-censored. Estimates were insensitive to the choice of priors (prior variances range 0.01–100). Parameter expansion was used to avoid poor mixing if variance component estimates were close to zero. All models were run for 100,000 iterations, preceded by a burn-in of 10,000 iterations. Estimates of parameters were stored every 25th iteration to reduce autocorrelation. We tested the statistical support of the fixed effect by evaluating whether their posterior distributions (95% credible interval) overlapped zero. Repeatability (r) in latency to approach a predator was obtained separately for urban and rural birds as $r = \sigma_{\text{individual}}/(\sigma_{\text{individual}} + \sigma_{\text{units}})$. Latencies to approach predators resulted repeatable within individuals (rural birds: $r = 0.55$; 95% CI = 0.35–0.74; urban birds: $r = 0.33$; 95% CI = 0.19–0.50), independently of the predator species used (95% CI for the effect of the species of predator considered: rural birds: −0.37–0.16, urban birds: −0.18–0.30), so we used one randomly selected measure per individual for further analysis (see below).

Analytical Procedures

We first compared FID, and latencies to approach the new food source and the predators across birds from different habitats (i.e., urban vs. rural). Differences in FID (log-transformed) between urban and rural birds were assessed using Generalized

FIGURE 1 | Breeding burrowing owls were systematically approached by a researcher to measure their flight initiation distances when they were perching close to their nests (A), and then were sequentially exposed to a trap with a white laboratory mouse kept within a small cage **(B)**, and polyester reproductions of a Peregrine Falcon *Falco peregrinus* **(C)** and a Pampa Fox *Pseudalopex gymnocercus* **(D)** placed close to the entrance of their nests.

Linear Models (normal error distribution, identity link function). Because latencies were right truncated at different times for those birds that did not approach the new food or the predators, we applied survival analyses to handle such censored data appropriately, using the package *survival* in R. Latency in each test was analyzed as a function of habitat using survival curves estimated through Kaplan-Meier methods, which allowed us to not assume underlying probability distributions and compare between two groups (urban and rural birds). Survival curves for urban and rural birds were compared using log-rank tests.

We then investigated behavioral correlations in urban and rural birds, separately. As we had censored data (i.e., minimum latencies) for both predator and food approaches, we were not able to perform classical Structural Equation Models (Dingemanse et al., 2010) without turning to imputation. Due to the large number of missing values, this procedure can reduce our ability to detect relationships among behaviors. Thus, as we prefer to avoid this last procedure, we calculated the covariances and correlations between behaviors using multivariate MCMCglmm, which are ultimately the main parameters indicative of the existence of a relationship between two behaviors. Although both statistics deal with the relationship between two variables, covariance indicates whether two variables change in tandem while correlation measures how strongly this relationship is. Moreover, while in the first case values are highly sensitive to the scale of measurement, correlations are calculated as the covariance between two behaviors divided by the product of their variances, so they can be viewed as the scaled form of covariance. Since the experiments were designed to measure the covariances between behaviors, we fitted completely parameterized covariance matrices during modeling (us()). An uninformative prior for these models was an improper prior with V = diag(x) and nu = dim(x+1), where x is the number of dependent variables (Hadfield, 2010). Support for the presence of relationships between behaviors was based on the posterior distribution of the estimated covariances and the corresponding 95% credible intervals.

RESULTS

We measured fear of humans (FID) in 357 breeding burrowing owls. The number of individuals included in successive behavioral tests decreased, given the logistic limitations imposed by the duration of the nestling period and the fact that some offspring fledged or were predated before we could complete all the tests. Therefore, exploration was measured in 253 individuals, and latency in approaching a predator in 165 individuals, respectively.

Rural birds did not respond to the stimulus as fast as the urban birds and thus, censored data were rather common among the former, skewing the distribution of the data and providing some infinite values for the mean and the 95% credible intervals. However, Kaplan-Meier analyses allowed us to deal with this, showing that flight initiation distance, latency in approaching the new food, and latency in approaching a predator significantly differed between urban and rural birds (**Table 1**; **Figure 2**). Urban

TABLE 1 | Median log-transformed values (and 95% credible intervals: L95%CI: lower tail, U95%CI: upper tail) obtained for risk taking (measured as FID, in m), exploration (measured as latency in approaching a new food source, in minutes) and aggression toward predators (measured as latency in approaching a predator, in minutes) in rural and urban burrowing owls *Athene cunicularia*.

Behaviors	Rural birds					Urban birds						
	n	Events	Median	L95%CI	U95%CI	*n*	Events	Median	L95%CI	U95%CI		
Risk taking	192	–	52.5	48.70	56.30	165	–	17.00	15.14	18.86	$t = 0.03$	$p < 0.0001$
Exploration	141	111	1.00	0.90	1.30	112	105	0.30	0.18	0.48	$\chi^2 = 61.5$	$p < 0.0001$
Antipredator	85	43	1.11	0.70	Inf	80	52	0.58	0.30	1.00	$\chi^2 = 4.3$	$p = 0.0375$

Sample sizes (n, number of individuals evaluated; events: number of uncensored data) and statistical tests (t-test comes from the GLM and χ^2 from the log-rank test) are shown. Inf, value not calculated but tending toward infinity due to the skewness of the data.

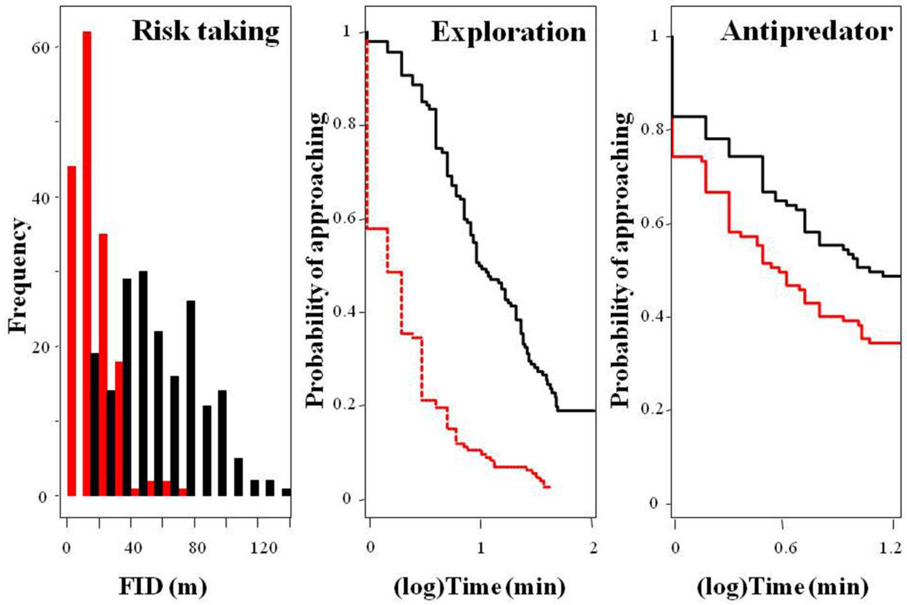

FIGURE 2 | Frequency distribution of fear of humans (risk taking behavior, measured as FID) and survival curves showing the probability of approaching a new feeding source (exploration) or a predator (antipredator) over time among urban (red) and rural (black) burrowing owls.

birds were more fearless in the face of humans and approached both the new food resource and the predator more quickly than their rural counterparts.

Moreover, urban and rural birds differed in their expression of behavioral correlations (**Table 2**; **Figure 3**). Among rural birds, which can be considered as the reference group, we found significant positive relationships between risk taking and the other two behaviors tested. Although covariances and correlations were rather low, they were all significantly higher than 0, meaning that rural individuals with large flight initiation distances when facing a human also show large latencies in approaching a predator and a new food item. It should be noted that the credible interval for the correlation between risk-taking and antipredatory behavior slightly overlap 0, while the bulk of the distribution is highly skewed toward positives values. This means that we can assume that the probability of having a correlation between these behaviors equal or lower than zero is negligible. Finally, the 95% credible interval for the correlation between exploration and response toward predators widely overlapped with zero (**Table 2**), suggesting that these 2 traits could be measuring independent aspects of the individual phenotype. All together, differences between these covariances and correlations suggest that latencies in the presence of a new food are manifestations of a behavioral trait related to the exploration/avoidance axis, which is not completely independent of the bold/shy axis represented by the latency to approach a predator and individual's fear of humans.

When considering the same relationships between behaviors among urban birds, however, all of them change. On the one side, the relationship between risk-taking and exploration became stronger in the urban population, as shown by the increase in the correlation coefficient, while exploration and latency to approach a predator coupled (positive correlation and 95% CI that does not overlap 0). Risk-taking and antipredatory behavior slightly covaried, but the correlation is not significantly different to 0 (95% CI widely overlap 0). All this means that urban birds with shorter flight distances approach a new food resource more

TABLE 2 | Mean covariances and correlations, with their 95% credible intervals in brackets, obtained for risk taking (measured as FID), exploration (measured as latency in approaching a new food source) and antipredatory behavior (measured as latency in approaching a predator) in rural and urban burrowing owls *Athene cunicularia*.

Behaviors	Rural birds		Urban birds	
	Covariance	Correlation	Covariance	Correlation
Risk taking - Exploration	**0.03 (0.00–0.06)**	**0.16 (0.00–0.32)**	**0.05 (0.03–0.09)**	**0.33 (0.17–0.48)**
Risk taking - Antipredator	**0.03 (−0.01–0.09)***	**0.15 (−0.05–0.34)***	**0.03 (−0.02–0.08)***	0.12 (−0.14–0.25)
Exploration - Antipredator	−0.09 (−0.21–0.06)	−0.12 (−0.35–0.11)	**0.08 (0.00–0.21)**	**0.23 (0.03–0.46)**

Correlation values take place between −1 and +1 while covariance values lie between −∞ and +∞. In bold, covariances and correlations different to 0 (i.e., 95% CI does not overlap 0).
**The lower tail of the 95% credible interval is very close to 0, so the parameter was interpreted as statistically different to 0.*

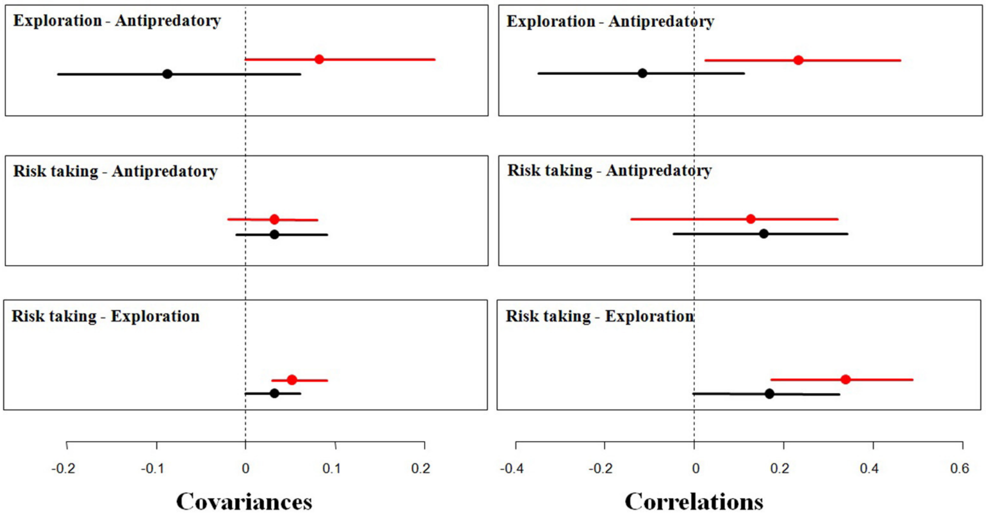

FIGURE 3 | Mean covariances and correlations (points), with their 95% credible intervals (segments), between fear of humans (risk taking behavior, measured as FID), antipredatory response and exploration among urban (red) and rural (black) burrowing owls. The vertical, dashed line represent 0 covariance and correlation.

rapidly while more explorative birds also approach more quickly the predator (**Table 2**).

DISCUSSION

We found differences in three main behaviors between urban and rural burrowing owls, namely exploration, aggression toward predators and risk taking, in particular fear of humans. Our results show that rural birds were, in general, less fearless when facing humans, less explorative and less aggressive toward predators than their urban counterparts. Moreover, these behavioral traits did not vary independently of each other among these birds, as shown by the significant and positive correlations between them. Thus, rural individuals with larger flight initiation distances when facing a human also showed longer latencies in approaching a predator and a new food item. However, when the same relationships were investigated among urban birds, we found a significant covariance between fear of humans and exploration and between latency to approach a predator and a new feeding source, suggesting that selection pressures acting during urban invasion and while living in the city may be dismantling the behavioral correlations existent among individuals occupying more natural environments.

Two main hypotheses have been proposed to explain the existence of behavioral correlations. The *constraint hypothesis* assumes that a shared proximate link between personality traits, e.g., physiological or genetic factors (Ketterson and Nolan, 1999; van Oers et al., 2005), is responsible for the behavioral correlations. Conversely, the *adaptive hypothesis* states that correlations between personality traits emerge when selection favors correlated behaviors in particular environments, this correlation being adaptive itself (Bell, 2005). Here, we show how behavioral syndromes detected among rural birds changed when considering their urban counterparts, supporting the adaptive nature of these behavioral correlations (Scales et al., 2011; Bókony et al., 2012).

In the most natural scenario, burrowing owls that tolerate humans at closer distances were also more reactive toward predators, and approached new feeding sources sooner, the last two behavior remaining independent. However, in urban environments, burrowing owls face different ecological pressures compared to those present in rural ones. On the one hand, they are constantly interacting with people (Carrete and Tella, 2011), which may represent an important selective factor. Indeed, differences in the fear of humans between urban and rural populations seem to arise as a consequence of selective pressures precluding frightened individuals from colonizing urban areas and/or favoring their emigration/mortality in urbanized areas (Evans et al., 2010; Møller, 2010; Carrete and Tella, 2011; Atwell et al., 2012). At the same time, predation pressure also differs between urban and rural areas, with predators being much less abundant and diverse in the former than in the latter (Rebolo-Ifrán et al., in press). Avoidance of predation is an important determinant of fitness in many animals (Godin, 1997; Ruxton et al., 2004). However, when isolated from predators, costly or no longer functional antipredator behaviors can be selected against or their effectiveness cannot be selected any more, as occurs in species occupying islands (Blumstein and Daniel, 2002) or individuals bred in captivity (Carrete and Tella, 2015). Thus, urban individuals living in a predator-free area where humans are constantly present can lose their antipredator behavior while breaking its links to the fear of humans. Moreover, urban birds can take advantage of the predator release effect by gaining higher breeding success than their rural counterparts (Rebolo-Ifrán et al., in press), such that the heritabilities of the fear of humans (Carrete et al., 2016) and antipredator behavior (Bize et al., 2012) can subsequently reinforce the behavioral differences between urban and rural populations initially resulting from selection. Whatever the mechanism causing the decoupling between fear of humans and antipredator behaviors in urban individuals, our results—in line with those obtained by Myers and Hyman (2016)-challenge previous interpretations of flight initiation distances (FID). While FID has been frequently used as an experimental measure of the response of individuals facing a predator (Díaz et al., 2013), our results suggest that FID actually measures their response toward humans which, in some but not in all cases (i.e., urban populations), correlates with responses to predators. Therefore, FID could be interpreted as a measure of fear of humans rather than a broader measure of response when facing predators.

Exploration positively correlated with fear of humans, more strongly among rural than among urban birds. However, exploration and antipredatory behavior were positively related among urban birds, but not among their rural counterparts. Exploration and fear of humans have been shown to be positively correlated in domestic red junglefowls (Agnvall et al., 2012), although studies relating exploration and antipredator behavior yielded inconclusive results (Jones and Godin, 2010; Couchoux and Cresswell, 2011). Explorative behaviors can be advantageous when invading novel habitats such as urban areas (Martin and Fitzgerald, 2005), neophilic/explorative phenotypes having advantages in exploiting novel food resources or food available in novel contexts (Tryjanowski et al., 2016). Recently, it has been shown that exploration is repeatable and heritable (Dingemanse et al., 2002; Mazué et al., 2015), with permanent environmental (maternal) effects explaining most of the resemblance between parents and offspring (Schuett et al., 2013). Thus, bold and explorative individuals that successfully raise chicks in both urban and rural sites may transmit these behaviors to their progeny, even increasing the correlation between both behavioral traits in urban areas.

A major complication of assessing the consequences of human disturbance on wildlife is that those consequences are not always directly visible. For instance, even if seemingly unaffected (i.e., behaviorally calm), animals might undergo profound physiological changes in response to anthropogenic disturbances, or even to the mere presence of human observers. Our results show that fear of humans, a key behavior during urban invasion, correlated with other behaviors (antipredatory and exploratory behaviors) in birds living in natural environments. However, changes in selection pressures faced by urban individuals change these relationships, maintaining only those that are adaptive themselves (Bell, 2005). These results support the idea that differences among populations in the strength or direction of a behavioral correlation imply that these correlations can change during the evolutionary divergence of populations and are mainly due to the adaptive nature of each behavioral trait involved, demonstrating how human disturbances have the potential to contribute to population differentiation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Argentinean wildlife agencies and the owners of private properties. The protocol was approved by the Ethic committee of CSIC.

AUTHOR CONTRIBUTIONS

MC and JT conceived the idea. JT and MC conducted field work. MC analyzed the data. JT and MC wrote the paper and discussed the results and commented on the manuscript.

ACKNOWLEDGMENTS

M. Santillán helped with field work. Field work was conducted under permits from Argentinean wildlife agencies and the owners of private properties, and was funded by Canal Sur TV, Fundación Repsol and Projects RYC-2009-04860, CGL2012-31888 and CGL2015-71378-P from MINECO (Spain). D. Blumstein and an reviewer greatly helped to improve the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 08 August 2017
doi: 10.3389/fevo.2017.00092

An Approach to Distinguish between Plasticity and Non-random Distributions of Behavioral Types Along Urban Gradients in a Wild Passerine Bird

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The impact of urbanization has been widely studied in the context of species diversity and life history evolution. Behavioral adaptation, by contrast, remains poorly understood because empirical studies rarely investigate the relative importance of two key mechanisms: plastic responses vs. non-random distributions of behavioral types. We propose here an approach that enables the simultaneous estimation of the respective roles of these distinct mechanisms. We investigated why risky behaviors are often associated with urbanization, using an urban nest box population of great tits (*Parus major*) as a study system. We simultaneously and repeatedly quantified individual behavior (aggression and flight initiation distance) as well as environmental factors characterizing level of urbanization (numbers of pedestrians, cars and cyclists). This enabled us to statistically distinguish plastic responses from patterns of non-random distributions of behavioral types. Data analyses revealed that individuals did not plastically adjust their behavior to the level of urbanization. Behavioral types were instead non-randomly distributed: bold birds occurred more frequently in areas with more cars and fewer pedestrians while shy individuals were predominantly found in areas with fewer cars and more pedestrians. These novel findings imply a major role for behavioral types in the evolutionary ecology of urban environments and call for the full integration of among- and within-individual variation in urban ecological studies.

Keywords: among-individual variation, phenotypic plasticity, personality, urbanization, great tit, within-individual variation

Edited by:
Caroline Isaksson,
Lund University, Sweden

Reviewed by:
Wayne C. Zipperer,
USDA Forest Service, United States
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Specialty section:
This article was submitted to
Urban Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *30 March 2017*
Accepted: *24 July 2017*
Published: *08 August 2017*

Citation:
Sprau P and Dingemanse NJ (2017)
An Approach to Distinguish between
Plasticity and Non-random
Distributions of Behavioral Types
Along Urban Gradients in a Wild
Passerine Bird. Front. Ecol. Evol. 5:92.
doi: 10.3389/fevo.2017.00092

INTRODUCTION

Urbanization represents one of the fastest environmental changes of our times (United Nations, Department of Economic and Social Affairs, and Population Division, 2017) and poses novel challenges to wildlife (Sih et al., 2011). Urban habitats are characterized by a loss of natural resources (e.g., food) and increased anthropogenic levels of disturbance (e.g., pedestrian and vehicular traffic, and industrial noise). There is a growing body of literature describing how animal populations are affected by urbanization (Marzluff, 2008). In birds, for instance, breeding densities are often higher in urban compared to natural habitats (Beissinger and Osborne, 1982). Furthermore, several studies have reported advanced lay dates, smaller clutch sizes and decreased offspring production in urban compared to rural habitats (reviewed in Chamberlain et al., 2009). More recently, research has focussed on behavioral modifications in urban wildlife (Lowry et al., 2013). Yet, the mechanisms responsible for associations between behavior and level of urbanization remain largely unknown.

Phenotypic and environmental variation are often associated (Stearns, 1989). Relationships between phenotypes and environments can be caused by various distinct mechanisms such as phenotypic plasticity, differential settlement, and/or the selective (dis)appearance of distinct types of individuals (Dingemanse et al., 2010). Several studies have demonstrated behavioral plasticity in response to urbanization (Lefebvre, 1995; Seferta et al., 2001; Bouchard et al., 2007; Levey et al., 2009). Song birds such as nightingales (*Luscinia megarhynchos*) and great tits (*Parus major*) have been shown to adjust song amplitude (Brumm, 2004) and frequency (Slabbekoorn and den Boer-Visser, 2006) to background noise. Phenotypes, however, also vary among individuals. Repeatable differences among individuals have been demonstrated for a variety of behaviors such as aggressiveness, boldness and exploration (Bell et al., 2009; Garamszegi et al., 2012; Holtmann et al., 2017). These particular types of behavioral traits have recently also been explicitly linked to urbanization. For instance, urban male song sparrows (*Melospiza melodia*) are bolder and more territorial than their rural conspecifics (Evans et al., 2010). Boldness reflects the tendency of an individual to take risks (Wilson et al., 1994; Réale et al., 2007). Accordingly, bold individuals are often considered to cope better with risky and novel situations and may therefore be favored in highly disturbed areas such as cities.

Importantly, behavioral traits vary simultaneously among and within individuals, due to individual variation (aka "personality") and plasticity (Dingemanse et al., 2010). Therefore, associations between behavior and urbanization can similarly originate from multiple processes. This can be usefully illustrated by viewing behavior in terms of reaction norms (Nussey et al., 2007; Dingemanse et al., 2010), where each individual is characterized by a reaction norm intercept (representing its average behavior in the average environmental condition, or behavioral "type"), and a reaction norm slope, representing its level of response (plasticity) to changes in urbanization (**Figure 1**). Viewing behavior in this way clarifies that the relationship between behavior and urbanization is the result of the combined effects of phenotypic plasticity and non-random distributions of behavioral types over urban gradients. Previous studies have not distinguished between these two mechanisms in

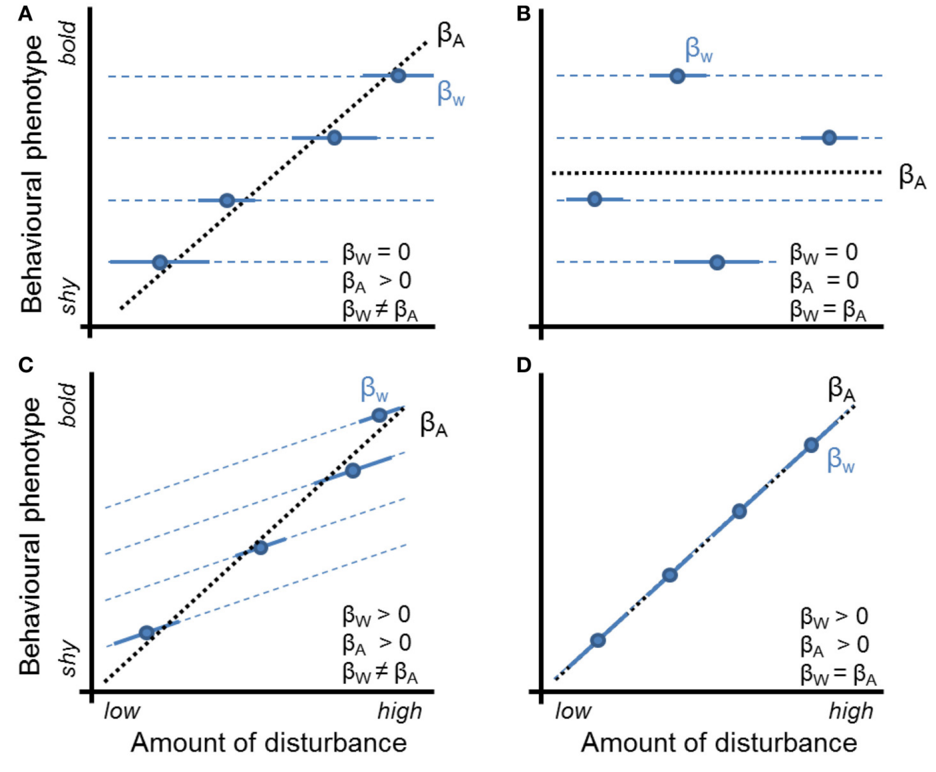

FIGURE 1 | Behavioral reaction norm plots, providing examples of possible relationships between anti-predator boldness (an exemplary behavioral phenotype) and an environmental gradient (e.g., amount of disturbance) among- vs. within-individuals. Each dot represents the average phenotype of an individual and each solid blue line represents its behavioral reaction norm over the range of environmental conditions (x-axis) that it occurred observationally. Among-individual variation in intercepts represents "personality variation" (*sensu* Dingemanse et al., 2010). Non-zero slopes represent within-individual plasticity (i.e., the observed relationship between y and x within the individual: β_W, blue lines). In contrast, blue dashed lines represent the hypothesized phenotype of each individual outside the range of environments in which it occurred observationally. The thick black dashed line represents the relationship between each individual's average behavior and average environment (β_A). Plots **(A–D)** show different scenarios for how behavior and the environment might be associated within and between individuals as detailed in the Introduction.

empirical demonstrations of relationships between behavior and urbanization.

We conceive four scenarios for how behavior and level of urbanization may be associated, though we recognize that further scenarios could be proposed. In the first scenario, individuals are non-randomly distributed over an urban gradient with respect to their reaction norm intercept (i.e., average behavior, or behavioral type) while they simultaneously do not respond plastically to changes in urbanization. This scenario is illustrated in **Figure 1A**, where individuals with high intercept values for boldness are more likely to occur in more disturbed areas, leading to a positive among-individual effect of disturbance on behavior (i.e., $\beta_A > 0$, A for "among") and a zero within-individual effect (i.e., $\beta_W = 0$, W for "within"). In the second scenario, individuals are instead randomly distributed over an urban gradient with respect to their intercept (i.e., behavioral type) while simultaneously not responding plastically to changes in urbanization. This scenario is illustrated in **Figure 1B**, showing a zero among- ($\beta_A = 0$) and a zero within-individual effect ($\beta_W = 0$). In the third scenario, individuals are both non-randomly distributed over an urban gradient with respect to their intercept while also responding plastically to changes in urbanization. This scenario is illustrated in **Figure 1C**, where individuals with high intercept values for boldness (i.e., bold behavioral types) are more likely to occur in more disturbed areas, leading to a nonzero among-individual effect of disturbance on behavior (i.e., $\beta_A > 0$), while individuals also alter their phenotype in response to changes in level of disturbance, leading to a nonzero within-individual effect (i.e., $\beta_W > 0$). In both scenarios 1 and 3, importantly, the effect of non-random distributions of behavioral types causes a difference between the among- and within-individual effect of the environmental gradient (van de Pol and Verhulst, 2006) (specifically, $\beta_W < \beta_A$ in both examples). In the fourth scenario, individuals do not differ in intercept (i.e., all have the same behavioral type) but they do respond plastically to changes in urbanization. This scenario is illustrated in **Figure 1D**, where individuals up-regulate their boldness with increasing levels of disturbance (i.e., $\beta_W > 0$), and where among-individual relationships between boldness and disturbance exist solely because each individual happens to experience a narrow range of environmental conditions, causing among-individual variance in the average level of disturbance experienced (i.e., $\beta_A > 0$). As a single mechanism causes variation at both levels, environmental effects on behavior do not differ between hierarchical levels (van de Pol and Verhulst, 2006) (i.e., $\beta_A = \beta_w$ in **Figure 1D**). In summary, our sketch of alternative scenarios clarifies that conclusions regarding non-random distributions of behavioral phenotypes over urban gradients warrants repeated observations of both behavior and environmental factors such that level-specific effects of urbanization on behavior can be statistically teased apart (**Figure 1**).

We investigated the mechanisms causing relationships between urbanization and behavior in great tits. We simultaneously and repeatedly quantified an individual's behavior and environment. That is, we repeatedly exposed the same individual to territorial intrusion experiments to measure aggressiveness (Araya-Ajoy and Dingemanse, 2014) and to flight initiation distance experiments to measure boldness (Blumstein, 2003), while quantifying aspects of urbanization during each test (numbers of pedestrians, cars and cyclists). This enabled us to statistically distinguish within-individual plasticity from patterns of non-random distributions of behavioral types (**Figure 1**). Human activity is known to alter behavioral phenotypes in animals (Fernandez-Juricic and Schroeder, 2003; Slabbekoorn and Peet, 2003). Based on previous literature (Brumm, 2004; Evans et al., 2010), we expected that birds would plastically up-regulate aggressiveness and down-regulate flight initiation distance with increasing levels of disturbance, and that more aggressive and bolder individuals would occur predominantly in areas with higher levels of disturbance (**Figure 1C**).

METHODS

Study Site

We studied an urban nest box population of great tits consisting of 157 nest boxes along urban-to-rural gradients in the city of Munich, Germany (48° 8′ 6.45" N 11° 34′ 55.132" E) during the breeding seasons of the years 2014 and 2015 (Sprau et al., 2016). Nest boxes were located in the entire city area of Munich (20×27 km^2) and covered a large range of human disturbance from highly disturbed habitats in the city center to relatively undisturbed habitats in sub-urban areas. All nest boxes were checked at least once per week from mid-March onwards and key fitness components quantified (e.g., lay date, clutch size, brood size, and number of fledged offspring). When the nestlings were 7–9 days old, parents were caught with a spring trap in the nest box, measured, and ringed if not previously captured.

Experimental Protocol

We quantified two behaviors both of which were assayed repeatedly for the same set of breeders: aggressiveness and boldness (measured as flight initiation distance; FID). Simulated territorial intrusions (i.e., aggression tests) were performed for all first broods found in our nest boxes by simultaneously presenting the male owner with a visual stimulus (a taxidermic mount of a male great tit) and an acoustic stimulus (a playback song) (as detailed in reference Araya-Ajoy and Dingemanse, 2014). In each year, each male was subjected to three aggression tests (between 7.30 and 15.00 h) when its mate was in the egg-laying phase (1, 3, and 5 days after its first egg was observed). The taxidermic mount was presented 1 m away from the subject's nest-box at 1.2 m height. We subsequently recorded the behavior of the subject for a period of 3 min after it had entered a 15-m radius around the nest box. Details of the experimental set up, and assayed behaviors, are provided in reference Araya-Ajoy and Dingemanse (2014). In short, an aggressive response was characterized by intensive alarm calling, approach to the stimulus, and, in the most extreme case, jumping and pecking of the cage that protected the mount. Here and elsewhere (Araya-Ajoy et al., 2016; Araya-Ajoy and Dingemanse, 2017) we used the subject's minimum approach distance to the mount as a measure of aggressiveness because previous work implied that this behavior represents a reliable predictor of the intensity of aggression. Subjects that did not arrive within 10 min were scored as non-responsive, and those data were thus not taken forward for analyses (Araya-Ajoy and Dingemanse, 2014, 2017). We used six mounts and 11 playback song stimuli (recorded from German great tits populations; sampling frequency: 44.1 kHz; resolution: 16 bit). One mount and one song stimulus (broadcasted with a Ligno Xtatic V2 Digital Soundsystem) were randomly allocated to each test (following Araya-Ajoy and Dingemanse, 2014). Songs were played back at 85 dB (measured at one meter from the sound source (Brumm, 2004). One of nine observers performed the experiment at a distance of 15 m. We performed 333 aggression tests with 107 unique males.

The occupants of each nest box were also subjected to three FID-tests during the nestling phase of their first brood (10, 12 and 14 days after the nestlings had hatched). FID-tests were conducted between 8:00 and 16:00 h. After identifying (by color ring combination) the focal individual as the male or female parent, FID was measured by walking at a constant speed toward a bird from a starting distance of 15 m away from the nest box (Blumstein, 2003). We used a laser distance meter (Bosch PLR 25) to quantify distance to the mount during the aggression tests, and start and flight initiation distance. Overall, we performed 308 flight initiation tests, on 59 females and 54 males. Note that because of nest failure prior to the onset of the FID-tests, the number of FID-tests is lower than the number of the aggression tests. Five and four individuals were assayed, respectively, for aggressiveness and flight initiation distance in both years of study. This study was carried out in accordance with the ethical guidelines of the Tierschutzgesetz (TierSchG, German animal protection law), and approved by the Regierung Oberbayern (55.2-1-54-2532.2-7-07).

The level of urbanization at each nest box was quantified by measuring human activity (the number of pedestrians, cyclists and cars, Table S1). Human activity was measured within a range of 15 m from the nest box for 2 min following each behavioral assay (detailed above).

Statistical Analyses

We performed a principal component analysis (PCA) with varimax rotation ("prcomp" function of Package "stats" version 3.1.27 of R version 3.1.2) to ask whether our indexes of human activity (number of pedestrians, bikes and cars) could be summarized into a single axis (principal component) representing an urban gradient (Table S1).

We fitted univariate mixed-effect models to simultaneously estimate sources of variation in behavior within and among individuals (Dingemanse and Dochtermann, 2013) ("lmer" function of Package "lme4"version 1.1-10 of R). We investigated sources of variation in each of the two focal behaviors (aggressiveness and flight initiation distance) separately. Random intercepts were included for subject and observer identity, enabling us to partition the total variance into variance attributable to individual, observer, and within-individual-within-observer residual. Start distance (covariate: only for flight initiation distance; meter), year (factor: 2014 vs. 2015), time of day (factor: morning vs. afternoon trial), and test sequence (covariate; within-individual test-day number; first vs. second vs. third test day) were fitted as fixed effects. The PCA resulted in two components (PC1 and PC2) describing two orthogonal axes of human activity. As detailed in the Introduction (**Figure 1**), we considered that environmental effects on behavior could vary within and among individuals (van de Pol and Wright, 2009). Specifically, a within-individual effect of the environment on behavior represents evidence for within-individual phenotypic plasticity, while the difference between among- and within-individual effects represents statistical evidence for non-random distributions of behavioral types over environments (van de Pol and Wright, 2009; Dingemanse and Dochtermann, 2013). We thus calculated (1) each individual's average value ($\bar{x}_j$) for each of the two environmental variables (PC1 and PC2) as well as (2) each observation's deviation of these individual average values ($x_{ij} - \bar{x}_j$) effects, and fitted both as part of the statistical model detailed above (van de Pol and Verhulst, 2006; van de Pol and Wright, 2009). We then reformulated the model to test whether the effect of the focal environmental axis differed between the within- and among-individual levels. Therefore, instead of fitting $\bar{x}_j$ and ($x_{ij} - \bar{x}_j$) and each individual's average value ($\bar{x}_j$) instead, such that the former estimated the within-individual effect (β_W) (see formulae 3 in van de Pol and Wright, 2009). This enabled us to statistically assess the evidence for non-random distributions of behavioral types over environments (which would be the case provided that ($\beta_A - \beta_W$) $\neq 0$). We note that the within-subject centering approach used here has been criticized because values of $\bar{x}_j$ are estimated with error, which causes estimates of the among-individual slope (β_A) to be biased toward the within-individual slope (β_W) in datasets with a low numbers of repeats per individual (Ludtke et al., 2008), such as ours. This means that differences (Δ) between among- and within-individual effects (i.e., $\beta_A - \beta_W$), as well as associated levels of significance, represent conservative estimates. We assumed a Gaussian error distribution for aggression and boldness, which was confirmed by visual inspection of model residuals. All covariates were further centered on their mean value (Kreft et al., 1995). For each specified relationship, we calculated the parameter estimate with its associated 95% credible interval (calculated using the function "quantile"; Package "stats"version 3.1.2 in R). Credible intervals not including zero indicate statistical significance (i.e., $p < 0.05$) in the frequentist's sense.

RESULTS

Axes of Environmental Variation

PCA applied to summarize variation in the number of pedestrians, cyclists, and cars, resulted in two significant principle components (PCs) that jointly explained 81% of the variance (Table S2). PC1 (Eigenvalue: 1.40; explained variance: 47%) loaded negatively on the number of bikes, cars, and pedestrians (Table S1); high values of PC1 were thus indicative of lower levels of human activity in general. PC2 (Eigenvalue: 1.04; explained variance: 35%) loaded negatively on the number of cars (−0.76) but positively on the number of pedestrians (0.65), and thus seemed to differentiate between streets differing in the primary means of transportation (e.g., larger streets suitable for cars vs. smaller streets suitable for pedestrians).

Phenotypic Plasticity and Non-random Distributions of Behavioral Types

Our analyses of the sources of variation in behavior, which focussed on the simultaneous estimation of within-individual (β_W) and among-individual (β_A) effects of environmental variables related to urbanization (**Figure 1**), demonstrated that individuals did not plastically adjust their aggressiveness nor their flight initiation distance (FID) in response to within-individual-among-day variation in PC1 or PC2 (Table 1). All models controlled for variation induced by aspects of the experimental design (starting distance, test sequence, time of day, and year), which were generally not of major importance (**Table 1**).

As a next step, we re-parameterised our models to directly estimate the difference (Δ) between the among- and within-individual effects ($\beta_A - \beta_W$) of each focal gradient as a test for non-random distributions of behavioral types (van de Pol and Verhulst, 2006; van de Pol and Wright, 2009). This analysis produced strong evidence for non-random distributions of behavioral types with respect to FID because the difference (Δ) in effect of PC2 among- vs. within-individuals was associated with 95% CIs that did not overlap zero (mode: 0.28; 95% CIs: 0.03, 0.56; **Table 1**). The analysis implied that individuals that allowed observers to approach closer (i.e., "bolder" birds) were overrepresented in areas with more cars and fewer pedestrians, whereas "shyer" birds were more likely found in areas with fewer cars and more pedestrians (**Figure 2**). By contrast, there was no strong evidence for non-random distributions of aggressiveness types as the 95% CIs overlapped zero for all tested differences (Δ) between the within- and among-individual levels for aggressive behavior (**Table 1**). Notably, there was some support for non-random distributions of aggressiveness types with respect to PC1 as the CIs associated with the difference (Δ) between levels for this gradient slightly overlapped zero (95% CIs: −0.001, 0.38) (**Table 1**), suggesting that aggressive types were perhaps overrepresented in areas with more cyclists, cars, and pedestrians (**Table 1**).

DISCUSSION

Our study revealed that behavioral types, with respect to flight initiation distance, were non-randomly distributed over an urban gradient while individuals did not plastically adjust their behavior in response to changes in urban gradients experienced across repeated observations (days). Bolder birds (i.e., birds that could be approached by humans closely) were overrepresented in areas with more cars and fewer pedestrians, whereas shyer birds were more likely found in areas with fewer cars and more pedestrians (**Figure 2**). These findings imply that associations between behavior and urban gradients vary across hierarchical levels, in this case within and among individuals, and that meaningful conclusions regarding non-random distributions of "personality" types over urban environments thus require repeated measures study designs and variance partitioning approaches, as applied in this study.

TABLE 1 | Sources of variation in boldness and aggressiveness.

	Boldness (Flight initiation distance)		**Aggressiveness (Minimal approach distance)**	
Fixed effects	**Estimate**	**95% CIs**	**Estimate**	**95% CIs**
Intercept	−0.57	−0.93, −0.19	0.05	−0.22, 0.34
PC1				
Within individuals	−0.14	−0.34, 0.06	−0.06	−0.23, 0.13
Among individuals	0.07	−0.08, 0.22	0.13	−0.02, 0.27
Δ (Among−within)	0.19	−0.01, 0.42	0.19	−0.001, 0.38
PC2				
Within individuals	−0.12	−0.41, 0.16	0.12	−0.23, 0.46
Among individuals	0.17	0.04, 0.30	−0.05	−0.22, 0.10
Δ (Among - within)	0.28	0.03, 0.56	−0.17	−0.55, 0.17
Start distance	−0.11	−0.22, 0.01	n.a.	n.a.
Year	−0.13	−0.57, 0.30	0.12	−0.15, 0.38
SEQUENCE (FIRST TEST AS REFERENCE)				
Second test	0.25	−0.04, 0.54	−0.05	−0.37, 0.23
Third test	0.23	−0.07, 0.56	−0.08	−0.4, 0.21
Time of day	−0.09	−0.26, 0.07	0.04	−0.09, 0.17
Random effects	**σ^2**	**95% CIs**	**σ^2**	**95% CIs**
Individual	0.013	0.01, 0.02	0.017	0.01, 0.02
Observer	0.20	0.12, 0.42	0.04	0.02, 0.08
Residual	0.65	0.56, 0.84	0.91	0.80, 1.14

We test here for within- and among individual effects of cyclists, cars, and pedestrians (summarized in PC1 and PC2, see Table S1). All models control for variation induced by various aspects of the experimental design (starting distance, test sequence, time of day, and year) and included random intercepts for subject individual and observer identity. We also present the difference (Δ) between among and within-individual effects derived from the same statistical model reformulated following reference (van de Pol and Wright, 2009). Parameter estimates and are provided with 95% credible intervals (CIs).

Human activity is known to affect behavior and abundance of animals (Gill et al., 1996; Fernandez-Juricic, 2000); there is considerable evidence that birds living in highly disturbed areas are more tolerant of humans than their conspecifics living in less disturbed areas (Moller, 2008; Evans et al., 2010; Scales et al., 2011; Clucas and Marzluff, 2012). In a similar vein, recent studies have shown that birds in more disturbed environments may display higher levels of territorial and defensive behaviors (Cilento and Jones, 1999; Evans et al., 2010; Fokidis et al., 2011; Scales et al., 2011). Most of these studies, however, have solely focused on differences in behavioral phenotypes at the population level. Researchers have only recently begun to assess individual variation in the context of urban ecology (Miranda et al., 2013). In this study we shed new light on relationships between urbanization and behavioral phenotypes by partitioning variation in behavioral phenotypes into within- and among-individual components; this enabled us to investigate the relative roles of distinct mechanisms causing such associations. The applied approach allowed us to simultaneously assess whether individuals responded plastically to urban gradients and whether behavioral types were non-randomly distributed over urban gradients (**Figure 1**). Our findings reveal that behavioral types were indeed non-randomly distributed along a key axis of

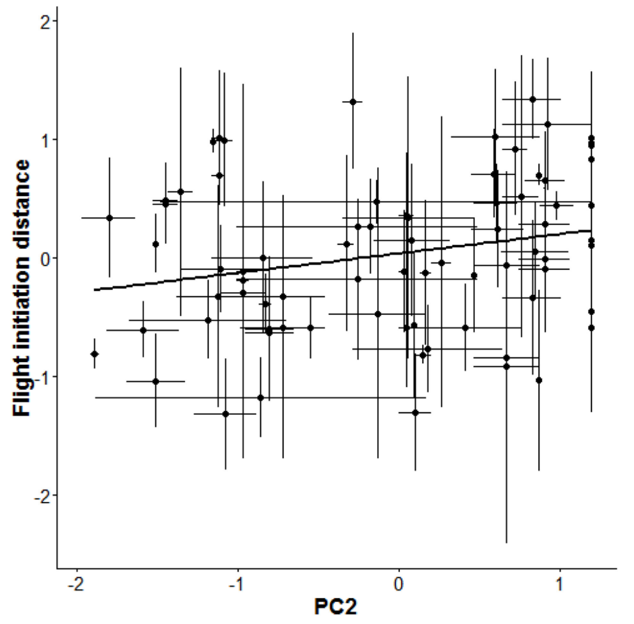

FIGURE 2 | Among-individual variation in flight initiation distance (FID) as a function of the compound environmental gradient PC2 that combines number of pedestrians and cars (more positive loadings represent more pedestrians and lower loadings more cars). As individuals do not plastically respond to changes in PC2 (**Table 1**), the relationship implies a non-random distribution of individual-level behavioral type along this urban gradient. The thick black line presents the regression line between FID and PC2 based on the fitted GLMM (**Table 1**). Shown are average scores ± SE.

urbanization (**Figure 2**): bolder individuals, i.e., birds that could be approached by humans closely, were predominantly found in areas with more cars, whereas shyer individuals were found more often in areas with more pedestrians. The documented effect might be explained by sensory constraints caused by traffic noise. We tested this *post-hoc* explanation by analyzing noise measurements that were taken during each test (detailed in the Supplementary Material), which demonstrated that noise neither affected FID nor aggression (Table S3).

Non-random distributions of behavioral types along environmental gradients have previously been documented in eastern chipmunks (*Tamias striatus*) where more explorative and docile individuals occupy habitats that experience the highest rates of human disturbance (Martin and Réale, 2008). Accordingly, bold animals might have an innately higher disturbance tolerance level than shy individuals. In urban environments, cars usually impose high risks as evident from high numbers of road kills (Spellerberg, 1998; Benitez-Lopez et al., 2010). In contrast, pedestrians may impose lower disturbance levels because birds quickly habituate to humans. Because bold individuals are often considered to cope better with risky situations (Smith and Blumstein, 2008), bold phenotypes in our study may be selected for in high-traffic environments because bolder individuals are more successful in colonizing such environments. Alternatively, bold phenotypes might be outcompeted by shy phenotypes for preferred types of territories and hence settle in these types of areas. It is also possible that prolonged exposure to particular environments permanently affects an individual's behavioral type, resulting in birds becoming bolder in high-traffic environments by means of developmental or other forms of plasticity with permanent effects. Experimental tests are therefore now required to address whether the non-random distributions of behavioral phenotypes documented in our study were caused by non-random settlement, habitat- and type-specific survival, or irreversible plasticity in response to long-term exposure to urban environmental effects. Similarly, we studied a very specific component of the urban environment, focusing on human traffic. Whether the relationships between behavior and aspects of urbanization shown in this paper apply generally to other components of urbanization remains to be evaluated by future studies.

Surprisingly, great tits did not show any sign of a plastic response to day-to-day variation in urban environmental gradients. It is possible that exposure to high levels of human disturbance for prolonged periods triggers habituation (see above) and consequently reduces short-term plastic responses. Such effects may suggest that birds experience only minor fluctuations in environmental conditions, and that each bird's characteristic level of urbanization is relatively stable. In urban environments, such a scenario seems unlikely as the numbers of cars, pedestrians and cyclists, in fact, varied substantially, for instance, between workdays and weekends. Temporal variation between workdays and weekends has in fact previously been shown to cause plastic adjustments in other behaviors (Brumm, 2004). We therefore conclude that phenotypic adjustments to day-to-day variation in human disturbance might well differ between behavioral traits, perhaps because the costs or limits associated with phenotypic plasticity are trait-specific (DeWitt et al., 1998; Auld et al., 2010). Our recent studies on aggressiveness, for example, demonstrated that this particular behavior (which birds did not plastically adjust to changes in human disturbance; **Table 1**) is also not plastically adjusted to population density (Araya-Ajoy and Dingemanse, 2017) or perceived predation risk (Abbey-Lee et al., 2016). Overall, the lack of evidence for within-individual plasticity suggests that its role in urban ecology may be more modest than previously anticipated (Lowry et al., 2013). At the same time, urbanization seems to drive non-random distributions of behavioral types via mechanisms yet to be revealed. Our study thereby demonstrates the importance of partitioning behavioral variation across hierarchical levels (Han et al., 2016; Moirón et al., 2016; Nicolaus et al., 2016), both in urban and other behavioral ecological studies, and the novel insights that may be gained by doing so.

In conclusion, we showed for great tits breeding in the city that behavioral types were non-randomly distributed over an urban environmental gradient. Based on these findings, future research should investigate whether non-random distribution of types is caused by selective appearance (i.e., differential settlement), selective disappearance (i.e., natural selection) or urbanization-related behavioral modification (i.e., developmental or other forms of irreversible plasticity).

AUTHOR CONTRIBUTIONS

PS and ND conceived and designed the study, conceived the statistical approach, and wrote the manuscript

ACKNOWLEDGMENTS

We thank past and current members, as well as field assistants and students of the Behavioral Ecology Group (Ludwig-Maximilians University Munich) and the research group Evolutionary Ecology of Variation (Max Planck Institute for Ornithology), as well as all citizen scientists for input, discussion, and/or help with data collection. Werner Schimmel and Dietfried Molter are acknowledged for technical support and Petri Niemelä for stimulating discussion. PS was funded by the Deutsche Forschungsgemeinschaft (SP 1450/3-1) and ND by the Max Planck Society.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00092/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 21 June 2017
doi: 10.3389/fevo.2017.00067

First to Flush: The Effects of Ambient Noise on Songbird Flight Initiation Distances and Implications for Human Experiences with Nature

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Throughout the world, birds represent the primary type of wildlife that people experience on a daily basis. However, a growing body of evidence suggests that alterations to the acoustic environment can negatively affect birds as well as humans in a variety of ways, and altered acoustics from noise pollution has the potential to influence human interactions with wild birds. Birds respond to approaching humans in a manner analogous to approaching predators, but the context of the interaction can also greatly influence the distance at which a bird initiates flight or escape behavior (i.e., flight initiation distance or FID). Here, we hypothesized that reliance on different sensory modalities to balance foraging and threat detection can influence how birds respond to approaching threats in the presence of background noise. We surveyed 12 songbird species in California and Wyoming and categorized each species into one of three foraging guilds: ground foragers, canopy gleaners, and hawking flycatchers and predicted FIDs to decrease, remain the same and increase with noise exposure, respectively. Contrary to expectations, the canopy gleaning and flycatching guilds exhibited mixed responses, with some species exhibiting unchanged FIDs with noise while others exhibited increased FIDs with noise. However, FIDs of all ground foraging species and one canopy gleaner decreased with noise levels. Additionally, we found no evidence of phylogenetic structure among species' mean FID responses and only weak phylogenetic structure for the relationship between FIDs and noise levels. Although our results provide mixed support for foraging strategy as a predictor of bird response to noise, our finding that most of the species we surveyed have shorter FIDs with increases in noise levels suggest that human observers may be able to approach ground foraging species more closely under noisy conditions. From an ecological perspective, however, it remains unclear whether these mixed responses translate into lost foraging opportunity for hypervigilant birds that flee a threat too soon or greater predation risk due to impaired surveillance for those that only respond once approaching threats are near.

Keywords: anthropogenic noise, birdwatching, escape behavior, predation risk, vigilance, masking, threat detection

OPEN ACCESS

Edited by:
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Lund University, Sweden

Reviewed by:
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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *20 January 2017*
Accepted: *06 June 2017*
Published: *21 June 2017*

Citation:
Petrelli AR, Levenhagen MJ, Wardle R, Barber JR and Francis CD (2017) First to Flush: The Effects of Ambient Noise on Songbird Flight Initiation Distances and Implications for Human Experiences with Nature. Front. Ecol. Evol. 5:67. doi: 10.3389/fevo.2017.00067

INTRODUCTION

Anthropogenic noise encroaches on many natural landscapes (Barber et al., 2011; Francis et al., 2011; Lynch et al., 2011; Mennitt et al., 2013). A growing body of research indicates such noise can detrimentally affect wildlife (Barber et al., 2010; Francis and Barber, 2013; Shannon et al., 2016b) and might create a negative feedback process that degrades humans' experiences of nature (Francis et al., in review). In the context of birdwatching, an increasingly popular recreational activity (La Rouch, 2003; Carver, 2013), the ability to approach birds in the wild is a valued human experience that may be threatened by anthropogenic noise. Although there is typically a limit to how close a human can approach a wild animal before the animal initiates an escape, the distance at which flight is initiated varies across taxa and could depend on the animal's acoustic environment.

The acoustic environment serves as a critical medium through which many species, including humans, interact with their surroundings. Humans are motivated by natural sounds, such as bird songs and sounds, to seek out and experience natural places (Haas and Wakefield, 1998; Marin et al., 2011). Recent work has also demonstrated that listening to birdsong has the potential to enhance personal experiences with nature (Newman et al., 2013), improve stress recovery (Ratcliffe et al., 2013), and renew cognitive abilities after mental exertion (Abbott et al., 2016). In addition to these benefits to casual nature-seekers, birdsong is a valued tool for birdwatchers, who can acoustically localize wild birds for viewing or identification. However, in areas of elevated background sound, an observer's ability to detect birds is constrained by masking, the process through which background noise interferes with the perception of an acoustic signal (Pacifici et al., 2008; Ortega and Francis, 2012). Like humans, masking by noise can interfere with birds' abilities to detect and discriminate biologically relevant cues. For example, elevated ambient noise may influence how birds detect and respond to the threat of an approaching predator or human observer, which they perceive in the same manner (Frid and Dill, 2002). Thus, from the human perspective, where the quality of seeing and hearing a bird can depend upon the proximity of an approach, changes to the acoustic environment could indirectly influence the quality of the human experience with birds through changes in bird behavior.

Broadly speaking, anthropogenic noise can affect wildlife in many ways beyond threat detection (Francis and Barber, 2013). Previous research has demonstrated that increased noise can lead to decreased reproductive success (Halfwerk et al., 2011; Kight et al., 2012; Mulholland, 2016), impact community structure and ecological interactions (Francis et al., 2009), and degrade habitat quality (Francis et al., 2009; McClure et al., 2013; Ware et al., 2015). Most relevant to this study are the many ways that noise affects avian behavior (Shannon et al., 2016b), especially aspects of risk assessment and antipredator behavior. Karp and Root (2009) found that free-living hoatzins (*Opisthocomus hoazin*) increased alertness and flush more quickly when tourists approach while conversing loudly compared to silent approaches. Samia and Blumstein (2015) suggested that escape behavior in birds is largely explained by the flush early and avoid the rush (FEAR) hypothesis, which posits that prey will flee soon after predator detection to avoid costs associated with monitoring the predator. Considering this finding, it might be expected that birds should flush quickly unless threat detection is delayed or otherwise impaired due to interfering circumstances.

Flushing behavior is commonly measured as the distance between an observer and an animal when it flushes (termed "flight initiation distance," hereafter FID) and is often used as a proxy for a species' tolerance of predators as well as the presence of humans. Previous studies have determined that species identity, starting distance, individual body size, and vegetation cover are all important predictors of FID (Fernández-Juricic et al., 2002; Blumstein, 2003; Blumstein et al., 2003). The influence of noise on FID has been investigated in two non-avian systems. Chan et al. (2010a,b) demonstrated that Caribbean hermit crabs (*Coenobita clypeatus*) are slower to respond to an intruder when noise is played during the approach. Based on this finding, Chan et al. (2010a) proposed the distracted prey hypothesis, which suggests that animals have finite attention and become distracted from ecologically relevant cues when a stimulus such as background noise occupies some of that attention. Shannon et al. (2016a) found the opposite outcome; black-tailed prairie dogs (*Cynomys ludovicianus*) flushed more quickly when a human intruder approached under higher sound levels generated from speakers broadcasting roadway noise. The prairie dogs in this study were hypervigilant, committing more time to detecting potential threats through visual surveillance when background sound levels were high (Shannon et al., 2016a). Birds are also known to increase visual alertness when their auditory abilities are impaired by ambient noise. In a study of how noise influences reproductive success, Meillère et al. (2015) found that free-living house sparrows (*Passer domesticus*) flee their nests more quickly (i.e., increased FID) in disturbed, noisy areas compared to quiet, control areas, possibly due to increased visual vigilance. Quinn et al. (2006) demonstrated that chaffinches (*Fringilla coelebs*) in a laboratory setting spent more time visually scanning for predators than actively foraging during playbacks of white noise, a stimulus not found in nature, when compared to quiet conditions. Ware et al. (2015) confirmed these findings in a study of white-crowned sparrows (*Zonotrichia leucophrys*) foraging during playbacks of traffic recordings, a stimulus many free-living birds experience. However, these studies introduced an acute, high intensity noise stressor to measure short-term changes in vigilance and, therefore, provide limited insight to how chronic ambient noise influences the daily lives of free-living birds. Furthermore, to our knowledge, there are no previous studies that have examined how hypervigilance exhibited in captive birds translates to detection of and response to approaching threats in nature. Thus, we sought to test whether study of bird FIDs can provide evidence to clarify the contradicting hypotheses of distraction and hypervigilance. Furthermore, we sought to examine FID responses in light of the different avian feeding ecologies that have the potential to influence detection of approaching threats via auditory and visual surveillance.

Here, we categorize our study species into three foraging guilds based on foraging behavior: ground foragers, canopy gleaners, and hawking flycatchers. We then use existing literature on the sensory ecology linked to each foraging strategy to formulate predictions of how ambient noise might influence FIDs for our foraging guilds.

Most birds rely on vision for both foraging and vigilance and can maintain vigilance while searching for food through peripheral vision and frequent movement of the head and eyes to maximize the visual field (Lima and Bednekoff, 1999; Fernández-Juricic et al., 2008). These behaviors, as well as adapted visual fields, are thought to be primarily determined by feeding ecology (Martin, 2007). Ground foraging birds generally have wide lateral visual fields and engage in frequent head movements to compensate for time spent head-down looking for food (Fernández-Juricic et al., 2008, 2010, 2011). Thus, we hypothesize that ground foraging species rely heavily on acoustic cues for threat detection while foraging and predict that they are more susceptible to the effects of masking in noisy conditions and will exhibit decreasing FIDs as noise increases (**Figure 1**).

To our knowledge, no previous study has specifically investigated the sensory ecology of species that glean arthropods and fruit in the canopy. However, like the ground foragers, canopy gleaners also rely heavily on vision to both forage and scan for predators. Other potential mechanisms of predator detection, such as con- or hetero-specific alarm calls, might also be influenced by background noise (e.g., Templeton et al., 2016) and could also contribute to slower responses of these species. Yet, because these species often forage high in the canopy rather than on the ground, we predict either a weak negative influence of noise or no change in FID in response to humans approaching at the ground level (**Figure 1**).

In contrast, flycatching species that sally out from a perch to catch flying insects on the wing visually scan for prey. Gall and Fernández-Juricic (2009) determined that the vision of the flycatching black phoebe (*Sayornis nigricans*) is primed for tracking active prey in three dimensions, but, to our knowledge, no studies have examined audition of any flycatching species in the context of foraging. With their constant visual vigilance for seeking prey, we hypothesize that these species may also be able to detect approaching threats as an epiphenomenon of scanning for volant prey. Based on this foraging strategy and the FEAR hypothesis (Samia and Blumstein, 2015), we predict flycatching species exhibit unchanged FIDs because they would flush upon first visual detection of an approaching threat while visually scanning for prey regardless of noise level (**Figure 1**). Alternatively, if flycatching species also compensate for reduced auditory surveillance for threats with increased visual vigilance, FIDs may increase with noise levels because more frequent visual scans for threats would lead to earlier detections (**Figure 1**). It is important to note that our expectation of varied responses for different foraging guilds is speculative due to limited literature on the visual fields and sensory ecology of species outside the ground foraging guild. However, we ultimately focus on the implications of ambient noise altering avian behavior in the context of human-wildlife interactions. Importantly, changes in FID for common songbird species in response to noise might influence how close human observers can approach wild birds, and thus, alter the quality of an experience with wildlife and nature.

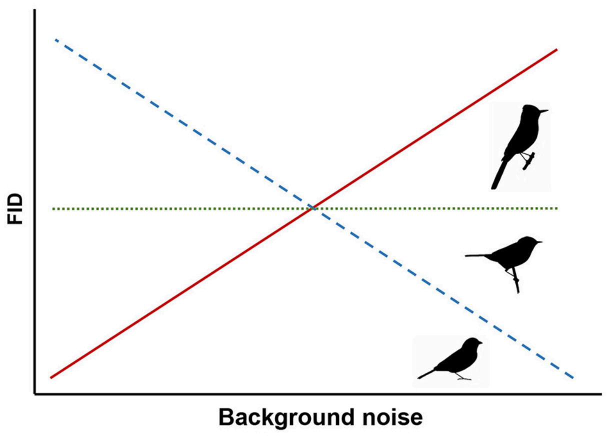

FIGURE 1 | Predicted responses for three foraging guilds of songbirds. We expect ground foragers to have shorter FIDs in noise (dashed line), canopy gleaners to have shorter or unchanged FIDs (dashed or dotted lines), and flycatchers to have unchanged or longer FIDs in noise (dotted or solid lines).

MATERIALS AND METHODS

We studied avian FIDs in relationship to background noise in urban parks and protected natural spaces throughout San Luis Obispo County, Muir Woods National Monument, California, and Grand Teton National Park, Wyoming. Our sites represented a range of background sound levels to capture natural variation in ambient noise and we did not manipulate the acoustic environment during our observations. Following previously described methods (e.g., Blumstein et al., 2003), a single observer located an individual bird and recorded the species, time, and initial distance between the observer and the target bird (Starting Distance) using an optical range finder (Nikon Aculon, Nikon Vision Co., Japan; TruPulse 360 R, Laser Technology, Inc., Colorado, USA). We targeted birds that were foraging, preening, or otherwise undisturbed by the intruder at the starting distance and not interacting with con- or hetero-specifics. While looking directly at the bird, the observer walked at a standard rate of 0.5 m/s along a straight path toward the target bird and dropped a marker at the distance where the bird flew away from the intruder (Flight Initiation Distance, measured as the distance between this marker and the bird's last perch). Immediately following the bird's escape, the observer measured time-averaged sound levels (Leq; A-weighted L_{eq}, *fast response*, re. 20 μPa) for at least 1 min at the bird's last perch with a Larson Davis 824 or 831 sound pressure meter or a MicWi436 (MicW Audio, China) microphone paired with the SPLnFFT Sound Meter v6.2 iPhone application (FL's Audio Apps, France), a measurement kit equivalent to a type two sound level meter (Kardous and Shaw, 2014). We also measured wind speed with a Kestrel 4,000 weather meter (Kestrel Meters, USA). During the sound pressure level measurement, the observer scanned the surroundings and counted any pedestrians and their distances to the bird's last perch. We utilized pedestrian activity and whether each site was predominately human-modified (e.g., presence of buildings and other built surfaces, heavily-managed urban parks) to categorize each site as either a predominantly developed or natural area. Because almost all trial locations were within 0.5 km of roadways, sound levels were primarily from anthropogenic sources and did not systematically differ between developed [49.35 ± 4.70 SD dB(A)] and natural areas [50.26 ± 9.55 SD dB(A); Welch's two sample *t*-test, $t = 0.87$, $df = 155.66$, $p > 0.38$]. The observer then used a surveyor's tape or laser range finder to measure the FID, the distance between the bird's last perch to the dropped marker. If the bird was perched above the ground, we measured the Euclidean distance as the square root of the sum of the squared horizontal distance and squared perch height (Møller et al., 2015). Finally, the observer categorized surrounding vegetation as open, medium, or dense. To avoid sampling the same individual more than once, the observer moved at least 250 m from the first survey before locating a subsequent individual. We also visited sites throughout each location only once to avoid resampling individuals.

Data Analysis

We used a $\log_{10}$ transformation on all distance data to normalize their distributions. We assigned each of the surveyed species to one of three foraging guilds based on foraging behavior: ground foragers, canopy gleaners, and hawking flycatchers (Ehrlich et al., 1988). Because we were most interested in the influence of noise on FIDs, using the entire dataset we first calculated adjusted FIDs as the residuals of a linear regression model where raw FIDs were explained by vegetation category and starting distance, two variables known to strongly influence FID (e.g., Fernández-Juricic et al., 2002; Blumstein, 2003; Blumstein et al., 2005). We then used linear mixed effect models using the *lme4* package (Bates et al., 2014) in R with vegetation and starting distance-corrected FID (henceforth adjusted FID) for each foraging guild as a response variable and background sound level, wind speed, Julian date, time of day, developed vs. natural habitat, species and an interaction between sound level and species as fixed effects. Developed vs. natural habitat was used as a covariate to capture exposure to human activity and account for the possibility of habituation to human approach. Models for canopy gleaners did not include developed vs. natural habitat because all individuals were sampled in areas categorized as natural. In all models, we also treated location as a random intercept. We used Akaike information criterion corrected for small sample size (AIC_c) in model selection. Because of recent criticisms of model-averaging (Cade, 2015), we considered all models with $\Delta\mathrm{AIC}_c < 2$ to be equivalent (Boersma et al., 2016). To gauge the influence of individual predictors, for each model with $\Delta\mathrm{AIC}_c < 2$, we concluded that a predictor variable had a strong effect on adjusted FID when its 95% confidence interval (95% CIs) did not overlap zero.

Finally, foraging guilds may also reflect shared evolutionary histories that could influence variation in FID values among guilds. For example, all species categorized as hawking flycatchers are suboscines in family Tyrannidae. Thus, we tested for phylogenetic structure in mean FID values among species and species-specific model-estimated effect sizes for the influence of background sound levels on adjusted FIDs, including standard errors, using the phylosig function in the R package phytools (Revell, 2012), which incorporates the method from Ives et al. (2007) to account for sampling error. For our phylogenetic hypotheses, we used phylogenies from Jetz et al. (2012) and available from Birdtree.org. However, due to phylogenetic uncertainty among the Jetz et al. set, we used 100 randomly selected trees to calculate mean values for two common metrics for phylogenetic signal: Pagel's λ (Pagel, 1999) and Blomberg's K (Blomberg et al., 2003). Pagel's λ values vary from zero to one. High λ values indicate that closely related species have very similar trait values (i.e., high phylogentic structure). Low λ values indicate that trait values are unrelated to phylogeny. Blomberg's *K*-values > 1 suggest strong phylogenetic signal and values from zero to 1 suggest no phylogenetic signal to weak phylogenetic signal.

RESULTS

We surveyed a total of 197 individuals of 12 species of songbird across 27 locations from January to July 2016. Due to wind speed exceeding Category 3 on the Beaufort scale, we excluded one observation from the dataset to prevent potential bias introduced to sound measurements (Francis et al., 2011). Of the resulting 196 individuals, 105 were ground foragers, 52 canopy gleaners, and 39 flycatchers (**Table 1**). These individuals were sampled across 46 developed and 150 natural sites. We conducted trials throughout the day (0,600–1,630); however, we conducted most our observations (179 of 196) between 0,600 and 1,200 h. Wind speed ranged from 0 m/s to 5.5 m/s, with an average of 0.68 ± 0.93 m/s. Background sound levels ranged from 17.0 to 77.4 dB(A) with an average of 50.04 ± 8.68 dB(A). Starting and flight initiation distances averaged 26.76 ± 15.01 and 10.77 ± 7.32 m, respectively. We found no evidence for phylogenetic structure for mean FID (Pagel's $\lambda = 0.01$, $sd = 0.1$; Blomberg's $K = 0.41$,

TABLE 1 | We observed 196 individuals from 12 songbird species and grouped the species into three foraging guilds.

Foraging Guild	Common name	Scientific name	Sample size
GROUND			
	American Crow	*Corvus brachyrhynchos*	12
	California Scrub Jay	*Aphelocoma californica*	13
	California Towhee	*Melozone crissalis*	12
	Dark-eyed Junco	*Junco hyemalis*	23
	Golden-crowned Sparrow	*Zonotrichia atricapilla*	7
	White-crowned Sparrow	*Zonotrichia leucophrys*	38
CANOPY			
	Pacific Wren	*Troglodytes pacificus*	12
	Wilson's Warbler	*Cardellina pusilla*	9
	Yellow Warbler	*Setophaga petechia*	31
FLYCATCHING			
	Black Phoebe	*Sayornis nigricans*	19
	Dusky Flycatcher	*Empidonax oberholseri*	15
	Pacific-slope Flycatcher	*Empidonax difficilis*	5
Total	**12 species**		**196**

$sd = 0.03$), but evidence for a weak to moderate phylogenetic signal for the effect of noise on adjusted FID (Pagel's $\lambda = 0.50$, $sd = 0.30$; Blomberg's $K = 0.77$, $sd = 0.07$).

Among the top models for ground foraging species, the parameters background noise, diet, and species had strong effects, where 95% confidence intervals did not overlap zero (**Table 2**). The model that only included the random effect of location (i.e., null) was 2.00 ΔAIC_c from the top model. We visualized our results using the most parsimonious top model, which demonstrates an overall negative influence of background noise level on FIDs for all ground foraging species (**Figure 2B**). However, several ground foraging species differed in their overall response distances (i.e., different intercepts per species; **Figure 2A**, **Table 3**). Our results indicate that the individuals from the ground foraging guild were generally slower to respond to an observer's approach with elevated background sound and that omnivorous species flush at farther distances than *granivorous* species.

Among the top models for canopy gleaning species, the parameters with strong effects, included background noise, species, and an interaction between the two (**Table 2**). The null model (location as random intercept only) was 38.42 ΔAIC_c lower than the top model. Wilson's warbler (*Cardellina pusilla*) adjusted FIDs were positively influenced by background noise, Pacific wren (*Troglodytes pacificus*) adjusted FIDs were negatively affected by noise and yellow warblers (*Setophaga petechia*) appear uninfluenced by noise (**Figures 2C–E**, **Table 4**).

In the flycatching guild, the best model contained species, background noise, and an interaction between the two as fixed effects (**Table 2**). It was 29.6 ΔAIC_c better than the null model that only included the random effect of location and there were no other models with $\Delta\text{AIC}_c < 2$. Background noise levels had a strong positive influence on black phoebe (*S. nigricans*) adjusted FIDs (**Figure 2F**), but adjusted FIDs for both the Pacific-slope flycatcher (*Empidonax difficilis*) and dusky flycatcher (*Empidonax oberholseri*) were negatively affected by noise, albeit weakly (**Figures 2G,H**, **Table 5**).

TABLE 2 | This model selection table reports all the models within 2.00 ΔAICc in addition to the null model (intercept only) for each foraging guild.

Foraging Guild	Model	K	df	AIC_C	ΔAIC_C	weight
GROUND						
	dB + Species	3	9	10.6	0.00	0.139
	dB + Diet + Species	4	9	10.6	0.00	0.139
	Diet	2	4	10.9	0.31	0.119
	dB + **Diet**	3	5	11.4	0.77	0.095
	dB + Habitat + **Species**	4	10	11.8	1.25	0.075
	dB + Diet + Habitat + Species	5	10	11.8	1.25	0.075
	Null (intercept only)	1	3	12.6	2.00	0.051
CANOPY						
	dB + Species + dB*Species	4	8	−37.5	0.00	0.389
	dB + Species + Wind speed + **dB*Species**	5	9	−36.5	1.06	0.229
	Null (intercept only)	1	3	0.9	38.42	0.000
FLYCATCHER						
	dB + Species + dB*Species	5	8	−37.9	0.00	0.443
	Null (intercept only)	1	3	−29.6	8.28	0.007

*All models include location as a random effect. K indicates the number of parameters in the model. Model parameters include background sound level (dB), diet (e.g., omnivorous), habitat (either developed or natural), species, wind speed, and an interaction between background sound and species (dB*Species). Bolded variable names indicate predictors with 95% confidence intervals that do not overlap zero.*

DISCUSSION

To our knowledge, this report is the first to specifically address changes in songbird FIDs in response to noise, with implications for how noise influences predation risk and missed foraging opportunity as well as bird behavior relevant to casual nature-seekers and birdwatchers. Through our observations of wild songbird FIDs in varying acoustic conditions, we found evidence that noise influences bird FIDs in a variety of ways. However, we found no evidence that level of habitat development influenced FIDs of ground foraging and flycatching birds, suggesting that habituation to human presence may not strongly influence FID or the relationship between FID and background sound level. We also found no evidence that mean FID values were influenced by relatedness of species in our sample. However, the effect of noise on adjusted FIDs had some phylogenetic signal, likely reflecting that model estimates found identical relationships between noise and adjusted FID among all Emberizid species and because the congener flycatchers demonstrated similar adjusted FID responses with increasing noise. In general, however, our results indicate mixed responses across songbirds that can be species-specific and that might be explained in part by foraging behavior and, possibly, height of perch in the canopy.

All species within the ground foraging guild exhibited shorter FIDs in noise, as we predicted, such that the observer could approach closer to the target bird before it flushed. Shorter FID responses in noisy conditions may be explained by the distracted prey hypothesis, which posits that background noise occupies the target bird's finite attention and thus distracts the individual from other potentially important stimuli (Chan et al., 2010a). Although the distracted prey hypothesis may explain shortened FIDs in noise for some of these bird species, it is impossible to uncouple distraction from the effects of energetic masking. The high background sound in some areas might have masked the sounds of an approaching observer, leading to a slower response from the target bird. Of course, both mechanisms could operate simultaneously. Regardless, the result is that noise reduces an individual's ability to detect approaching threats and likely elevates an individual's risk of predation (Krause and Godin, 1996; Simpson et al., 2015); failure to detect predators at a sufficient distance could be lethal.

Although our results suggest ground foraging birds may be more at risk to predation in noisy environments, the effects of masking and noise on predator abundance and hunting ability must also be considered. Opportunistic avian nest predators avoid noisy areas in a natural gas extraction field (Francis et al., 2009, 2011) and owls, which are specialized acoustic predators, have trouble localizing or foraging in noisy conditions found in

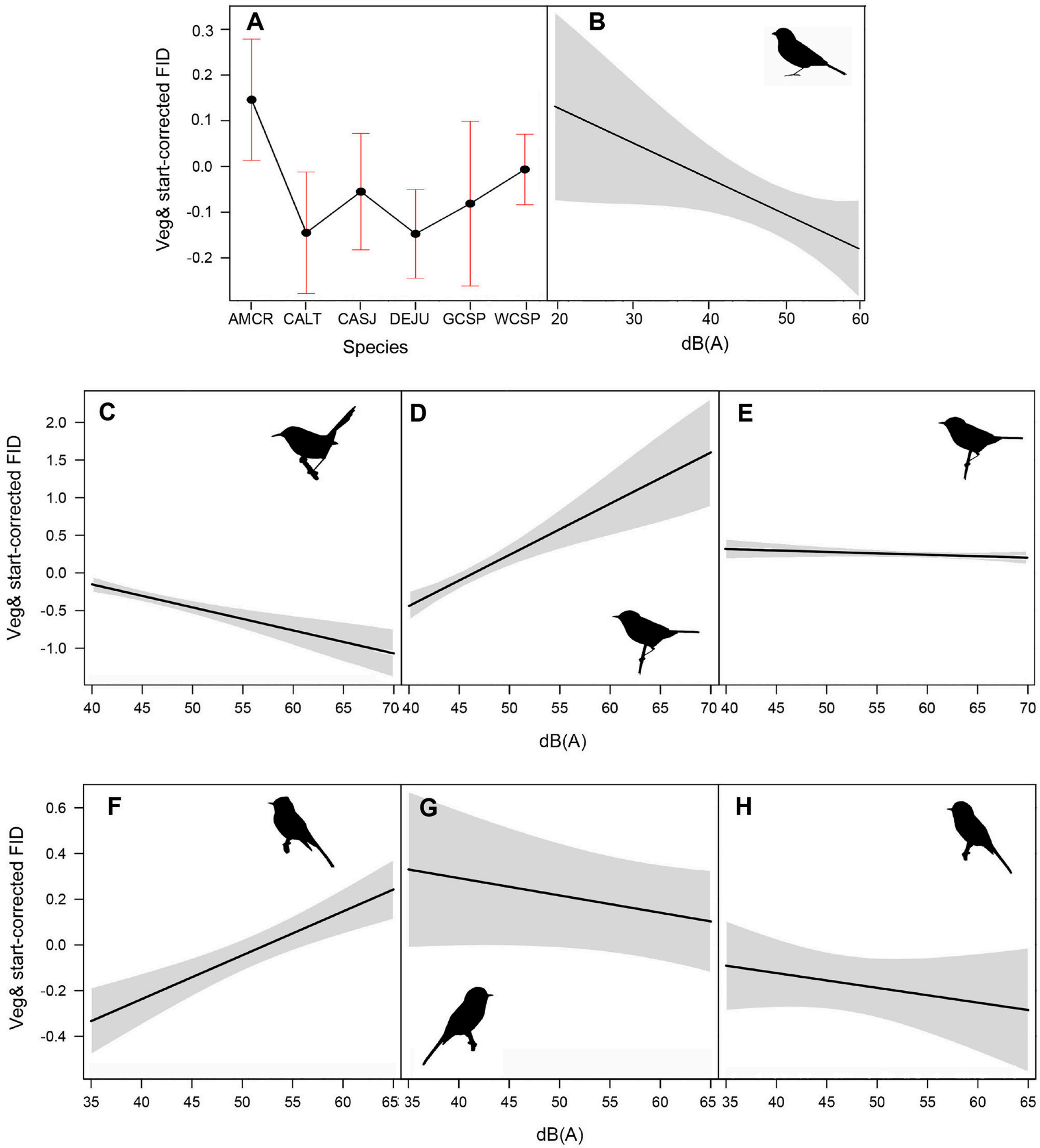

FIGURE 2 | **(A)** illustrates mean adjusted FID and standard error for each species in the ground foraging guild. Species depicted are American crow (AMCR), California towhee (CALT), California scrub jay (CASJ), dark-eyed junco (DEJU), golden-crowned sparrow (GCSP), and white-crowned sparrow (WCSP). **(B–H)** illustrate the influence of ambient noise on vegetation and start distance-corrected FID values for all ground foraging species **(B)**, canopy gleaning species Pacific wren **(C)**, Wilson's warbler **(D)**, yellow warbler **(E)**, and flycatching species black phoebe **(F)**, dusky flycatcher **(G)**, and Pacific-slope flycatcher **(H)**. For plotting purposes, we utilized the top-ranking model (lowest AICc and fewest parameters) for each of the foraging guilds (**Table 2**).

gas fields and near roadways (Mason et al., 2016; Senzaki et al., 2016). In laboratory settings, both Quinn et al. (2006) and Ware et al. (2015) demonstrated that ground foraging species spend significantly more time visually scanning for predators when ambient noise is high compared to quiet conditions. If ground foraging birds in our study also increase visual vigilance with

TABLE 3 | The influence of fixed effects on adjusted FID for ground foraging species.

Variable	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	0.464	0.169	0.129	0.798
dB	**−0.007**	**0.003**	**−0.013**	**−0.000**
Species CALT	**−0.292**	**0.095**	**−0.479**	**−0.104**
Species CASJ	**−0.205**	**0.093**	**−0.389**	**−0.021**
Species DEJU	**−0.291**	**0.083**	**−0.456**	**−0.126**
Species GCSP	**−0.228**	**0.114**	**−0.453**	**−0.003**
Species WCSP	**−0.153**	**0.077**	**−0.305**	**−0.001**

Presented are effect sizes, standard error, 95% confidence intervals from the top-ranking model (i.e., lowest AICc value and/or fewest parameters). The species included in this group are: American crow (Intercept), California towhee (CALT), dark-eyed junco (DEJU), golden-crowned sparrow (GCSP), white-crowned sparrow (WCSP), and California scrub jay (CASJ). Lower and upper confidence intervals represent 95% confidence. Bolded parameters have confidence intervals that do not overlap zero, indicating a strong effect.

TABLE 4 | The output for a linear mixed effect model of the canopy gleaning guild.

Variable	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	1.064	0.303	0.459	1.669
dB	**−0.030**	**0.007**	**−0.044**	**−0.017**
Species WIWA	**−4.225**	**0.746**	**−5.713**	**−2.736**
Species YEWA	−0.585	0.366	−1.316	0.146
dB*Species WIWA	**0.098**	**0.016**	**0.066**	**0.131**
dB*Species YEWA	**0.027**	**0.007**	**0.012**	**0.041**

The species included in this guild are: Pacific wren (Intercept), Wilson's warbler (WIWA), and yellow warbler (YEWA). The variables include background sound (dB), species, and interactions between background sound and species. Lower and upper confidence intervals represent 95% confidence. Bolded parameters have confidence intervals that do not overlap zero, indicating a strong effect.

TABLE 5 | The output for a linear mixed effect model of the flycatching guild.

Variable	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	−0.974	0.207	−1.081	−0.526
dB	**0.019**	**0.004**	**0.018**	**0.021**
Species DUFL	**1.594**	**0.402**	**0.712**	**1.890**
Species PSFL	1.149	0.366	NA	1.336
dB*Species DUFL	**−0.026**	**0.007**	**−0.031**	**−0.012**
dB*Species PSFL	**−0.025**	**0.008**	**−0.028**	**−0.012**

The species included in this guild are: black phoebe (Intercept), dusky flycatcher (DUFL), and Pacific-slope flycatcher (PSFL). The variables include background sound (dB), species, and an interaction between the two. Lower and upper confidence intervals represent 95% confidence. Bolded parameters have confidence intervals that do not overlap zero, indicating a strong effect.

noise, our results suggest that they were still unable to detect and respond to approaching threats as quickly as in quiet conditions, despite compensating with visual scans. Notwithstanding the potential ultimate costs of failing to detect predators at an appropriate distance, for human observers seeking to experience wild birds at close range, species that flee at shorter distances in noisy conditions would be easier to approach, view and hear. Finally, diet was also a significant predictor of FID for ground foraging species; species with omnivorous diets exhibited longer FIDs than species with *granivorous* diets irrespective of noise levels. Although limited evidence can help explain this trend, Francis (2015) demonstrated that the abundance of most avian species decline in noisy areas, species with plant-based diets appear to be less sensitive than those with animal-based diets. It is also possible that other traits unique to the two omnivorous species, which are both corvids, could explain their longer FIDs relative to *granivorous* species. For example, various measurements of brain size are positively associated with FIDs (Symonds et al., 2016) and corvids are known to have relatively large brains (Emery and Clayton, 2004). Greater cognitive capacity could also potentially mitigate distraction by noise and other stimuli (i.e., distracted prey hypothesis) by permitting individuals to process multiple streams of sensory information and respond to approaching threats appropriately. Future work should explore the relative contributions of cognitive abilities and foraging modalities to explain FIDs or sensitivities to changes in background acoustics in general.

For the canopy gleaning guild, we predicted that FIDs would decrease as a result of distraction or masking due to increased ambient noise. However, we suspected that because these species often utilize high tree perches, they might only exhibit weakly decreased or unchanged FIDs to a human approaching on the ground due to vertical relief from the threat. Although these predictions were not supported for all canopy gleaning species, we observed a trend of decreased FIDs with increasing noise for Pacific wrens (*T. pacificus*) and a pattern of unchanged FIDs for yellow warblers (*S. petechia*). Similar to the ground foraging species, Pacific wrens may be more susceptible to the effects of masking or distraction due to frequent sensory modality shifts as they remain acoustically vigilant while visually foraging. Pacific wrens are known to forage in the low canopy and, thus, experience little vertical relief from ground-level threats; however, yellow warblers frequently utilize high perches, which may explain the lack of FID response for the species. Additionally, although our robust sampling of yellow warblers indicates that this species exhibits consistent FID behavior across medium to high sound levels [46.9–77.4 dB(A)], we were unable to conduct any approaches under relatively low ambient sound conditions [<40 dB(A)]. Future work should focus on observing yellow warblers in environments with less ambient noise, as even acoustic cues at these relatively quiet levels can elicit responses in other taxa (Shannon et al., 2016b) and perhaps there is a threshold below which noise might influence FID in yellow warblers. In contrast to our predictions for the canopy gleaning guild, Wilson's warblers (*C. pusilla*) exhibited a trend of increased FIDs with increasing noise. Wilson's warblers primarily forage by gleaning insects in the canopy, but they are also known to hawk for flying insect prey (Ehrlich et al., 1988) and, thus, might utilize sensory modalities differently when compared to other canopy gleaning species. However, yellow warblers are also occasional flycatchers (Ehrlich et al., 1988) and, yet, do not exhibit the same increased FIDs of Wilson's warblers. Thus, engaging in flycatching behavior cannot solely explain the pattern consistent with hypervigilance in noise among Wilson's warblers. Finally, although two of the canopy gleaning species studied here fit our predictions of decreased FIDs or no response, future research should explicitly include individual perch height as a potential predictor of variation in FIDs both within and across species.

Our observed results for Wilson's warblers follow our prediction for species of the flycatching guild such that these species would exhibit unchanged or increased FIDs in noise due to their foraging strategy of constant visual and aural vigilance that might allow these species to scan for predators and prey simultaneously. However, only one species of the flycatching guild, the black phoebe (*S. nigricans*), provided support for this prediction with increasing FIDs with increases in noise levels. The other two species of this guild, dusky flycatchers (*E. oberholseri*) and Pacific-slope flycatchers (*E. difficilis*), exhibited a weak trend of decreased FIDs with increasing noise. This muted response might be explained by our small sample size or perhaps like the canopy gleaning yellow warblers, by these flycatchers' shared tendency to utilize high perches, thereby relieving the threat of a ground-level approaching human. In the case of the black phoebe, however, our robust sampling in a wide range of ambient noise conditions indicates that this species is generally more likely to flush sooner if background noise is high. Such increased FIDs in noise might be explained by heightened vigilance in noisy conditions, which was demonstrated in laboratory settings with chaffinches (Quinn et al., 2006) and white-crowned sparrows (Ware et al., 2015) as well as in a field experiment with free-ranging prairie dogs (Shannon et al., 2016a). Furthermore, flushing more quickly in noise coincides with FEAR hypothesis, which suggests that birds are likely to flush quickly after detection in order to avoid the need to monitor an approaching threat (Blumstein, 2010). This response might allow for more foraging time if the bird flushes to an area with available food; however, fleeing from a beneficial foraging site may result in lost foraging opportunity and could incur the cost of increased energy expenditure.

In the context of human experience of wildlife, our results indicate that background noise may influence the quality of a birdwatching experience. Due to their hawking behaviors, both Wilson's warblers and black phoebes can be exciting birds to observe. Both species exhibited longer FIDs in noise, indicating that birdwatchers may experience difficulty when seeking these species and perhaps other hawking species in noisy conditions. However, most of the species in our sample trended toward shorter FIDs in noise, which would allow birders to approach closer. This may lead to a surprisingly positive outcome of anthropogenic noise pollution, under which birdwatching experiences are improved through field observations at closer range in noisier conditions. Particularly for new birdwatchers, this close viewing of wild birds may foster a personal and lasting connection with wildlife and lead to increased support for wildlife conservation. Of course, this potential benefit to nature-seekers of visually experiencing birds at a closer range must be viewed in the context of the quickly growing body of literature reporting the negative effects of noise and human disturbance to wild animals (Ellison et al., 2012; Francis and Barber, 2013; Shannon et al., 2016b). Nevertheless, our conclusions offer new perspective on the coexistence of humans and wildlife in an increasingly noisy world.

ETHICS STATEMENT

This study was carried out in accordance with the guidelines of the Committee of the Care and Use of Laboratory Animals Resources, National Research Council, and the research protocol was approved by the California Polytechnic State University, Institutional Animal Care and Use Committee (#1519).

AUTHOR CONTRIBUTIONS

AP, ML, and RW conducted the fieldwork. JB and CF conceived of the project. All authors contributed to analysis and interpretation of the data. AP was primarily responsible with writing the manuscript added by CF and all other authors contributed to writing, editing and, ultimately, approving of the manuscript for publication.

ACKNOWLEDGMENTS

We thank Gita Kolluru, Crow White and two reviewers for constructive feedback on earlier versions of this paper. This project was funded by National Science Foundation Grant (CNH-1414171) to JB and CF.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 18 December 2017
doi: 10.3389/fevo.2017.00163

Urban Great Tits (*Parus major*) Show Higher Distress Calling and Pecking Rates than Rural Birds across Europe

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Specialty section:
*This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution*

Received: *05 June 2017*
Accepted: *29 November 2017*
Published: *18 December 2017*

Citation:
Senar JC, Garamszegi LZ, Tilgar V, Biard C, Moreno-Rueda G, Salmón P, Rivas JM, Sprau P, Dingemanse NJ, Charmantier A, Demeyrier V, Navalpotro H and Isaksson C (2017) Urban Great Tits (Parus major) Show Higher Distress Calling and Pecking Rates than Rural Birds across Europe. Front. Ecol. Evol. 5:163. doi: 10.3389/fevo.2017.00163

Environmental change associated with urbanization is considered one of the major threats to biodiversity. Some species nevertheless seem to thrive in the urban areas, probably associated with selection for phenotypes that match urban habitats. Previous research defined different "copying styles" in distress behavior during the handling of birds. These behaviors vary along a continuum from "proactive" to "reactive" copers. By studying avian distress behaviors we aimed to broaden our understanding of the relationship between coping styles and urbanization. Using a large-scale comparative study of seven paired rural and urban sites across Europe, we assayed distress behaviors during handling of urban and rural-dwelling populations of the great tit *Parus major*. We detected no consistent pairwise differences in breath rate between urban and rural habitats. However, urban great tits displayed more distress calling (fear screams) and higher pecking rate (handling aggression) than rural birds. These findings suggest that urban great tits have a more proactive coping strategy when dealing with stressful conditions. This finding is in line with previous studies implying that urban great tits are more explorative, less neophobic, and display shorter flight distances than their rural counterparts, representing further aspects of the same "proactive," coping strategy. Future research should investigate whether reported differences in distress behavior are due to local adaption caused by natural selection or due to phenotypic plasticity.

Keywords: urbanization, distress behavior, handling, coping strategy, distress calling, pecking rate, local adaptation, great tit

INTRODUCTION

Urban environments are expanding worldwide with an unprecedented speed (United Nations, 2014); environmental change associated with urbanization is being considered as one of the main current threats to biodiversity (Turner et al., 2004). However, urbanization also represents a potential source of selection and a new avenue to study evolutionary processes (Donihue and Lambert, 2014; Isaksson, 2015; Alberti et al., 2017; Hendry et al., 2017).

For wildlife, urban habitats differ in many respects from rural habitats, including microclimate, food abundance, pollution, abundance of exotic species, and predation risk (Luniak and Pisarski, 1982; Marzluff et al., 2001; Gaston, 2010; Gil and Brumm, 2014). Many of these factors induce stress, defined in a broad sense as changes away from an organisms' physiological homeostasis that emerge in response to a change in the environment (sensu Badyaev, 2005; Tuomainen and Candolin, 2012). This is especially the case for factors that represent, or are associated with increased perceived predation risk, such as elevated disturbance due to the presence of humans, pet mammals, and cars (Buchanan and Partecke, 2012). The obvious hypothesis based on this known disturbance or perceived threat is that urban animals would have an enhanced stress response than rural-dwelling animals. However, the results are mixed. For example, some studies reveal higher corticosterone levels (avian stress hormones) in urban compared to rural bird populations (Schoech et al., 2007; Fokidis et al., 2009; Zhang et al., 2011), whereas other studies found the opposite pattern or no significant differences (Partecke et al., 2006; French et al., 2008; Abolins-Abols et al., 2016).

Nevertheless, whatever the direction of the effects, they are likely to influence aspects of behavior, physiology, and life history of urban-dwellers in their coping response to the urban environment (Tuomainen and Candolin, 2012; Sprau and Dingemanse, 2017). Consequently, stress-responses, i.e., the behavioral and physiological means by which animals cope with environmental stress, play an important role in local adaptation (Badyaev, 2005). Hence, comparing stress-responses between urban- and rural-dwelling populations can shed light on the mechanisms underpinning adaptation to urban habitats. For example, predatory attack is most likely one of the highest stresses that a prey animal can experience, and this is irrespective of whether the animal is habituated to the urban environment. Behavioral responses to such threats are commonly referred to as distress behaviors (Møller and Ibáñez-Álamo, 2012). Previous research has shown that distress behaviors displayed during handling are parts of the same phenotypic syndrome that encapsulates an entire suite of behavioral and physiological traits that individual display in order to adaptively respond to stressful situations (Koolhaas et al., 1999, 2010; Carere and van Oers, 2004; Coppens et al., 2010; Carvalho et al., 2013; Class et al., 2014; Kluen et al., 2014). Responses can be categorized into different "coping styles," varying along a continuum from "proactive" to "reactive" copers, and they relate to the way organisms deal with stressors. Summarizing, proactive individuals are more aggressive, explorative, neophilic, and risk-prone than reactive individuals (Carere et al., 2010).

Accordingly, birds displaying a high frequency of distress behaviors represent proactive individuals. We focus in this paper on three distress behaviors easily recorded during handling: distress calling, handling aggression, and breath rate. Distress calling rate has previously been related to proactivity: in black-capped chickadees *Poecile atricapillus*, distress calling rate was positively related to exploration rate, which is one of the main characteristics of the proactive coping style (Guillette and Sturdy, 2011). In siskins *Carduelis spinus*, individuals uttering more often distress calls, displayed also bolder behaviors in front of a novel object (Mateos-González and Senar, 2012; Pascual and Senar, 2014). In the Japanese quail *Coturnix japonica*, distress calling was positively related to the excretion of corticosterone metabolites, implying that high levels of distress calling are related to proactivity (Niall Daisley et al., 2005). Distress calling has therefore been used as a proxy of proactivity in several studies (Andersen, 2012; Pascual and Senar, 2014; Thorsteinsen, 2015; Richardson et al., 2016). Aggression has also been regarded as a typical response of proactive copers to stressful situations (Koolhaas et al., 1999; Carere et al., 2010), and pecking rate during handling is classically used to compare level of proactivity among individuals (Brommer and Kluen, 2012; van den Brink et al., 2012a,b; Class et al., 2014; Kluen et al., 2014; Dubuc-Messier et al., 2017). Breath rate has also been suggested as an indicator of coping style, with breath rates being higher in proactive individuals than in reactive ones (Carere and van Oers, 2004; van Oers and Carere, 2007; Torné-Noguera et al., 2014; Charmantier et al., 2017). Additionally, breath rate has been found to correlate positively to exploration rate, which as previously stated, is one of the main characteristics of the proactive coping style (Charmantier et al., 2017). The three behaviors have also been found to be heritable (Koenig et al., 1991; Brommer and Kluen, 2012). Therefore, these three distress behaviors can be used as an accurate proxy of coping style and their study may allow a broader understanding of the role of coping style mediating processes of urbanization in birds.

Recent work has found that distress behaviors during handling differ between urban and rural population of birds. For example, an interspecific comparison revealed that urban birds showed higher frequencies of distress calling when handled than their rural counterparts (Møller and Ibáñez-Álamo, 2012). Aggression, another typical response to stressful situations (Koolhaas et al., 1999), has also been found to differ in inter-specific comparisons between urban and rural individuals during handling (Møller and Ibáñez-Álamo, 2012). At the intra-specific level, urban birds have also been shown to display different breath rates compared to their rural counterparts during handling (Torné-Noguera et al., 2014; Abolins-Abols et al., 2016; Charmantier et al., 2017). Yet, this relationship may differ between species. To date, studies comparing distress behaviors between urban and rural bird populations (Møller and Ibáñez-Álamo, 2012; Torné-Noguera et al., 2014) have lacked sufficient replication to draw firm conclusions regarding the relationship between urbanization and avian distress behavior (Evans et al., 2009).

The main aim of the present paper was to investigate whether urban and rural populations of the great tits (*Parus major*) differ consistently in their behavioral response to an acute perceived threat, assayed during capturing and handling (Møller and Nielsen, 2010; Markó et al., 2013; Class et al., 2014; Kluen et al., 2014; Dubuc-Messier et al., 2017). To acquire a robust insight into the relationship between urbanization and distress behavior, we assayed three different distress behaviors (distress calling rate, pecking rate, and breath rate) in seven replicate pairs of urban and rural/forest populations across Europe. Previous work on this species has revealed many differences between urban and rural great tits in terms of morphology (Horak et al., 1995; Senar et al., 2014; Biard et al., 2017), physiology (Andersson et al., 2015; Salmón et al., 2016; Toledo et al., 2016), genetics (Björklund et al., 2010; Riyahi et al., 2015), life-history (Berressem et al., 1983; Schmidt and Einloft-Achenbach, 1984; Isaksson and Andersson, 2007; Hedblom and Soderstrom, 2012; Bailly et al., 2015; Vaugoyeau et al., 2016; Charmantier et al., 2017), population dynamics (Horak and Lebreton, 1998), and behavior (Slabbekoorn and Peet, 2003; Salaberria and Gil, 2010; Riyahi et al., 2017). Urban great tits have been found to be more explorative and less neophobic than rural tits (Tryjanowski et al., 2016; Charmantier et al., 2017; Riyahi et al., 2017), to display shorter flight initiation distances (Møller et al., 2015), and to show a higher problem solving performance than rural birds (Preiszner et al., 2017). Based on this growing body of literature, which suggests that a proactive coping strategy fits urban conditions, we predict that urban great tit populations show a higher distress calling rate, pecking rate and breath rate compared to their rural conspecifics.

MATERIALS AND METHODS

Study Areas and Sampling

Great tits were caught in seven distinct cities paired with a close rural site: Granada (Spain), Barcelona (Spain), Montpellier (France), Munich (Germany), Paris (France), Malmö (Sweden), and Tartu (Estonia; **Table 1**). Paired sites within localities were separated with distances ranging from 5 to 106 km (**Table 1**). Natural habitats were either forest or rural areas, but we refer to them as rural habitats for simplicity. To quantify the degree of urbanization in each locality we used aerial images from Google Maps following the methods previously described to assess the effect of the degree of urbanization on wild bird populations (Seress et al., 2014; Vincze et al., 2017). Briefly, each locality was represented by a 1 × 1 km^2 rectangular area around the birds' capture site. The content in each rectangle was evaluated by dividing the image in 100 × 100 m^2 cells and considering three land-cover characteristics in each: proportion of buildings, vegetation (including cultivated fields), and paved surfaces. The different land-cover measures obtained per site fitted into a principal component analysis (PCA) to estimate an urbanization score (PC1) per site (**Table 1**). The PC1 values were multiplied with −1 to obtain more positive values in the more urbanized localities.

Great tits were captured using spring traps, mist nets or funnel traps (Senar et al., 1997). Though trapping methods differed among localities, they were identical in six out of seven urban/rural pair sites (only Granada differed). Sampling was conducted during the main breeding season at each site (April–June), except for the Barcelona population, which was sampled from January to April. Overall, we quantified distress behaviors of 1,539 individual great tits. All individuals represent independent data points as re-captures were not included in our analyses comparing distress behavior between habitats (see below). However, behavioral information of individuals recorded in multiple years was used to estimate repeatability (68 birds for breath rate and 22 for pecking and distress calling rates, see below). All birds were sexed and aged (yearling vs. adult) according to plumage characteristics (Svensson, 1992).

Behavioral Tests

We performed measurements of distress behavior within 5 min after capture. We recorded three behaviors known to reflect coping style and stress responsiveness and/or fighting propensity during handling (Carere and van Oers, 2004; Fucikova et al., 2009; Laiolo et al., 2009; Markó et al., 2013; Torné-Noguera et al., 2014). Breath rate, distress call rate, and pecking rate were recorded in the same consecutive order. The target traits were not

TABLE 1 | Sampling localities of urban and rural great tits (*Parus major*) across Europe.

		Rural				Urban				
Locality	**Period sampled**	**Latitude**	**Longitude**	**N**	**PC1**	**Latitude**	**Longitude**	**N**	**PC1**	**Distance (km)**
Granada	2015–2016	36º56′N	3º30'W	19	−0.99	37º12′N	3º36′W	10	1.29	27
Barcelona	2015–2016	41º23′N	2º10′E	77	−2.40	41º23′N	2º09′E	49	2.76	5
Montpellier	2014–2016	43°40′N	3°40′E	97	−1.60	43°36′N	3°52′E	273	1.99	20
Munich	2014–2015	47° 58′N	11° 14′E	442	−1.55	48° 8′N	11° 34′E	208	3.05	31
Paris	2015–2016	48°16′N	2°41′E	55	−2.58	48°51′N	2°20′E	48	2.27	80
Malmö	2015	55°40′N	13°31′E	37	−2.56	55º36′N	13º02′E	41	2.03	35
Tartu	2015–2016	58°7′N	25°5′E	134	−2.17	58º23′N	26º43′E	49	0.46	106

We print the name of each site, year of sampling, locations, sample size (n), urbanization index (PC1), and the distance between the rural and the urban site. The lower the urbanization index, the more urbanized the population's habitat; Munich being the most and Tartu the least urbanized habitats among the cities.

recorded in all localities: in Montpellier and Munich only breath rate was recorded.

Breath rate was recorded by counting the number of breast respiratory movements within 30 s, while holding the wings fixed and also ensuring reduced disturbance by visual and sound cues (Markó et al., 2013; Torné-Noguera et al., 2014). Pecking rate and distress calling rate were subsequently quantified by counting the number of pecks and distress calls that were emitted during the next 15 s of handling (Fucikova et al., 2009; Laiolo et al., 2009). Pecking rate refers to the number of pecks against a straight finger positioned at 1–2 cm from the beak of the focal bird while holding its legs (Markó et al., 2013). Distress calling rate was defined as the number of vocalized distress calls recorded during the same pecking rate trial (Markó et al., 2013). Breath rate was measured by multiple observers in Montpellier ($n = 4$) and Munich ($n = 14$). In the other localities, a single observer recorded all data in both the rural and urban site.

Statistical Analyses

To test for general differences in distress behavior between the urban and rural habitats across the seven localities, data were analyzed using univariate linear mixed-effects models (LMM), in which breath rate, pecking rate, or distress calling rate were sequentially included as the response variable. As fixed effects, we included habitat (rural vs. urban), sex and age (yearling vs. adult). Locality ($n = 7$ levels) and population ($n = 2$ populations* 7 localities = 14 populations) were included as random effects. LRT refers to a likelihood ratio test. As breath rate was measured by multiple observers in two of the localities (see above), we also included observer as random effect (23 levels, nested within locality) in models explaining variation in breath rate. Since the seven localities represented a wide range of different habitats, a habitat-specific residual variance was fitted to account for unequal residual variances between the two types (rural vs. urban). Breath rate was analyzed assuming a Gaussian error distribution, while pecking rate and distress call were analyzed assuming a Poisson error distribution.

Bivariate LMM were used to estimate among-population and among-individual-within-population covariances (and correlations) between each unique combination of two behaviors, where the two focal behaviors were entered as response variables (following procedures detailed by Dingemanse and Dochtermann, 2013).

Only *a priori* considered combinations of predictor variables were entered into the statistical models, which was based on biological thinking. This approach to the explicit test of *a priori* formulated hypotheses has been suggested to allow more general inferences than more traditional exploratory analyses in which various possible interactions are tested sequentially or based on statistical algorithm (Dochtermann, 2010). Accordingly, we were interested in the interaction between habitat and locality, because the difference between urban and rural habitats might differ among localities (see also below). We also included the interaction between habitat and sex, because previous analyses revealed sex differences in the expression of some of the focal behaviors (Markó et al., 2013). We also included an interaction term between habitat and age to test for age-specific differences between urban and rural populations (Markó et al., 2013). In cases where the interaction between sex and habitat was significant, we additionally modeled the sexes separately, in order to understand for which sex the differences between habitats were statistically supported.

We assumed that previous "capture history" did not affect distress behavior, an assumption that was confirmed for the Barcelona site, where the number of preceding captures had no effect on distress behaviors measured during the present study [distress calling rate: $F_{(1,\,119)} = 1.4$, $p = 0.32$; pecking rate: $F_{(1,\,119)} = 0.6$, $p = 0.52$; breath rate: $F_{(1,\,119)} = 0.2$, $p = 0.73$]. Furthermore, we can infer that "capture history" should not be an important confounder, as the data used in our analyses were largely based on first captures.

Adjusted repeatability was calculated following Nakagawa and Schielzeth (2010) using series of univariate LMMs where individual identity and population identity were fitted as a random effects, and where sex, age, and habitat as fixed effects. We repeated these analyses for rural and urban birds separately to calculate repeatability within each habitat type (urban vs. rural).

All statistical analyses were carried out using the package lme4, version 1.1-12 (Bates et al., 2011) and MCMCglmm version 2.23 (Hadfield, 2010) within the R (version 3.3.1) computing environment (R Development Core Team, 2017). For the modeling based on MCMCglmm, we ran Markov chains up to 230,000 iterations with 30,000 iterations of burn in and with 500 iterations of thinning interval (longer runs, such as 1,300,000 iterations, did not improve the results). For prior definition, we followed a technique called *parameter expansion* as was suggested by Hadfield (2010), and set V = 1, nu = 0.02, and alpha.V = 1,000. We repeated each run for each model 3–4 times to check the stability of results. After each run, the trace and distribution of all estimated parameters were checked visually, as well as autocorrelation between iterations. Furthermore, mixing and convergence were checked with Gelman-Rubin statistics (Gelman and Rubin, 1992).

RESULTS

The selected urban areas had significantly higher urbanization scores than the selected rural areas [$F_{(1,\,13)} = 96.08$, $p < 0.0001$] based on the satellite data, confirming that our assignments were appropriate.

Distress calling rate showed a low and non-significant between-year repeatability within individuals (**Table 2**). However, repeatabilities of pecking and breath rates were of higher magnitude, and were similar between rural and urban populations (**Table 2**). Distress calling rate and pecking rate were positively correlated within populations (**Table 3**) but distress calling and pecking rates did not correlate significantly with breath rate (**Table 3**), suggesting that breath rate was independent from the two other traits. None of the between-population correlations were statistically significant (**Table 3**).

Distress calling rate was higher in birds from urban compared to rural habitats (**Figure 1**, **Table 4**). There was a non-significant

TABLE 2 | Within-individual and between-year repeatability of distress calling, pecking, and breath rates.

	Individual repeatability				
	r	SE	−95%CI	+95%CI	*p*
DISTRESS CALLING RATE					
Unadjusted repeatability					
All birds	0.02	0.08	0	0.28	0.27
Adjusted repeatability					
All birds	0.02	0.09	0	0.32	0.26
PECKING RATE					
Unadjusted repeatability					
All birds	0.59	0.18	0.13	0.84	<0.001
Rural birds	0.59	0.3	0	0.97	0.09
Urban birds	0.54	0.23	0	83	0.04
Adjusted repeatability					
All birds	0.61	0.19	0.15	0.87	<0.001
Rural birds	0.38	0.32	0	0.91	0.04
Urban birds	0.55	0.22	0	0.84	0.04
BREATH RATE					
Unadjusted repeatability					
All birds	0.48	0.10	0.27	0.65	<0.001
Rural birds	0.43	0.2	0	0.76	0.01
Urban birds	0.49	0.11	0.25	0.68	<0.001
Adjusted repeatability					
All birds	0.35	0.10	0.14	0.54	<0.001
Rural birds	0.39	0.21	0	0.74	0.08
Urban birds	0.37	0.12	0.13	0.59	<0.001

Univariate linear mixed-effects models were performed fitting bird identity as a random effect. First, we present models where effects of sex, age, habitat, locality, and population, were not included (i.e., estimates represent the unadjusted repeatability). Second, we present models where the mentioned effects were controlled for (i.e., estimates represent adjusted repeatability; sensu Nakagawa and Schielzeth, 2010). We also present repeatability estimates separately for the urban and rural habitat (though for distress calling rate, variance and the sample size was too small to analyze the two habitats separately). Repeatability (r), standard error (SE), lower and upper confidence interval (CI), and significance (p-value) is provided. Sample size for breath rate: $n_{rural} = 32$, $n_{urban} = 104$ individuals measured twice in different years, for pecking and distress calling rates: $n_{rural} = 12$, $n_{urban} = 32$ individuals measured twice in different years.

TABLE 3 | Correlations coefficients at different levels among distress calling, pecking, and breath rates.

	Correlations					
	Among-populations			Within-populations		
	r	−95%CI	+95%CI	*r*	−95%CI	+95%CI
Distress calling vs. Pecking rate	0.54	−0.07	0.85	0.58	**0.49**	**0.68**
Distress calling vs. Breath rate	0.14	−0.41	0.69	−0.01	−0.13	0.03
Pecking rate vs. Breath rate	0.01	−0.63	0.65	0.04	−0.06	0.16

We print the correlation coefficient (r) and the credible intervals (CI) between-population (the correlation of population means of the individual traits across different populations) and within-population (the correlation of individual-specific measurements within populations). When the upper and lower CI's does not cross over zero the correlation can be considered as significant (high-lighted in bold face).

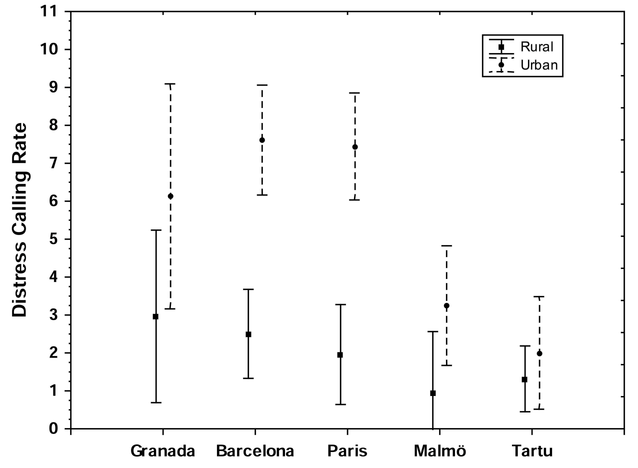

FIGURE 1 | Between-individual variation (mean with standard error) in distress calling rate along habitats (urban vs. rural) and localities (see **Table 4**; both sexes combined). Distress calling rate is number of calls during 15 s.

trend toward an interaction between sex and habitat on distress calling rate (**Table 4**). Though the difference in distress calling rate between urban and rural areas tended to be stronger in females compared to males, urban great tits of both sexes had higher distress calling rates compared to their rural conspecifics. The interaction between locality and habitat could not be tested because models incorporating this fixed effect did not converge.

In the full model for pecking rate, there was no significant effect of habitat, age or their interaction (habitat: LRT = 2.75, $p = 0.10$; age LRT = 0.00, $p = 0.98$; habitat × age: LRT = 0.29, $p = 0.59$). Both locality and population were significantly related with great tit pecking rate (locality: LRT = 7.24, $p = 0.01$; population: LRT = 2.41, $p < 0.01$). In addition, males displayed higher pecking rates than females (sex: LRT = 4.63, $p = 0.03$). However, we also found a non-significant trend for an interaction between sex and habitat on pecking rate (sex × habitat: LRT = 3.34, $p = 0.07$). A *post-hoc* analysis separating the sexes, revealed that there was an effect of habitat on pecking rate in males (LRT = 4.79, $p = 0.03$, **Figure 2**, **Table 5**) but not in females (LRT = 0.01, $p = 0.95$). The interaction between locality and habitat was non-significant (LRT: 0.34, $p = 0.95$) in the model for males indicating that the higher pecking rate of urban than rural males was consistent across localities (**Figure 2**).

Breath rate did not differ between urban and rural habitats when controlling for sex, age, locality, population, and observer effects (**Table 6**). The interaction between locality and habitat was also non-significant (LRT < 0.001, $p = 0.99$), indicating that this lack of difference was consistent across localities.

DISCUSSION

The present study reveals consistent differences in distress calling rate between urban and rural populations of great tits, across

TABLE 4 | Statistical summary of the analysis of distress calling rate between rural and urban habitats.

	LRT	p	ß	−95% CI	+95% CI
FIXED EFFECTS					
(Intercept)			−1.94	−3.13	0.80
Sex [male]	3.46	0.06	0.92	0.18	1.67
Age [yearling]	2.54	0.11	−0.44	−1.20	0.33
Habitat [urban]	15.44	< 0.001	2.72	1.25	4.04
Habitat*Sex [urban, male]	3.44	0.06	−1.08	−2.05	0.10
Habitat*Age [urban, yearling]	0.56	0.46	−0.06	−1.05	0.95
RANDOM EFFECTS					
			Variance		
Locality	< 0.001	0.99	< 0.001	0.00	2.01
Population	3.19	0.07	0.01	0.00	1.69
Error			4.40	2.09	8.60

The table shows the results of LMMs, in which the significance of terms was evaluated by likelihood-ratio test (LRT). Means of the parameter estimates (ß), variance components and their respective 95%CI intervals were obtained from the posterior distribution of the corresponding MCMCglmm. In square bracket we indicate to which factor level the parameter estimate corresponds.

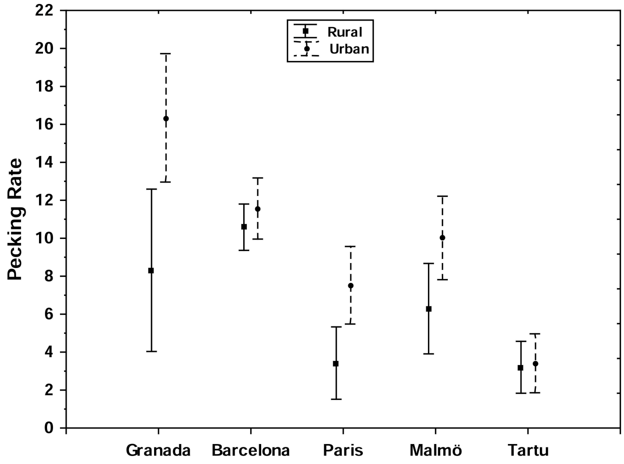

FIGURE 2 | Between-individual variation (mean with standard error) in pecking rate along habitats (urban vs. rural) and localities (see **Table 5**). Pecking rate is number of peckings during 15 s.

TABLE 5 | Statistical summary of the analysis of pecking rate for male great tits between rural and urban habitats.

	LRT	*p*	ß	-95% CI	+95% CI
FIXED EFFECTS					
(Intercept)			1.55	0.64	2.61
Age [yearling]	0.76	0.38	−0.07	0.21	0.32
Habitat [urban]	4.79	0.03	−0.46	0.02	1.09
Habitat*Age [urban, yearling]	0.11	0.75	−0.02	−0.38	0.35
RANDOM EFFECTS					
			Variance		
Locality	4.14	0.04	0.04	0.00	3.59
Population	4.78	0.03	0.01	0.00	0.52
Error			0.32	0.13	0.57

The table shows the results of LMMs, in which the significance of terms was evaluated by likelihood-ratio test (LRT). Means of the parameter estimates (ß), variance components, and their respective 95%CI intervals were obtained from the posterior distribution of the corresponding MCMCglmm. In square bracket we indicate to which factor level the parameter estimate corresponds.

TABLE 6 | Statistical summary of the analysis of breath rate between rural and urban habitats.

	LRT	*p*	ß	−95% CI	+95% CI
FIXED EFFECTS					
(Intercept)			62.64	60.35	65.89
Sex [male]	0.649	0.42	−0.16	−1.51	1.38
Age [yearling]	1.186	0.28	0.83	−0.76	2.49
Habitat [urban]	0.432	0.51	1.25	−2.44	4.10
Habitat*Sex [urban, male]	0.696	0.40	−0.71	−2.62	1.37
Habitat*Age [urban, yearling]	0.353	0.55	−0.53	−2.31	2.22
RANDOM EFFECTS					
			Variance		
Locality	0.001	1.00	<0.001	0.00	19.10
Observer	0.321	0.57	2.954	0.00	10.92
Population	16.500	< 0.001	7.632	2.35	15.68
Error			84.28	76.63	95.40

The table shows the results of LMMs, in which the significance of terms was evaluated by likelihood-ratio test (LRT). Means of the parameter estimates (ß), variance components and their respective 95%CI intervals were obtained from the posterior distribution of the corresponding MCMCglmm. In square bracket we indicate to which factor level the parameter estimate corresponds.

multiple European localities, with urban birds having, in general, a higher distress calling rate than their rural conspecifics. In line with this finding, pecking rate showed similar tendencies, but only for males. By contrast, we did not find a general difference in breath rate between urban and rural birds.

Distress behaviors displayed during handling have been hypothesized to reflect the coping style of the different individuals in the presence of an acute stressful situation (Brommer and Kluen, 2012; Class et al., 2014; Kluen et al., 2014; Class and Brommer, 2016). Among the three measured distress behaviors, only distress calling rate showed a clear habitat effect. The higher distress calling rate of urban great tits compared to their rural counterparts is consistent with findings of previous studies that compared bird species (Møller and Ibáñez-Álamo, 2012). Given that high distress calling rates have been related to a proactive coping style (Guillette and Sturdy, 2011; Pascual and Senar, 2014), our results support the view that urban birds are more proactive than rural birds.

Because distress calling and pecking rates were correlated, we expected similar habitat differences for the two traits. Indeed, pecking rate was related to urbanization, but only for males. This sexual difference has previously been observed (Markó et al., 2013), and is congruent with the general observation that males are more aggressive than females. Under this general view, the higher aggression of urban male great tits could be boosted by intense intra-specific competition for a limited number of territories and a potentially higher breeding density in urban areas (Chamberlain et al., 2009). In line with this finding, elevated levels of male territorial aggression have been found in urban song sparrows *Melospiza melodia*, compared to rural male conspecifics. However, this pattern is not necessarily supported in other species (Newman et al., 2006; Bókony et al., 2010; Atwell et al., 2014; Hasegawa et al., 2014). Furthermore, bird species living in urban landscapes have been shown to peck in lower frequencies in response to handling than those inhabiting rural habitats (Møller and Ibáñez-Álamo, 2012). Yet, Møller and Ibáñez-Álamo (2012) did not discriminate between males and females, which hampers a straightforward comparison with our findings. Taken together, these inconsistent results do not allow assessing whether pecking rate is more related to aggression and the latter trait that differ between habitat types, or if it is *per se* a distress behavior that is subjected to selection. In any case, both aggression and behavior upon handling have been regarded as typical indicators of the same proactive coping style (Koolhaas et al., 1999; Carere et al., 2010; Class et al., 2014; Kluen et al., 2014; Dubuc-Messier et al., 2017). Accordingly, our data on great tit pecking rate suggests that urban males are more proactive than rural males. The interaction between habitat and locality was not significant, indicating that the pecking rate differences among habitats was consistent across localities.

In songbirds, breath rate has been proposed as an indicator of acute stress resulting from a predatory attack, and more generally, as an indicator of stress sensitivity and coping style (Carere and van Oers, 2004; van Oers and Carere, 2007; Torné-Noguera et al., 2014). Indeed, some studies found higher breath rates in urban great tits compared to their rural counterparts (Torné-Noguera et al., 2014; Charmantier et al., 2017). Our study suggests that this finding does not represent a general pattern. In other species, such as dark-eyed juncos *Junco hyemalis*, urban birds even had a lower breath rate than their rural conspecifics (Abolins-Abols et al., 2016). Our study further reveals that breath rate did not correlate with any of the other behaviors in great tits. This is surprising, since a positive association between pecking rate and breath rate has previously been documented in the closely related blue tit *Cyanistes caeruleus* (Brommer and Kluen, 2012). However, breath rate has been shown to be affected by seasonal, annual and other ecological factors, hence it may be especially prone to micro-climatic and habitat variation which may mask links to both general urbanization and other behaviors (Torné-Noguera et al., 2014; Charmantier et al., 2017).

The relatively low repeatability of distress calling, potentially due to the low number of repeats per individual (Dingemanse and Dochtermann, 2013; Markó et al., 2013), points to a conservative measure of differences among habitats. Despite the low repeatability, there existed strong differences in distress calling among rural and urban individuals, what points to important differences among habitats, suggesting that distress calling may have evolved in response to urban-induced selection pressures. The between-year repeatability estimates for pecking and breath rate that we recorded, were generally higher than repeatabilities for other behaviors reported in literature (Bell et al., 2009). Given that male pecking rate was different across the habitats and that the repeatability was high, this particular trait seems part of the "urban behavioral phenotype." This finding is also supported by a recent study indicating a non-random distribution of behavioral types along urban gradients, but little behavioral plasticity in response to within-individual variation to urbanization (Sprau and Dingemanse, 2017).

The combined datasets from different European projects across the distribution of great tits enabled us to conduct a cross-population comparison over a large geographic scale. It entailed study-specific differences in capture time, trapping methods, and observer effects. Nevertheless, we were able to detect consistent differences between habitats, implying that effects reported should be conservative. Altogether, our data suggest that urban great tits, especially males, have a more proactive coping strategy when dealing with stressful conditions than rural birds. This finding is in line with studies showing that urban great tits are more explorative and less neophobic than their rural counterparts (Tryjanowski et al., 2016; Charmantier et al., 2017; Riyahi et al., 2017), and display shorter flight initiation distances (Møller, 2008, 2012), which represent other aspects of the proactive coping strategy. Findings on the great tit are also in line with data from other bird species, for which urban populations have also been found to display proactive behaviors (Evans et al., 2010; Carrete and Tella, 2017), but these studies compared few populations (Miranda et al., 2013). Our study included 14 populations from a large geographic area and revealed consistent results across localities, allowing to generalize the impact of urbanization on great tit coping styles.

This proactive strategy of urban great tits could be advantageous in the urban environment. Proactive individuals, for instance, more rapidly discover and use new food resources (van Overveld and Matthysen, 2010), and are less afraid of novel objects or environments than reactive birds (Tryjanowski et al., 2016; Charmantier et al., 2017; Riyahi et al., 2017), which could help urban dwellers to take profit of the new feeding opportunities that a city can provide (e.g., Fisher and Hinde, 1949). Our study therefore implies that urban birds may adapt to human disturbance by a process of local adaptation, as suggested previously (Partecke et al., 2006; Sol et al., 2013). Future research should investigate whether the differences in distress behaviors reported here have an underlying genetic base, hence are due to local adaption caused by divergent natural selection, or originate from phenotypic plasticity.

ETHICS STATEMENT

Behavioral data was recorded during ringing operations. Capture and ringing was performed under individual ringing permits delivered by the ringing office within each country.

AUTHOR CONTRIBUTIONS

The study was conceived by JCS, LG, VT, CB, GM-R, and CI. The data were collected by VT, CB, GM-R, PaS, JR, PhS, ND, AC, VD, and HN. The statistical analyses were conducted by JCS and LG. The manuscript was written by JCS and LG with input from all other co-authors. Contributed funding and materials: JCS, VT, CB, GM-R, PhS, ND, AC, CI.

FUNDING

This work was supported by funds from the Ministry of Economy and Competitivity, Spanish Research Council (CGL-2016-79568-C3-3-P; to JCS. CGL2015-70639-P; to LG. CGL2014-55969-P; to GM-R), from the National Research, Development and Innovation Office (Hungary) (K-115970; to LG), from the Estonian Ministry of Education and Research (institutional research funding IUT no. 34-8; to VT), from the European Research Council (ERC-2013-StG-337365-SHE; to AC), from the OSU-OREME (to AC), from the Deutsche Forschungsgemeinschaft (DFG) (SP 1450/3-1; to PhS), from the European Union a Marie Curie Re-Integration grant (CIG 322217; to CI) and from the Swedish Research council (C0361301; to CI).

ACKNOWLEDGMENTS

We thank the Institute of Parks and Gardens of Barcelona (Parcs i Jardins), the mairie de Montpellier and the cities of Paris and Rueil-Malmaison, for allowing us to sample birds in their city parks. We also thank the National Forest Office (ONF) for allowing us to survey tit populations breeding in state-owned forest, the late Leopoldo Gil, who always supported our work, for allowing us to sample birds in Can Catà forest area, and Hermanitas de la Asunción for allowing us to sample birds in the Sarria area. We also thank Lluïsa Arroyo, Arnaud Grégoire, Marcel Lambrechts, Mar Comas, Nuria Mora, Abelardo Requena, Alexia Mouchet, Maria Moiron, Robin Abbey-Lee, Natalia Perez Ruiz, Mattia Bessone, Rui Machado, Lucila Fernandez, Alexander Hutfluss, Diogo Barros, Gülperi Stenhouse, Josefa Bleu, Simon Agostini, Beatriz Decencière, Erika Beaugeard, Jeanne Dupuy, Eva Du Tien Hat, Laura Grosvalet, Lucie Mathieu, Juliette Rabdeau, Marine Ramirez, Baptiste Vancostenoble, Antonin Waterschoot, Audrey Fournier, Estelle Garrouste, Marilou Keck, Alice Le Priol, Cécilia Mondet, Amélie Pinel, and Quentin Pionneaud for help during field work. Anders P. Møller also provided useful advice during the early stages of the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

REVIEW
published: 07 April 2017
doi: 10.3389/fevo.2017.00024

Garden Bird Feeding: Insights and Prospects from a North-South Comparison of This Global Urban Phenomenon

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Intentional feeding of wild birds in gardens or backyards is one of the most popular forms of human–wildlife interactions in the developed world, especially in urban environments. The scale and intensity of bird feeding are enormous with mainly birdseed consumed daily by a range of species. This represents a subsidy to natural diets of birds attracted to the feeders and typically involves novel dietary components. Yet, relatively little is known about how it influences the behavior and ecology of the species visiting feeders. In part, research has been hampered by logistical difficulties of working in urban areas but studies have demonstrated powerful influences on behavior and phenology of avian breeding, the spread of disease, and the structure of avian communities. Here, we compare bird feeding between Northern and Southern Hemispheres as a means of exploring how similarities and differences in avian responses might inform knowledge of this global urban phenomenon. We start by tracing its origins to north-western Europe and how its expansion has occurred before considering how geographical differences in feeding practices and attitudes map onto bird feeding "on the ground." We explore some of the major emerging themes of recent interest, including why citizens are motivated to feed birds, whether birds become fully dependent on food supplements, the role of feeding in avian disease transmission, and how feeding changes urban bird communities. By proposing that scientists work in collaboration with the public providing food to birds, we pose key research questions that need to be answered urgently and suggest accompanying experimental approaches to do so. These approaches are essential if we are to improve our understanding of how bird feeding shapes the behavior, ecology, movements, and community structure of urban birds. Our hope is that through such citizen science we will be able to provide advice as to location-relevant practices that should maximize benefits to both urban biodiversity and human well-being, and minimize potential adverse impacts. We demonstrate that bird feeding is important for urban biodiversity conservation, community engagement, and in establishing personal connections with nature and their associated benefits.

Keywords: carry-over effects, citizen science, community structure, dependency, disease transmission, food supplements, human well-being, source-sink dynamics

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *31 January 2017*
Accepted: *21 March 2017*
Published: *07 April 2017*

Citation:
Reynolds SJ, Galbraith JA, Smith JA and Jones DN (2017) Garden Bird Feeding: Insights and Prospects from a North-South Comparison of This Global Urban Phenomenon. Front. Ecol. Evol. 5:24. doi: 10.3389/fevo.2017.00024

INTRODUCTION

The world is urbanizing rapidly (United Nations, Department of Economic and Social Affairs, Population Division, 2014) and, as a result, human–wildlife interactions will become ever more commonplace. One of the most popular and globally common of such interactions is the feeding of wild birds in residential gardens or backyards (hereafter referred to as "garden bird feeding") that is widespread across many parts of the developed world (Jones, 2017). This pastime is increasingly becoming the subject of scientific and societal scrutiny (Jones and Reynolds, 2008; Robb et al., 2008a). Bird feeding is variously advocated as an essential conservation activity, a simple way for people to connect with nature in an urbanizing world, and a means for enhancing environmental awareness and psychological well-being (Schoech et al., 2008; Davies et al., 2012; Cox and Gaston, 2016). However, it has also been implicated in the spread of catastrophic avian diseases (e.g., Dhondt et al., 2007; Robinson et al., 2010), altering ecosystem structure (Galbraith et al., 2015), benefiting invasive species (Galbraith et al., 2015), changing predator-prey dynamics (Malpass et al., 2017), and even contributing to rapid evolutionary change (Bearhop et al., 2005). Furthermore, the possibility that birds may become dependent on anthropogenic food is a primary concern of both opponents to, and proponents of, bird feeding (e.g., Howard and Jones, 2004; Jones, 2011). These issues constitute a far from exhaustive list and clearly a more complete understanding of this interaction between birds and humans should be a significant research priority. This is especially pertinent within the context of urban ecology (Robb et al., 2008a) as it is becoming clear that many animal and plant populations exhibit "phenotypic signatures" associated with the urban areas in which they live (Alberti et al., 2017). We have previously described bird feeding as a supplementary feeding experiment on a global scale but without due consideration of its effects on the behavioral, community, and population ecology of the birds consuming supplements (Jones and Reynolds, 2008). However, despite the above concerns, scientific investigations into most aspects of the practice are fragmentary and geographically limited.

Here, we employ a comparison between Northern and Southern Hemispheres to compare and contrast the impacts of bird feeding on the biology of birds, and on avian populations and communities. We identify gaps in knowledge that require bridging as a matter of urgency because bird feeding appears to be growing in popularity (Jones, 2017). Ultimately, we will identify key research priorities that should be targeted by researchers and citizen scientists with the ultimate aim of promoting avian conservation efforts globally.

DEFINITIONS, SCALE, AND GEOGRAPHY

Our first task is to define what we mean by garden or backyard "bird feeding." The focus of this review is the intentional feeding (i.e., food supplementation) through the provision of food to free-living birds (**Figure 1**). It does not include the incidental feeding of birds congregating at locations where food sources are available as a result of disposal (e.g., landfill, fish discards from fishing vessels) or feeding of non-avian companion taxa such as dogs *Canis lupus familiaris* and cats *Felis catus silvestris*. We considered the growing interest in bird feeding in the scientific literature by performing a literature search (**Figure 2**) of the ISI Web of Science in January 2017 entering "food supplement* AND bird*" as a search term. We defined the start of this literature search as being the year when the first major review was published in the primary scientific literature by Boutin (1990) who considered the impacts of feeding on free-living terrestrial vertebrates, including birds. The search included articles, proceedings papers, reviews, notes, and editorial materials, and was restricted to the "Ornithology" Web of Science category; this search yielded 206 items. **Figure 2** reveals a slow but steady increase in the scientific outputs related to bird feeding, demonstrating that the subject is of increasing interest.

The scale of bird feeding by a range of different criteria is remarkable. In the United Kingdom (UK), various studies have found that more than 60% of households regularly feed birds in their gardens, spending US\$188–226 million on 60,000 tons of birdseed annually (Fuller et al., 2008). Robb et al. (2008a) estimated that sufficient seed was distributed for blue tits *Cyanistes caeruleus* alone to support five times the national population of the species. In the United States (US), the 2011 National Survey of Fishing, Hunting, and Wildlife-Associated Recreation reported that 52.8 million households practiced some form of bird feeding, giving a countrywide rate of 73% (U.S. Fish Wildlife Service and U.S. Census Bureau, 2011) spending a total of US\$4 billion on birdseed and an additional US\$10 billion on related hardware and peripherals, annually. The annual amount of bird food supplied in the US is ∼500,000 tons, enough to feed 300 million chickadees *Poecile* spp. if they consumed nothing else (Robb et al., 2008a).

Although bird feeding is commonly practiced through much of North America and parts of Europe, relatively little is known about its extent and scale in countries other than Germany, Norway, Sweden, the UK, and the US (Jones, 2017). Anecdotally, there has long been a general impression that it is a far more established practice in the more northern European countries, a conclusion confirmed by a recent informal qualitative survey (Jones, 2017). The widespread practice of bird feeding is directly linked to geographical areas that experience prolonged or extreme climatic conditions during winter. Indeed, apart from the notable exceptions of the UK (Cox and Gaston, 2015) and Germany (Berthold and Mohr, 2006), bird feeding in northern Europe remains almost entirely a winter-only activity. In contrast, bird feeding is rarely practiced throughout the countries of southern Europe, although there are some enclaves—notably areas with residents from more northern European countries—where some bird feeding does occur (Jones, 2017).

It is noteworthy that bird feeding—at least as practiced in much of the Northern Hemisphere—is not just the preserve of "northern" countries but also of the "Western World"; bird feeding is apparently virtually unknown in most of Asia, including China, Korea, Japan, and most countries of the south-east (Jones, 2017). An important exception occurs in many parts of the Indian subcontinent and beyond where daily offerings,

FIGURE 1 | Examples of bird species feeding on food supplements in city gardens in (A) New Zealand (tui *Prosthemadura novaeseelandiae*) (Photo: J. Galbraith), **(B)** the US (house finch *Carpodacus mexicanus*) (Photo: B. Vuxinic), **(C)** the UK (blue tits *Cyanistes caeruleus*) (Photo: J. Galbraith), and **(D)** Australia (rainbow lorikeet *Trichoglossus moluccanus*) (Photo: D. Jones).

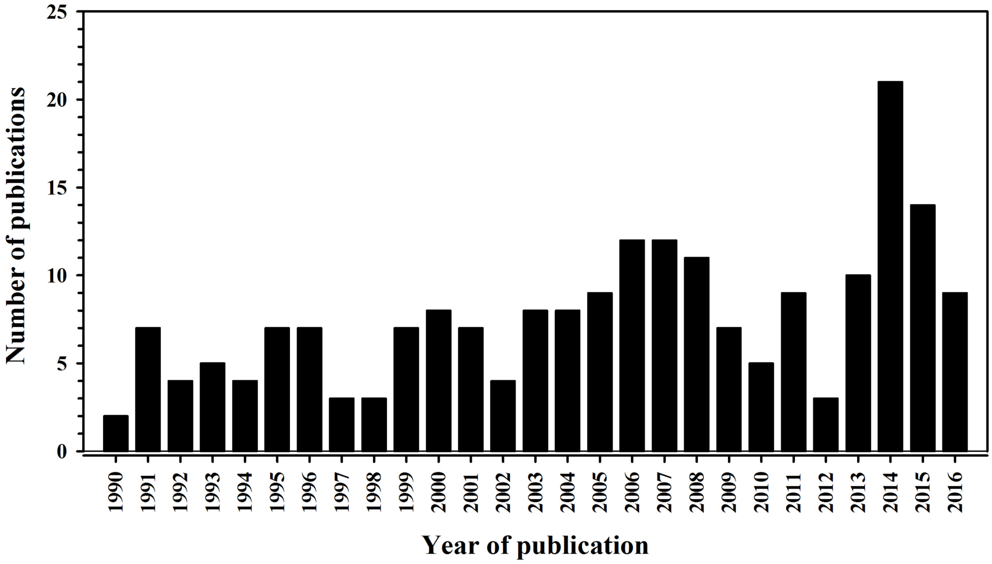

FIGURE 2 | Number of publications (including articles, proceedings papers, reviews, notes, and editorial materials) from a search of the ISI Web of Science conducted in January 2017 about food supplementation of birds. Note that the literature search ended in 2016 because 2017 was less than a month old when the search was performed.

traditionally of rice cakes, are made to birds (and other animals) in the practice of *Bhuta-Yajna*, a ritual observed by orthodox Hindus (Jones, 2017). Undoubtedly, ethno-biology, and more specifically ethno-ornithology (Tidemann and Gosler, 2010), are increasingly providing further insights into practices such as feeding of wildlife and how they explain the prominent role that animals play in human culture (Cocker, 2013). However, in the context of non-ritualistic feeding of birds within our towns and cities, it is important to remember that while we regard it as commonplace and familiar, it appears to be confined to parts of the world populated by people originally from north-western Europe (Jones, 2017).

NORTH VS. SOUTH

We are not the first to consider the impacts of bird feeding on avian biology (see Boutin, 1990; Reynolds et al., 2004; Robb et al., 2008a), but we break new ground in considering comparative spatial perspectives. Traditionally, attempts to explore the origins of bird feeding have emphasized the significance of harsh winter weather on the origins of the practice, with the humane response to the apparent suffering of birds being an obvious motivation (Fuller et al., 2008). However, this perspective has limited relevance to bird feeding in several major countries of the Southern Hemisphere (Chapman, 2015). In general, the main population centers of Australia, New Zealand, and South Africa do not experience the often prolonged and severe periods of cold typical of much of the Northern Hemisphere. Despite such differences in climate, the feeding of wild birds in Australia and New Zealand is on a similar scale to that of the Northern Hemisphere; recent surveys have revealed participation rates of households of 36–57% in New Zealand (Galbraith et al., 2014), and 63% in Australia (Chapman, 2015) (No similar studies have been conducted in South Africa despite the practice being popular among Europeans in cities such as Cape Town and Durban). Although there was evidence of a slightly higher frequency of feeding in winter in these two countries, by far the largest proportion of participants did so throughout the year and most provided food items daily. Nonetheless, many Australians also indicated that they were especially likely to feed or increase the amount of food supplements during challenging times for birds such as heat waves, and extended periods of drought and cold (Galbraith et al., 2014; Chapman, 2015).

Perhaps the most unexpected contrast in bird feeding characteristics between the hemispheres is provided by the bird species visiting feeders (**Table 1**; **Figure 1**). While species richness at feeders is high in all countries where garden bird feeding is a common pastime, by far the most frequent visitors in North America are black-capped chickadees *Poecile atricapillus* and Carolina chickadees *Poecile carolinensis*, and blue and great tits *Parus major* in the UK, all being members of the family Paridae (Toms and Sterry, 2008; Baicich et al., 2015). These species are typically small, weighing between 10 and 19 g (all body masses reported from Dunning, 2008). Extending the list to the top five species that are the most frequent feeder visitors, the heaviest in the US is the mourning dove *Zenaida macroura* (119 g) and in the UK it is the common blackbird *Turdus merula* (113 g). In contrast, the two most frequent species at feeders in Australia, the Australian magpie *Gymnorhina tibicen* and the rainbow lorikeet *Trichoglossus moluccanus*, weigh 212–360 and 84–169 g, respectively. Therefore, Australian feeder birds are far larger than those typically found at Northern Hemisphere feeders. In New Zealand, the situation is strongly influenced by the abundance of introduced species with the most common being house sparrows *Passer domesticus* (20–35 g), common blackbirds and common mynas *Acridotheres tristis* (82–140 g) with the only native species feeding on grain-based supplements being the silvereye *Zosterops lateralis* (9–17 g; Galbraith et al., 2015). In South Africa, while the diversity of birds visiting gardens is remarkably large—from diminutive waxbills (Estrildidae) to huge hornbills (Bucerotidae)—we could find no reliable data with which to compile a comparative list of the most common species observed at bird feeders.

While the predominant food supplements provided to wild birds in garden feeders throughout the world are seeds, especially sunflower *Helianthus* spp. and millet *Panicum* spp., nyjer *Guizotia abyssinica,* and various cereals, as well as peanuts *Arachis hypogaea*, several other supplements are frequently used, often triggered by changes in local weather (Berthold and Mohr, 2006; Toms and Sterry, 2008; Baicich et al., 2015). For example, various fat-rich items (typically suet balls, often with added peanuts) are commonly fed in winter. In areas with nectar-feeding species such as hummingbirds (Trochilidae), silvereyes, honeybirds (Indicatoridae), and lorikeets (Psittacidae), a wide variety of sugar- or honey-based solutions is provided in drinkers. Bread is also an extremely common food source provided in all countries where bird feeding is widespread (Jones, 2017).

As well as differences in avian community composition at feeders of Northern and Southern Hemispheres (**Table 1**), there are major differences in the types of food supplements provided, especially in Australia where meat is routinely fed to populous species such as Australian magpies, butcherbirds *Cracticus* spp., and kookaburras (Alcedinidae) that are all relatively large species and abundant in urban landscapes (O'Leary and Jones, 2006; Jones, 2011). The most commonly provisioned form of meat is raw beef mince (or ground beef), presumably because it is relatively inexpensive and readily available, although pieces of sausage, salami, ham, bacon, and cooked chicken are also provided (Ishigame and Baxter, 2007). This raises many concerns including the impacts on bird communities dominated by large and often predatory species, the hygiene of feeding structures, and the nutritional impacts if these food supplements were to constitute a large proportion of their diets. Rainbow lorikeets, for example, that are specialized consumers of pollen and nectar have learned to consume meat supplements too; this recent expansion of their foraging niche is resulting in much consternation among participants and ecologists (Gillanders et al., in press).

HUMAN MOTIVATIONS AND REASONS FOR FEEDING

Whether in Northern or Southern Hemispheres, at its most superficial level the simple act of feeding birds in one's private

TABLE 1 | The top 10 bird species visiting feeders in selected countries of the Northern and Southern Hemispheres.

Rank	Species name	Mean body mass (g)	Food sources
UK[a]			
1	House sparrow *Passer domesticus*	27.7	Seeds, buds, berries, and animal matter
2	Blue tit *Cyanistes caeruleus*	10.6	Seeds and insects
3	Common starling *Sturnus vulgaris*	86.0	Many and varied
4	Common blackbird *Turdus merula*	113.0	Invertebrates, worms, fruits, and seeds
5	Common wood pigeon *Columba palumbus*	490.0	Leaves, buds, flowers, seeds, berries, and grain
6	Common chaffinch *Fringilla coelebs*	27.7	Insects, seeds, and buds
7	European goldfinch *Carduelis carduelis*	16.0	Seeds, buds, flowers, flowers and fruits
8	Great tit *Parus major*	18.3	Insects, seeds, and fruits
9	Eurasian collared dove *Streptopelia decaocto*	149.0	Seeds and other plant material
10	European robin *Erithacus rubecula*	17.7	Invertebrates, fruits, seeds, small vertebrates, and carrion
US[b]			
1	Dark-eyed junco *Junco hyemalis*	18.6	Seeds and insects
2	Mourning dove *Zenaida macroura*	119.0	Seeds and grain
3	Blue jay *Cyanocitta cristata*	88.0	Nuts, seeds, eggs, nestlings, and insects
4	Black-capped chickadee *Poecile atricapillus*	10.8	Insects, buds, and seeds
5	Downy woodpecker *Picoides pubescens*	25.7	Insects, seeds, nuts, and berries
6	House finch *Carpodacus mexicanus*	21.4	Seeds, nuts, and grain
7	American goldfinch *Carduelis tristis*	12.8	Seeds and grain
8	Northern cardinal *Cardinalis cardinalis*	42.7	Seeds, fruit, buds, and insects
9	House sparrow	27.7	Seeds, grain, insects, and food waste
10	White-breasted nuthatch *Sitta carolinensis*	21.0	Acorns, seeds, nuts, and insects
AUSTRALIA[c]			
1	Australian magpie *Gymnorhina tibicen*	287.0	Insects
2	Rainbow lorikeet *Trichoglossus moluccanus*	133.0	Nectar, pollen, fruits, seeds, and insects
3	Parrots (mainly eastern rosella *Platycercus eximius*)	104.0	Seeds, blossoms, and insects
4	Crimson rosella *Platycercus elegans*	132.5	Seeds, insects, and blossoms
5	Crested pigeon *Ocyphaps lophotes*	204.0	Seeds, leaves, and insects
6	Gray butcherbird *Cracticus torquatus*	83.1	Small vertebrates, insects, fruits, and seeds
7	Sulphur-crested cockatoo *Cacatua galerita*	790.0	Seeds, grains, fruits, flowers, and insects
8	Laughing kookaburra *Dacelo novaeguineae*	334.5	Insects, worms, crustaceans, and small vertebrates
9	Galah *Eolophus roseicapilla*	307.3	Seeds
10	Spotted dove *Streptopelia chinensis*	159.0	Seeds
NEW ZEALAND[d]			
1	House sparrow	27.7	Seeds, buds, berries, and animal matter
2	Common blackbird	113.0	Invertebrates, worms, fruits, and seeds
3	Silvereye *Zosterops lateralis*	12.9	Insects, fruits, and nectar
4	Song thrush *Turdus philomelos*	67.8	Invertebrates and fruit
5	Common starling	86.0	Many and varied
6	Finches (mainly common chaffinch, common redpoll *Carduelis flammea*, European goldfinch, and European greenfinch *Chloris chloris*)	13.0–26.0	Insects, seeds, buds etc.
7	Tui *Prosthemadera novaeseelandiae*	107.3	Nectar, honeydew, fruits, and insects
8	Common myna *Acridotheres tristis*	116.0	Many and varied
9	Gulls (e.g., black-billed gull *Larus bulleri*, kelp gull *Larus dominicanus*)	230.5–941.0	Many and varied
10	New Zealand fantail *Rhipidura fuliginosa*	8.0	Invertebrates

Also included for each species are details of its body mass (means calculated across the sexes and across multiple locations as reported in Dunning, 2008), and its main dietary constituents (as described on the relevant Birdlife International website for each country). Data sources: [a]RSPB's Big Garden Birdwatch for 2014; [b]Barker and Griggs, 2000; [c]Chapman, 2015; and [d]Galbraith, 2016.

garden may appear to be little more than a way to see birds close at hand and to promote their persistence and welfare. However, several detailed and recent studies have expanded our knowledge of motives further. For example, in the UK, Cox and Gaston (2016) found that most participants felt relaxed and connected with nature, feelings that increased positively with the frequency of bird feeding activity. Furthermore, public perception of decreasing natural food supplies resulted in increased intensity of bird feeding. A recent investigation of the motivations of participants within the south-east of England (Clark, 2013) identified a complex suite of influences and drivers, with pleasure, enhancing survival, and a desire to nurture all being significant. These results suggest a strong relationship between perceptions—regardless of their veracity—and practice among people feeding birds, and illustrate the potential role this activity may play in promoting mental wellness. The physical, psychological, educational, and social benefits from interaction with nature through a wide variety of means are being increasingly recognized (Horvath and Roelands, 1991; Beck et al., 2001; Shanahan et al., 2014).

The above conclusion may be appropriate and relevant in a cultural setting in which garden bird feeding is popular and promoted, as is the case in the UK. However, in contrast, in Australia where although bird feeding occurs at similar levels to that in the UK, there is widespread antipathy toward the practice among many environmental and conservation groups, a stance broadly recognized (although largely ignored) by most participants (Howard and Jones, 2004; Jones, 2011, 2014). This opposition generates concern among participants about the potential impacts of feeding birds and a common reluctance to discuss it publicly (Jones, 2014). Nonetheless, the potential welfare benefits that garden bird feeding provides for humans connecting with nature are presumably equivalent between Australia and the UK. Given these contrasting societal contexts, exploring the salient motivations of participants from the two countries is sure to yield valuable insights. Adapting Kellert's widely used "wildlife values" to discern themes among participants, Chapman (2015) found unexpected similarities between the two groups. Among UK participants, the theme associated with care and responsibility for the birds they feed was by far the most predominant. Given the more equitable climate of Australia, this featured highly too for Australian participants and was second only to the enjoyment that they derived from the activity. Interestingly, enjoyment was the second most important motivator for feeding in the UK too. Participants from both countries were also motivated by reasons associated with a meaningful connection with nature as well as the more objective goal of observation (Chapman, 2015). Thus, despite seemingly significant differences in the status of the pastime between the two countries, their citizens share many of the same motivations for bird feeding.

FEEDING AND DEPENDENCY

The obvious popularity and widespread practice of garden bird feeding in the Northern Hemisphere is often acknowledged to have a generally positive value for both human participants and birds (e.g., see Baicich et al., 2015) with sometimes forceful arguments based on apparent welfare and conservation benefits being advanced in favor of the practice (Berthold and Mohr, 2006). Similar claims are also made by feeding proponents in the Southern Hemisphere (see Jones, 2011 for further details). Yet, Australia is notable in the extent to which the practice is opposed (Jones, 2017). While reasons given for this opposition are largely similar to concerns expressed elsewhere—potential dependency on anthropogenic foods, the spread of disease, inadequate nutrition, attracting predators and vermin, for example—despite their ubiquity, most are not based on robust empirical data (Jones, 2011; Murray et al., 2016). What little has been published has tended to demonstrate that these concerns, while justified, are often less straightforward than was initially hypothesized (Robb et al., 2008a). This can be illustrated with reference to the first three of these well-known issues. Numerous studies have found that the possibility of birds becoming dependent upon anthropogenic food supplements is a primary concern for both advocates and opponents of bird feeding (e.g., Rollinson et al., 2003; Jones and Reynolds, 2008). There are certainly examples of avian populations that are entirely reliant on supplementary food in winter, including tits in Finland (Jansson et al., 1981) and Anna's hummingbirds *Calypte anna* in British Columbia, Canada, that feed from heated feeders supplying sugar solution to them even in the coldest weather (Jones, 2017). In these cases, birds exhibit full dependence on food supplements with their survival through the winter not being possible without access to them.

Such examples also illustrate another unforeseen outcome increasingly being attributed to an abundance of, and dependency of birds on, anthropogenic food: the tendency of some individuals or groups to overwinter or alter their migration route (Courter et al., 2013). However, even among the well-studied examples such as Eurasian blackcaps *Sylvia atricapilla* (Plummer et al., 2015) and white storks *Ciconia ciconia* (Massemin-Challet et al., 2006), it is difficult to disentangle various other potential influences such as the effects of climate change.

In the few studies where dependency of supplementary food has been explicitly investigated in resident species, the predicted outcomes did not eventuate. In their study of wintering black-capped chickadees in Wisconsin in the US, Brittingham and Temple (1992) found that a population supplied with supplementary food for 25 years had an identical survivorship to that of an unfed (control) population nearby. In Australia, adult Australian magpies continued to provide natural foods to their nestlings even when large supplies of favored foods were readily available (O'Leary and Jones, 2006). There are numerous other studies from a wide variety of species that strongly suggest that in the vast majority of cases individuals that visit feeders do so in rather a sporadic fashion with the diet comprising mainly natural food sources (e.g., Harrison, 2010; Robb et al., 2011; but see Sauter et al., 2006). Nevertheless, the proportion of the diet constituted by food supplements can vary seasonally (e.g., Chamberlain et al., 2007).

FEEDING AND DISEASE TRANSMISSION

One of the most obvious characteristics of bird feeding is that, unlike natural food sources, food supplements are typically made available regularly, in a surfeit, and in the same location.

This has a consequence of concentrating many birds in one place, often including species that are unlikely to interact when foraging naturally, as they compete closely for access to food. As well as increasing aggressive interactions (e.g., Wojczulanis-Jakubas et al., 2015; Le Louarn et al., 2016; and presumably stress levels), these aggregations also provide ideal conditions under which infectious agents persist and spread. Perhaps the best-studied example is provided by the so-called House Finch Disease, a particularly virulent form of conjunctivitis spread by mycoplasmal bacteria (e.g., Dhondt et al., 2005). Within a few months of this disease's appearance among house finches *Carpodacus mexicanus* in the mid-1990s near Washington DC in the US it was reported by participants in the Cornell Lab of Ornithology's *Project FeederWatch*. This citizen science program already had a large and active network of members and, having been informed about the disease outbreak, they were able to provide real-time information on the spread of the epidemic as it moved rapidly through the eastern US (Bonney and Dhondt, 1997). Within just a few years, the house finch population in the eastern US had declined by a third, partly because of the gregariousness of the species, but especially because of the bacteria's capacity to remain viable on damp feeding structures (Adelman et al., 2015). Although there is evidence that access to feeders enabled some infected birds to survive for longer (by being able to access food despite being sight-impaired), their tendency to remain for prolonged periods on or near feeders undoubtedly increased the likelihood of infection (Adelman et al., 2015). No cure or antidote has been developed for this disease and incidents are still reported.

A similar disease phenomenon occurred in the UK in 2005–2006 involving a trichomoniasis epidemic among several finch (Fringillidae) species but primarily impacting European greenfinches *Chloris chloris*. This species had been one of the chief beneficiaries of the increase in the popularity of bird feeding over the preceding decades, its considerable increase in abundance having been attributed at least in part to its attraction to nyjer seed provided as a food supplement (Lawson et al., 2012a). Within a single year of the outbreak of this extremely contagious bacterial infection, the British greenfinch population declined by 35% (Robinson et al., 2010). A detailed picture of the spatio-temporal dynamics of the disease was only made possible through the network of participants in the British Trust for Ornithology's (BTO's) *Garden BirdWatch* citizen science program (Robinson et al., 2010). As for the House Finch Disease outbreak, the role of feeders as sites for disease transmission was called into question for the trichomoniasis outbreak. Prior to this, the disease had been unknown among finches; in mainland Europe, it was primarily associated with rural-dwelling columbids such as common wood pigeons *Columba palumbus* (Höfle et al., 2004). However, in recent decades this species traditionally found in the countryside, has increasingly become a resident of towns and cities, an unexpected move attributed both to declining food resources in rural areas and the proliferation of seed feeders within urban areas (**Table 1**; Lawson et al., 2012a). Although as yet unconfirmed, this suggests a potential source of cross-species transfer of infection that previously would have been unlikely.

These two disease outbreaks are highlighted mainly because of their scale and impact, but also because of the possible role bird feeding plays in increasing rates of infection in avian populations. Moreover, numerous other diseases may be similarly spread because of the interactions of birds at feeders. These include salmonellosis and Avian Pox, highlighting the importance of thorough and frequent cleaning of feeders as best practice when feeding birds (Lawson et al., 2012b). Despite the severity of these outbreaks, and the relatively high level of publicity associated with them, relationships between feeders and disease remain remarkably under-studied. In a rare exception, the presence of pathogens was investigated among common garden bird species in New Zealand, comparing individuals frequenting feeders with those that were not (Galbraith et al., 2017). All pathogens of interest—*Salmonella, Chlamydophila* and Avian Pox Virus—were detected in at least one of the species, with *Salmonella enterica* being present on ∼7% of all feeding structures examined. In addition, birds using feeders carried greater parasite loads than those that did not, with common blackbirds having more helminths and house sparrows more feather lice (Phthiraptera) (Galbraith et al., 2017).

FEEDING AND AVIAN COMMUNITY STRUCTURE

The importance of food resources in all aspects of the lives of animals is fundamental to understanding population and community dynamics. These complex interactions have been investigated experimentally in a vast number of supplementary feeding studies on many different species and in many biomes. However, remarkably little empirical data exist for comparison between cities and species. In part, this is due to the many challenges associated with undertaking scientifically robust studies in environments unavoidably occupied by high densities of people. Nonetheless, a growing number of important pioneering investigations are shedding light on the ecological influence of food supplements on the local community of birds. One recent study compared the changes in composition and abundance of the suburban bird community in Auckland, New Zealand over an 18-month period during which food was provided by householders, followed by its withdrawal (Galbraith et al., 2015). The results were dramatic for several of the species involved, with increased abundance of house sparrows and spotted doves *Streptopelia chinensis*—both introduced species—during the provisioning phase, while that of the native gray gerygone *Gerygone igata* was significantly lower. The influence of feeding in shaping avian communities was further emphasized when the community returned to its pre-supplementary feeding structure within only a few weeks of the cessation of feeding. An important (but rarely achieved) aspect of this study was the willing compliance of the participants to engage with the scientific objectives in stopping feeding birds when instructed to do so to allow the role of feeding to be investigated rigorously.

Although we have claimed that bird feeding is extremely popular and effectively ubiquitous, the timing, duration, and intensity of the practice can be markedly heterogeneous even over short spatial scales. For example, Lepczyk et al. (2004) found almost twice as many feeders in suburban as in rural areas of Michigan in the US. In the UK, Davies et al. (2012) attempted to discern patterns of participation in feeding at a national scale and found considerable variation, although the prevalence increased with the more detached house types and participant age. In a more detailed investigation within the city of Sheffield in the UK, Fuller et al. (2008) found a clear negative relationship between socioeconomic deprivation and the proportion of households participating in feeding. This study also found a notably robust positive correlation between the density of feeders and the overall abundance of birds. However, there was no such relationship between feeder density and species richness (i.e., feeding increases numbers of birds but not their diversity).

DIRECTIONS FOR FUTURE RESEARCH

The remainder of this piece focuses on research priorities that should concern all of us with interests in bird feeding, whether researchers investigating the biological effects of providing food supplements to birds to address targeted research questions, or members of the general public feeding birds over long temporal and wide spatial scales. The world is urbanizing ever more rapidly (United Nations, Department of Economic and Social Affairs, Population Division, 2014) and as a consequence our interactions with wildlife generally, and birds more specifically, are likely to increase in frequency and, in the case of bird feeding, intensity. That urbanization will inevitably influence all aspects of avian life has not gone unnoticed by ornithologists; research examining how urbanization influences the behavior and ecology of birds has been summarized in a number of books over the last 20 years (e.g., Bird et al., 1996; Marzluff et al., 2001; Lepczyk and Warren, 2012; Gil and Brumm, 2014; Marzluff, 2014; Jones, 2017). Many provide invaluable accounts of how urbanization (and sub-urbanization) impacts birds in terms of their behavior, ecology, physiology, abundance, and distribution. The next major challenge, however, is to determine how food availability, especially through the provision of food supplements, influences the biology of birds in our urban centers while contemporaneously being able to control extrinsic factors that influence the biology of urban birds equally strongly (e.g., predation—Gering and Blair, 1999; temperature—Stager et al., 2016; light pollution—Kempenaers et al., 2010; noise pollution—Arroyo-Solís et al., 2013).

In the case of garden bird feeding, we think that it is highly unlikely that the practice will decline in popularity in countries where it is well-established. In fact, there is evidence to suggest that it may intensify as human populations are ever more concentrated in cities in the future where feeder density will inevitably increase (Fuller et al., 2008). Therefore, it may be timely to harness further the power of citizen science (Dickinson and Bonney, 2012) to investigate how feeding influences individual birds, and avian populations and communities in a concerted and structured way. Citizen science has significantly advanced our understanding of various aspects of the breeding biology of birds (e.g., phenology, clutch size, productivity; reviewed in Cooper et al., 2015) and promises significant accumulations of further knowledge through carefully planned and coordinated research projects (Greenwood, 2007). We see no reason why those engaged in bird feeding would not embrace the opportunity to carry out similar projects that improve our understanding of its impacts on urban birds.

Below, we retain the comparative perspectives offered by ongoing research in the Northern and Southern Hemispheres to explain what we consider to be the key future research priorities. Such an approach allows us to compare and contrast the responses to feeding of species between different ecological (and avian) communities (**Table 1**), under different seasonal conditions and under different patterns for introduced species as well as native species sometimes in competition for food at feeders. Here, we pose a number of research questions that will allow us to gain further and new insights into how individuals, populations, and communities respond to bird feeding.

WHAT CONTRIBUTION DO FOOD SUPPLEMENTS MAKE TO A BIRD'S DIET?

Modern field ornithology has access to an increasing number of methodologies that allow this question to be answered effectively. They include, for example, the marking of individual birds with devices such as Passive Integrated Transponder (PIT) tags that quantify visitation rates to feeders where receivers have been incorporated into feeder access points (e.g., Aplin et al., 2013). However, the number of feeder visits that a bird makes may reflect intense defense of food, and therefore the value that the bird places on this resource, but it may not indicate levels of food consumption. More invasive protocols involving tissue sampling enable methods such as fatty acid signature characterization to be carried out (Andersson et al., 2015) while stable isotope analysis (SIA; Inger and Bearhop, 2008) allows comparisons of biomarkers within samples with dietary reference material. Such approaches have revealed that there is much variability in dietary intake of food supplements within populations of blue tits visiting feeders in the winter in Northern Ireland (Robb et al., 2011). They were also used to determine the breeding diet of adult great tits and blue tits in central England that contained only a small percentage (∼9%) of food supplements, suggesting that birds were only "snacking" during visits to feeders (Harrison, 2010). Furthermore, food supplements were estimated to constitute only ∼9% of nestling diet suggesting that adults were feeding natural foods such as Lepidopteran larvae to their offspring (Harrison, 2010). Statistical approaches that allow dietary reconstruction from SIA outputs using Bayesian modeling are growing ever more sophisticated (e.g., Parnell et al., 2013) and now extend to multiple food (reference) sources. These approaches promise much in improving knowledge of urban birds' dependence on food supplements.

PROPOSAL A

Examination of the patterns of feeder use by birds exposed to long-term food supplementation and how they relate to over winter-survival, recruitment into the breeding population, investment of energy, time, etc. to current vs. future breeding attempts. This could be achieved through surveys combining monitoring efforts of both feeders and nests, and intense tissue sampling of birds at feeders and of birds and/or their eggs at nests to describe diet composition. The challenge will be directly relating dietary composition to reproductive and life-history traits of birds when we know that they are sensitive to so many intrinsic and extrinsic factors.

DO INDIVIDUAL BIRDS OVER-COMMIT TO DEFENCE OF FEEDERS?

Feeding studies of birds across entire cities are well-suited to citizen science approaches and, indeed, many citizens have contributed winter observations of feeder-visiting birds to the BTO through the *Garden Bird Feeding Survey* (GBFS; 1970/1971 to the present day) in the UK (Chamberlain et al., 2005) and to the Cornell Lab of Ornithology through *Project FeederWatch* (1987/1988 to the present day) in the US (Bonter, 2012). Both surveys provide invaluable long-term species richness and abundance data over large spatial scales but neither is targeted specifically at urban areas and food availability is not manipulated in the sense that provisioning is not experimentally prescriptive. Manipulative feeding studies have attempted to mimic urban habitats by providing food supplements over long periods from feeders in high density. One such study (Robb et al., 2008b) was carried out over the winter and, despite feeding finishing 6 weeks before the breeding season began, blue tits produced on average one extra fledgling per breeding attempt compared with unsupplemented (control) birds. Harrison et al. (2010) supplemented birds in a UK deciduous woodland over the spring and early summer and found that brood sizes of both blue tits and great tits were reduced compared with unsupplemented (control) birds. We know that some urban bird species demonstrate reduced clutch size and productivity in urban landscapes (Chamberlain et al., 2009), but the findings of Harrison et al. (2010) were unexpected because it took place in a preferred breeding habitat for the focal species and typically food supplementation advances laying onset and either increases or has no statistically significant effect on clutch size (see Table 2 of Robb et al., 2008a).

PROPOSAL B

Examination of whether birds exposed to long-term food supplementation over-commit in defense of food sources and/or breeding habitat resulting in reduced investment of energy, time etc. in breeding attempts. This could be achieved through surveys combining monitoring efforts of both feeders and nests. The challenge will be recruiting sufficient participants to undertake monitoring of both nests and feeder use by breeding birds.

DO INDIVIDUAL BIRDS EXPERIENCE CARRY-OVER EFFECTS FROM SUPPLEMENTARY FEEDING?

Carry-over effects are defined as the outcome of processes experienced by an organism in one season that subsequently influence its performance in the following season. Harrison et al. (2011) reviewed the potential mechanisms through which such effects might be mediated, focusing especially on macronutrient availability and downstream effects such as reproductive success and survival. The feeding of urban birds appears to be an ideal "study system" to test some of their ideas. For example, much could be learned from an investigation of the differences in how birds access and use food resources in the pre-breeding season, how they translate into "state" differences between birds that make the transition into the breeding season, and ultimately whether these map onto fitness differences between birds (e.g., Bearhop et al., 2004; Gunnarsson et al., 2005; see Table 1 in Harrison et al., 2011 for further examples). Robb et al. (2008b) found evidence for such carry-over effects in blue tits fed over the winter that exhibited increased fledging success the following breeding season compared with unsupplemented (control) birds. More investigations need to take place within the urban context as a matter of urgency. Of course, it is one thing to examine how protracted feeding of birds influences their breeding performance within one annual cycle; it is quite another to study how food supplementation throughout the life of an urban bird influences its lifetime reproductive success (Newton, 1991).

PROPOSAL C

Examination of whether birds exposed to long-term food supplementation exhibit carry-over effects that result in their improved breeding performance and survival. This could be achieved through surveys combining monitoring efforts of both feeders and nests. The challenge will be controlling for major sources of variation between birds in breeding experience, age, and onset of senescence.

DOES FEEDING INFLUENCE MATING STRATEGIES AND GENE FLOW IN URBAN BIRD POPULATIONS?

Food supplementation is a potent tool in applied conservation of endangered species when it is employed to promote the establishment of new populations in areas that have been ecologically restored (e.g., Florida scrub-jays *Aphelocoma coerulescens*—Schoech et al., 2008), augment the nutritional status of a non-breeding fraction of a population to the point where more individuals recruit to the breeding population (e.g., kakapo *Strigops habroptilus*—Powlesland and Lloyd, 1994), and increase the productivity of translocated populations (e.g., stitchbirds [or "hihi"] *Notiomystis cincta*—Castro et al., 2003). However, it is not a panacea and in the case of kakapo, as well as promoting recruitment, food supplementation resulted in unpredicted adverse effects such as breeding adult obesity

(Powlesland and Lloyd, 1994) and an unwanted skew toward a male-biased sex ratio in offspring (Clout et al., 2002).

In light of the negative (as well as the positive) effects of intentional feeding of birds outlined above, we feel that it is critical that research be directed urgently toward understanding the impacts of feeding on the mating behaviors of urban birds. Our focus in this context is on the role that feeding plays in connecting bird populations within towns and cities through the movements of individual birds. After all, such connectivity may be of major importance in the processes of natural selection (i.e., survival), sexual selection (i.e., mate choice and, hence, mating systems), and gene flow. In turn, it has downstream effects on population dynamics, the distribution of species, the spatial distribution of genetic diversity, and urban ecosystem functioning (Unfried et al., 2013; LaPoint et al., 2015). Ultimately, it is essential for the long-term viability of metapopulations since it reduces local extinctions, accelerates recolonization, and controls the detrimental effects of inbreeding (Unfried et al., 2013).

One mechanism that would bring about increased genetic diversity in urban compared with rural populations of birds would be changes in mating systems of birds as a result of feeding. One could envisage that feeding might result in males guarding their social mates more intensely, resulting in reduced incidents of broods containing extra-pair young (EPY) and reduced proportions of broods containing EPY (e.g., O'Brien and Dawson, 2011); equally, the opposite may be just as likely with feeding resulting in better-fed males and females of social pairs seeking out more extra-pair copulations (EPCs) with the outcome being greater incidents of broods containing EPY and greater proportions of broods comprising EPY (e.g., Hoi-Leitner et al., 1999). To the best of our knowledge, no urban study has tested such hypotheses although Smith (2011) provided food supplements to a woodland population of birds throughout the spring and early summer. This study mimicked urban bird feeding and found that feeding had no direct effect on the proportion of EPY within broods (**Figure 3**). Clutch size had a significant effect on the incidence of EPY with the direction of this relationship influenced by supplementary feeding; the proportion of EPY increased with clutch size in broods raised in unfed (control) areas of the woodland, but decreased in areas where supplementary food was available (**Figure 3**). Although this suggests that food availability may influence the mating decisions of birds, supplementary feeding throughout the breeding season appears to have no direct effect on extra-pair paternity (EPP) rate (Møller, 1986). Clearly, many mechanisms by which feeding influences the mating strategies and gene flow in urban bird populations remain unresolved.

PROPOSAL D

Examination of whether birds exposed to long-term food supplementation exhibit changes in mating behavior that play out through changes in movements across cityscapes and in the degree of EPP found across and within broods. This could be achieved through heavy investments in banding (i.e., "ringing") in combination with intensive resighting programs across cities and in banding-retrapping studies at nests where both social adults and nestlings are all tissue sampled to provide genetic samples for parentage analyses. The challenge will be banding enough birds to generate meaningful movement and parentage data.

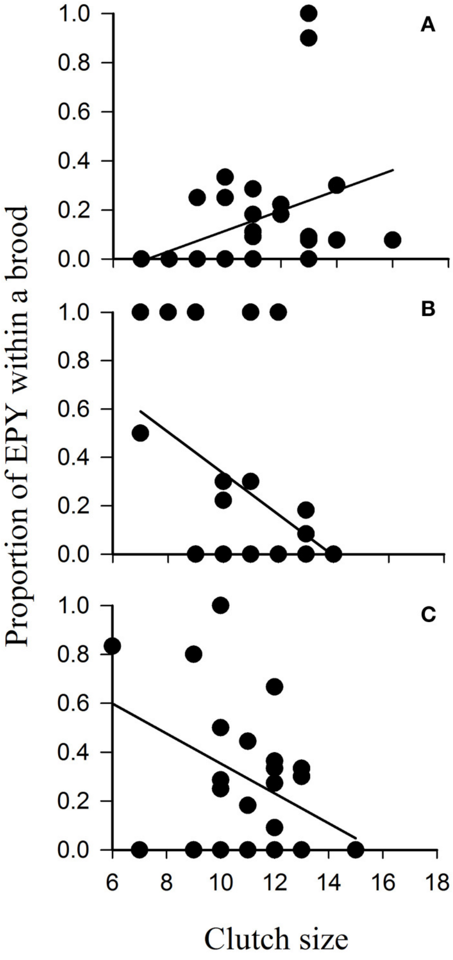

FIGURE 3 | Proportion of extra-pair young (EPY) within each brood in relation to clutch size of blue tits *Cyanistes caeruleus* breeding in Chaddesley Woods National Nature Reserve, Worcs., UK in 2008 and 2009 that were (A) unfed controls, **(B)** supplemented with peanut cake, or **(C)** supplemented with peanut cake and live mealworms *Tenebrio molitor*. Adapted from Smith (2011).

DOES FEEDING CREATE AN ECOLOGICAL TRAP FOR URBAN BIRD POPULATIONS?

Artificially increasing food availability through feeding birds in urban centers has the potential to act as an ecological trap (Schlaepfer et al., 2002). Feeding may provide a false environmental cue of habitat quality that invokes behaviors (e.g., choice of foraging locations, sedentariness) and life-history strategies (e.g., timing of breeding, clutch and brood sizes) that may prove to be maladaptive. In fact, bird feeding can be viewed more broadly as an evolutionary trap that can result in local extinctions because birds are unable to keep pace through adaptation with rapidly changing environmental conditions. The idea of adverse effects on urban bird populations mediated by ecological traps can be extended also to consider the idea of cities as habitat mosaics, some habitat types producing more birds than they lose through mortality (so-called "source" areas) while others lose more birds through local mortality than they gain through local breeding success (so-called "sink" areas; Pulliam, 1988). Dias (1996) described how source-sink dynamics can be played out in population regulation, outlining processes that might result in a re-distribution of birds across a cityscape driven by movements of birds from source to sink habitats. Importantly, such ideas explain why maladaptations persist in urban bird populations but also how local extinctions can occur rapidly. An appreciation of source and sink habitats driven by food availability (i.e., feeding in this context) informs conservation managers in assessing the importance, and the need for conservation, of source habitats within cities (Leston and Rodewald, 2006) because if only sink habitats are prioritized, local populations may be at risk of extirpation.

PROPOSAL E

Examination of whether feeding of urban birds creates ecological traps resulting in differential production, mortality, and migration between different areas of cities. This could be undertaken through heavy investments in banding-resighting programs across cities, in nest and feeder monitoring to assess annual productivity and to define potential source and sink habitats, respectively, and in estimating turnover of birds across cityscapes. The challenge will be investing sufficiently in banding and nest monitoring to generate a city-wide assessment of source-sink dynamics.

ARE FEEDERS IMPLICATED IN THE SPREAD OF DISEASE?

Perhaps the most concerning impact of bird feeding is its potential to spread disease. Although there is often strong defense of feeding in response to this concern (see Jones, 2017), numerous studies have strongly implicated feeders as mediating infection and exacerbating the virulence of outspread, most markedly in the case of house finch mycoplasma (Hartup et al., 2001) and trichomoniasis (Lawson et al., 2012a). The implications of this potential link between likelihood of epidemics and bird feeders are sufficiently significant to warrant a high level of concern and action among all people engaged in the pastime. The response may be as simple as regular hygienic practices (Cleary et al., 2016). However, the best such approaches are not "second nature" to the bird feeding public, and thus much remains to be developed both in terms of adding to knowledge and effective communication of pertinent research outputs to the "end user" (Galbraith et al., 2014). Moreover, the relationship between feeders and transmission of disease is far from clear or straightforward.

PROPOSAL F

Widespread surveillance of feeding platforms to assess the background levels of a range of common diseases and parasites, potentially through the existing networks of citizen scientists already engaged in programs such as *Project FeederWatch* and *Garden BirdWatch*. Simple protocols are already suitable for rolling out to address this research question (see Galbraith et al., 2017 for further details). The challenge will be building sufficiently strong relationships with the bird feeding public that they will buy into this research aspiration, one that could markedly change the way that birds are fed depending upon research findings.

DOES FEEDING INEVITABLY CHANGE URBAN BIRD COMMUNITY STRUCTURE?

We know that in the UK feeding in cities results in peaks in numbers of farmland bird species such as the yellowhammer *Emberiza citrinella* in the late spring in response to food deficits on farmland caused by changes in agricultural practise such as the loss of winter stubbles (Chamberlain et al., 2005). Such transient incursions into urban areas from surrounding countryside typically result in temporary changes to urban bird community structure. In contrast, changes in migration patterns of some species such as the Eurasian blackcap have led to more enduring changes to community structure because birds are now present throughout the winter in the UK. This is a trend that has grown in blackcaps in the last 60 years and appears to be closely related to the increased availability of feeders in gardens (Plummer et al., 2015). We expect that urban bird communities will continue to be sensitive to anthropogenic feeding practices resulting in foods being available year-round. This will increase the carrying capacity of urban habitats despite also potentially increasing the creation of many more ecological/evolutionary traps and sink habitats (Leston and Rodewald, 2006).

As well as changes in short-distance movements and seasonal migratory patterns of native species, feeding in urban centers has also favored the predominance of introduced species in New Zealand. Galbraith et al. (2015) found greater abundances of introduced house sparrows and spotted doves at feeders in Auckland and reduced abundances of native species such as gray gerygones. While feeders clearly attract greater numbers of the species using them, they may also reduce species richness in the avian community because of the predominance of introduced species. Certainly, in the UK and the rest of temperate Europe the introduced rose-ringed parakeet *Psittacula krameri* has established in many cities with its success at least in part associated with access to urban bird feeders (Clergeau and Vergnes, 2011). This species not only outcompetes many native species for food but also for nest sites (e.g., Strubbe and Matthysen, 2009). However, we urge caution in uncritically assuming that introduced avian species always have negative implications. For example, despite many local studies suggesting that common starlings *Sturnus vulgaris*, one of the most abundant introduced birds in the US, negatively impact native species, Koenig (2003) could find no evidence for this contention in a nationwide analysis of bird census data. Moreover, while many people feeding birds do not enjoy the presence of parakeets, others value them as exotic and colorful additions to the local avifauna (e.g., Menchetti et al., 2016).

PROPOSAL G

Monitoring of the community of birds that routinely forage at urban feeders to determine whether long-term feeding results in changes in the avian community. This could be achieved through heavy investments in feeder monitoring not only to record species richness but also to undertake behavioral observations between introduced and native species to detect early warning signs of competitive exclusion of the latter (Grarock et al., 2012). The challenge will be investing sufficiently in behavioral data collection to define adverse effects of feeding from the perspective of the avian community.

CONCLUDING REMARKS: WHAT NEEDS TO BE DONE TO ANSWER THESE RESEARCH QUESTIONS?

The research questions posed here are framed by the comparison of the impacts of feeding on urban birds between the Northern and Southern Hemispheres. Rather than establishing lines of investigation in countries where bird feeding is not well-established, we suggest that such research should be carried out in countries where many households are engaged in bird feeding activities. All of the research questions raised require sustained engagement with the bird feeding public who we feel would be readily recruited to citizen science programs to provide banding capacity, food supplements at urban feeders, and feeder monitoring (Fuller et al., 2012; Amrhein, 2014).

Target countries in the Northern Hemisphere include Belgium, Canada, Germany, Sweden, the UK, and the US that already have well-established banding programs (Balmer et al., 2008). In fact, banding takes place in most European countries with the European Union for Bird Ringing (EURING) coordinating banding schemes to ensure consistency in data collection and overseeing the exchange of banding data between different countries (Balmer et al., 2008). Furthermore, the UK, Canada, and the US also have well-established feeder monitoring programs that form a solid foundation for integration of banding and feeding activities into a comprehensive and rigorous urban citizen science network for data collection (Dickinson and Bonney, 2012).

There are fewer countries in the Southern Hemisphere in which banding and feeder monitoring programs run in parallel so the first step would be to establish a garden bird watch scheme in a country such as South Africa where banding is well established (Jones, 2017) but is not paralleled by a systematic survey of feeding practices by its citizens. South Africa could follow in the footsteps of New Zealand where the *New Zealand Garden Bird Survey* was started in 2007 (Spurr, 2012). This survey was modeled on the Royal Society for the Protection of Birds' (RSPB's) *Big Garden Birdwatch* that has taken place in the UK since 1979. Spurr (2012) provided an informative account of results from the first 4 years of the New Zealand scheme and found that citizens have a strong appetite for engaging in citizen science programs. For example, he found that 66% of survey returns came from gardens in which food supplements were provided and that 76% came from urban, as opposed to rural, locations. Of course, the latter observation probably simply reflects how the human population is distributed across the country but it also highlights the fact that there is potential in New Zealand to carry out research that we envisage. In Australia the *Birds in Backyards* program run by BirdLife Australia has recently included surveys among its members focused on feeding and watering of birds. It is now discussing research partnerships with several of the country's universities.

Much has been written about how to establish, coordinate, and collate research outputs from citizen science programs (e.g., Greenwood, 2007; Dickinson and Bonney, 2012). Therefore, it is not necessary here to "go over old ground." However, it is clear that our research questions cannot be answered by scientists alone. By taking a north-south perspective *and* working with urban-dwelling citizen scientists, as we propose, we believe that we will maximize our understanding of bird feeding, a phenomenon that is showing absolutely no signs of waning.

AUTHOR CONTRIBUTIONS

Conceived the review and wrote the paper: SR, JG, JS, and DJ. Contributed funding: SR and DJ. Wrote the paper: SR, JG, JS, and DJ.

FUNDING

This research was funded in part through Ph.D. studentships to JG and JS.

ACKNOWLEDGMENTS

We are most grateful to the three topic editors—Caroline Isaksson, Diego Gil, and Amanda Rodewald—for establishing this research topic, particularly the former for encouraging us to pursue the direction this paper has taken. We would also like to extend thanks to Jon Sadler, Victoria Pattison-Willits, and two reviewers for critical comments on earlier drafts of the manuscript. We also thank Robyn Bailey and Chelsea Benson at the Cornell Lab of Ornithology and Oliver Smart of Smart Images for assistance in sourcing images of birds at feeders as shown in **Figure 1**.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 02 August 2017
doi: 10.3389/fevo.2017.00081

Urban Bird Feeders Dominated by a Few Species and Individuals

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The practice of garden bird feeding is a global phenomenon, involving millions of people and vast quantities of food annually. Many people engage in the practice of feeding assuming that birds gain some benefit from the food they provide, yet recent studies have revealed the potential for detrimental impacts as well. However, there is still a paucity of information on the impacts of feeding, including the ubiquity of these impacts among and within feeder-visiting species. Consistency in feeder use among birds is likely an important determinant of this. Individual birds and species that make frequent use of feeders are more likely to experience both the benefits and detrimental impacts of supplementary food. We investigated patterns of feeder use by garden birds visiting experimental feeding stations in Auckland, New Zealand, with the specific aim of determining whether use of supplementary food was consistent or variable among individuals and species. We used camera traps as well as Radio Frequency Identification (RFID) technology to examine intra- and interspecific feeder visitation patterns and to discern species associations. Eleven bird species were detected using feeding stations, however, two introduced species (house sparrow *Passer domesticus* and spotted dove *Streptopelia chinensis*) dominated visitation events. These species were present at feeders most frequently, with the largest conspecific group sizes. Significant associations were detected among a number of species, suggesting interspecific interactions are important in determining feeder use. We also found within-species differences in feeder use for all focal species, with individual variation greatest in house sparrows. Furthermore, season had an important influence on most visitation parameters. The observed individual and species-specific differences in supplementary food resource use imply that the impacts of garden bird feeding are not universal. Crucially, particularly given the avifaunal context in New Zealand, resource dominance by introduced species could have potential negative outcomes for native species conservation in cities.

Keywords: competition, individual variation, interspecific interactions, resource use, supplementary feeding, urban wildlife

Edited by:
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Specialty section:
This article was submitted to Urban Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *01 March 2017*
Accepted: *11 July 2017*
Published: *02 August 2017*

Citation:
Galbraith JA, Jones DN, Beggs JR, Parry K and Stanley MC (2017) Urban Bird Feeders Dominated by a Few Species and Individuals. Front. Ecol. Evol. 5:81. doi: 10.3389/fevo.2017.00081

INTRODUCTION

Garden bird feeding is a phenomenally popular activity in many parts of the world, including in New Zealand, Australia, the UK, Europe, and the USA (Jones, 2017). Participation rates for engagement in bird feeding are consistently estimated at between one- to two-thirds of households (Jones and Reynolds, 2008; Galbraith et al., 2014; Orros and Fellowes, 2015b). In recent decades bird feeding has shifted from a predominantly winter-only activity to a pastime commonly practiced year-round (Jones and Reynolds, 2008; Horn and Johansen, 2013; Galbraith et al., 2014). Effectively a massive ecosystem-scale intervention, bird feeding has numerous potential implications for the biology and ecology of feeder-visiting birds (Jones, 2011), as well as the wider faunal community (e.g., Bonnington et al., 2014; Orros et al., 2015). Although studies of bird-feeding impacts in urban habitats are rare, there is mounting evidence to confirm that garden bird feeding can be profoundly influential for urban-dwelling bird communities (Amrhein, 2014). For example, feeding can alter body condition, reproductive outputs, adult survival, disease dynamics, community assemblages, and migration (Robb et al., 2008; Jokimäki and Kaisanlahti-Jokimäki, 2012; Galbraith et al., 2015, 2017; Orros and Fellowes, 2015a; Plummer et al., 2015; Wilcoxen et al., 2015). The vast body of scientific literature on the influence of supplementary feeding on a wide range of species and non-urban habitats corroborates many of these findings (e.g., Boutin, 1990; Clout et al., 2002; Ilarri et al., 2008; Schoech, 2009; Ruffino et al., 2014).

Whether the impacts of supplementary feeding are universal among and within feeder-visiting species remains largely unstudied. Certainly, food availability is an important factor acting to limit bird populations (Newton, 1980), affecting reproductive success and survival of many bird species in different systems (Martin, 1987). Despite the additional deliberate (i.e., bird feeding) and unintentional (e.g., refuse) food resources available to birds in urban systems, demand for food can be high due to high bird densities reducing the *per capita* amount of food available (Seress and Liker, 2015). This demand is illustrated by an experimental study in Arizona, USA, that found supplementary food in urban areas was depleted much faster than equivalent amounts provided in natural habitats (Shochat et al., 2004). Thus, competition for food resources in urban systems, including supplementary food, may be high. The competitive ability of animals, in discovering and dominating a resource, plays an important role in the structuring of numerous faunal assemblages (e.g., in ants; Parr and Gibb, 2012; Bertelsmeier et al., 2015). Intraspecific and interspecific asymmetries in competitive abilities commonly give rise to dominance hierarchies (Holway, 1999), dictating resource access and consequently determining which individuals or species gain benefits from the resource (French and Smith, 2005). Dominant competitors may displace others via interference (physical exclusion from a resource via aggression) or exploitative (rapid discovery and removal of a resource) competition (Bertelsmeier et al., 2015). Agonistic interactions are certainly common at bird feeders, with body size found to be a critical factor in determining outcomes (Tamm, 1985; Wojczulanis-Jakubas et al., 2015). Speed of novel food discovery (Tryjanowski et al., 2015) and bird densities at feeding locales (Galbraith et al., 2015) can also vary by species. Furthermore, in natural habitats, different foraging strategies are frequently used by different individuals within a species to optimize foraging efficiency (Gustafsson, 1988). Consequently, it is unlikely that the ability to exploit supplementary food is consistent among all feeder-visiting birds, both within and between species.

Intraspecific and interspecific differences in use of supplementary food could have important implications for population- and community-level impacts of urban bird feeding, potentially determining the mechanisms by which changes to survival, reproduction, migratory patterns, and community organization occur (Newton, 1980; Robb et al., 2008). Few studies, though, have looked at individual variation in supplementary food use or how species associations at feeders affect resource access in an urban context specifically (but see Cowie and Hinsley, 1988; Crates et al., 2016; Jack, 2016). Bird populations in urban habitats are subject to different pressures than those in more natural environments, frequently resulting in differences in ecology, behavior, and life history (Chace and Walsh, 2006; Chamberlain et al., 2009; Seress and Liker, 2015; Garcia et al., 2017; Lepczyk et al., 2017). Furthermore, urban areas, particularly in New Zealand, are hotspots for introduced (i.e., nonnative/exotic/alien) bird species (Day, 1995; Duncan et al., 2003; van Heezik et al., 2008; Spurr, 2012; Davis et al., 2014), with bird feeding implicated in the success of some of these species (Strubbe and Matthysen, 2007; Peck et al., 2014; Orros and Fellowes, 2015b). Thus, it is immensely important to study supplementary food use *in situ*, in urban areas where most bird feeding occurs, to gain a realistic understanding of the demand for these resources, the urban-specific competitive interactions which may be occurring, and the wider implications for urban birds.

Here we explore avian visitation patterns at experimental bird-feeding stations established in the gardens of volunteer households in Auckland, New Zealand. Via this experiment we have also investigated the impacts of bird feeding on avian disease dynamics (Galbraith et al., 2017) and avian community structure (Galbraith et al., 2015). We know from the latter that our feeding regime significantly altered bird communities at feeding locales, prompting a shift toward communities heavily dominated by introduced birds, primarily house sparrows (*Passer domesticus*) and spotted doves (*Streptopelia chinensis*). This study investigates whether there were intraspecific and interspecific asymmetries in feeder use that could be indicative of resource dominance, giving insight into the community-level observations of Galbraith et al. (2015). We also consider whether supplementary food use is modified by interspecific interactions or seasonality. The demand for supplementary food resources, and the associated competitive interactions at feeders, are likely to vary across seasons (e.g., Ottoni et al., 2009; Cox et al., 2016) due to fluctuations in natural food availability and physiological (thermoregulatory as well as reproductive) demands on birds. We used camera traps at feeding stations to identify species-level patterns of feeder use and examine species associations, and Radio Frequency Identification (hereafter "RFID") technology to explore the feeder-visitation patterns of individuals. Specifically, the objectives of our study were to: (1) examine whether feeder use varies among and within species to determine whether birds exploit supplementary food equally; (2) determine whether individuals and species are consistent in their use of supplementary food over time or if use varies seasonally; and (3) explore the associations between species at feeders that may modify access to supplementary food.

METHODS

Experimental Feeding Stations

Experimental feeding stations were established at 11 urban residential properties in northern Auckland, New Zealand, as part of a wider study of typical bird feeding practices (Galbraith et al., 2015, 2017). The study area is largely suburban residential, with a population density of 1,600/km^2 in 2006 (New Zealand Census data, www.stats.govt.nz). Properties representative of the study area were selected from a pool of 42 volunteered properties. Feeding stations (a low feeding table (40 × 80 cm, 17-cm high), a seed feeder, and a mesh bread-tube; Supplementary Figure 1A) were active for 18 months from March 2012 to September 2013, and householders were responsible for provisioning them on a daily basis for the duration of the study. The feeding regime consisted of 4–5 slices of bread and 1 metric cup of birdseed (white millet, Hungarian millet, hulled oats, and canary seed blend) per day; householders were asked to put the food out between 0700 and 0800 h NZST. This regime and the design of the feeding stations reflected typical feeding practices of the New Zealand public (Galbraith et al., 2014). The majority (75%) of feeding participants in New Zealand throw food directly onto the ground, rather than using structures or containers (Galbraith et al., 2014); however, for the present study it was necessary to use a fixed structure and food containers to standardise experimental feeding among properties and to enable data collection. Properties were a minimum of 900 m apart (Supplementary Figure 2), with no detections of banded birds at properties other than their place of capture. For full details of the experimental setup see Galbraith et al. (2015).

Species Visitation and Association Data

The visitation patterns of feeder-visiting species and species associations were examined using camera traps. Camera trap data were collected at all feeding stations ($n = 11$) over four sampling periods: Austral winter 2012 (June–July; non-breeding season), spring 2012 (October–November; early breeding season), summer 2013 (February; late breeding season), and winter 2013 (June–July). In each sampling period, three cameras (ScoutGuard SG570V, HCO Outdoors, USA; 1.5 m from the feeding station, at a height of 0.5 m) were rotated around the feeding stations over a 4-week timeframe, and operated for 4–6 nights at each feeding station depending on weather. Three full days of recordings were scored for analysis. The cameras were programmed to record upon motion-activation, with a 10-min delay period following a recording event to increase independence of observations. Three consecutive photographs, stamped with the date and time, were taken at 2-s intervals upon motion activation. These photo sets were considered as one observation event.

We used co-occurrence at feeding stations to identify associations among species. For scoring, we counted the maximum number of individuals of a species observed simultaneously in an event. For analysis we included only those recordings with at least one "feeder visitor," henceforth referred to as a "visitation event." Birds on the feeding station structure itself (i.e., on the feeding table or feeders) were considered feeder visitors; birds on the ground or elsewhere nearby were disregarded. We also noted the level of food remaining at the time of the visitation event: bread, seed, or both food types remaining (visible in the containers) or none (no food visible, negligible quantities available).

Individual Visitation Data

To investigate individual patterns of feeder use we used RFID and Passive Integrated Transponder (hereafter "PIT") tag technology. At three study properties birds were captured via mistnetting as part of our wider study of feeding impacts (for full details see Galbraith et al., 2017). We PIT-tagged a subset of captured individuals, and fitted RFID antennae to feeding stations (Supplementary Figures 1A, 2). The RFID reader setup (Microchips Australia Pty Ltd) consisted of a 30 × 25 cm (inner dimensions) coil antenna attached via an RS232 serial cable to a LID-650N decoder (Trovan Ltd, UK) mounted on nearby posts that also supported a 20 W solar panel (Supplementary Figure 1B). RFID readers were powered with a 26 A h, 12 V sealed lead acid battery (HAZE Solar Gel, USA) in weatherproof housing. Acrylic walls supported antennae and encouraged birds to pass through the antenna to reach the food containers (Supplementary Figure 1A). Antennae were intentionally designed to allow multiple birds access to the feeding stations simultaneously, reflecting a typical bird-feeding situation. Readers recorded the individual code (ID) of all PIT-tagged feeder-visitors and the time and date of the visit, with a 1-s read-delay between consecutive reads of the same ID, and were active between 0600 and 2100 h NZST from 22 September 2012 until the end of the study (13 September 2013). Readers did not distinguish between arrival/departure movements.

We PIT-tagged five species (Supplementary Table 1) under the conditions of our animal ethics permit (University of Auckland Animal Ethics Committee Permit R921). Birds were tagged over three capture rounds conducted at 6-monthly intervals, with the monitoring duration (number of days from PIT-tag implantation to the end of the experiment) among returning individuals varying between 134 and 377 days (mean = 291.5 d ± 10.7 SE). Sample sizes depended largely on capture rates of those species. PIT-tagging took place on only those days where two experienced researchers were present in the field. Only adult birds, without apparent injuries or clinical signs of illness, were PIT-tagged. We used Trovan Unique ID100 implantable PIT tags (2.12 × 11.5 mm; 0.1 g; Trovan Ltd, UK) with a unique 5-byte code. These were injected subcutaneously in the back of birds above the scapula following the methods of Nicolaus et al. (2008). The tags are pre-sterilized by the manufacturer, and come ready to use with a disposable needle. During each procedure, one person cleaned the insertion site and injected the PIT tag, while a second person held the bird and gently pulled up the skin at the top of the back to facilitate injection. The perforation of the skin at the insertion site was closed by applying a small quantity of surgical adhesive (Vetbond, 3M, St. Paul, MN, USA). This method has been used on small passerines without obvious negative effects on the birds (Nicolaus et al., 2008; Nomano et al., 2014). Five PIT-tagged individuals were recaptured during subsequent days or capture rounds and we found all tags to be in place with no visible problems for these individuals (Supplementary Figure 1C).

For analysis of PIT-tag data we omitted readings from the same individual that occurred within 2 s of each other, to obtain a more conservative estimate of feeder visitation rates. Two key parameters were calculated for analyses: the total number of reads per day for each individual, and the presence/absence of each individual at the feeder for each day that individual was monitored.

Statistical Analyses

We used basic descriptive and multivariate statistics to examine feeder visitation parameters. These visitation parameters may be considered a proxy for food resource consumption, though our data do not allow for the exact relationship between consumption and visitation to be explored. Note for logistical reasons data collection periods for camera trap and PIT-tag data differed, hence in the analyses treatment of season differed for each. Specifically, because collection of camera trap data encompassed two winters, seasonal period (season ID) was used for analysis of this dataset to enable variation between winters to be examined (in the absence of sufficient data to include year as a fixed effect). Means are shown with their standard errors (SEs) ($x \pm$ SEM), and the critical α level was 0.05 for all tests.

Species Visitation and Co-occurrence (Camera Trap Data)

To explore species associations at feeders (camera trap data) we initially used a probabilistic model approach (Veech, 2013) to test for overall patterns of co-occurrence between species pairs (across all sites and seasons). This approach, implemented in the *cooccur* package (Griffith et al., 2015) in R 3.4.0 (R Core Team, 2017), uses presence/absence data to calculate an expected frequency of co-occurrence between species pairs if they were distributed independently of one another across sites, or observations in this case. The model then calculates the probability that the observed co-occurrence frequency is greater than the expected frequency (a positive co-occurrence association), less than the expected frequency (negative association), or random. Here the model tests the probability of co-occurrence at the level of the visitation event. For this analysis we used feeder-visiting species whose occupancy across study properties was >50% (Supplementary Table 2; determined via bird surveys conducted over the study duration; Galbraith et al., 2015).

We then examined the composition of and variation in bird assemblages visiting feeding stations simultaneously using the PERMANOVA+ add-on to PRIMER (Anderson et al., 2008). We calculated Bray-Curtis distances on fourth-root-transformed data as there were large differences in the baseline abundance of species. We then performed a non-metric multidimensional scaling (NMDS; Kruskal, 1964) on the distance-matrix which is displayed as a reduced space plot where each point represents the bird species composition at each feeding site and time point. To visualise species associations we superimposed vector lines, the length of which represents how much weight a species carried in determining the position of the points in the plot. The degree to which two lines are aligned with each other show the extent to which two species are correlated, with lines pointing in opposite directions meaning negative correlation. To explore the impact of seasonal period and food availability on species composition at feeding stations, a permutational analysis of variance (PERMANOVA) was used. Initially we fitted a PERMANOVA model with four factors: season ID (winter 2012, spring 2012, summer 2013, and winter 2013), food remaining (bread + seed remaining, bread remaining, seed remaining, no food remaining), property identity (ID), and observation day (1, 2, or 3). Observation day was found to be non-significant with minimal variance and was removed from the final model. Property ID was treated as a random factor while season ID and food availability were treated as fixed factors, and all factors were crossed.

To investigate predictors of feeder use at the species level we used a mixed model approach, focusing on the six most frequent feeder-visiting species in the camera trap data (Supplementary Table 3). Generalized Linear Mixed Models (GLMMs) implemented in R were used, accounting for the repeated measures structure of the data. Two feeder-use parameters, abundance at feeders and number of daily visitation events (daily visitation rate), were modelled as the response variables for each species. Abundance models tested whether the number of conspecifics (individuals of the same species) during a visitation event was influenced by the abundance of other visiting species and/or varied among seasonal periods. Visitation rate models tested whether the number of daily visitation events for a focal species varied among seasonal periods. In abundance models, we included season ID, food remaining, the abundances of each of the other five focal species in the visitation event, minimum daily temperature (°C), and rainfall (mm) as fixed effects. In visitation rate models, we included season ID, duration food available (min day^{-1}), minimum daily temperature, and rainfall as fixed effects. Temperature and rainfall data were obtained from the NIWA National Climate Database (for the Albany, North Shore, Auckland weather station; https://cliflo.niwa.co.nz/, accessed 15 November 2016). While avian foraging patterns typically vary with time of day, food was not consistently available throughout the day in our study due to depletion (reflecting the typical feeding practices of the public). As such, feeder visitation across the day was likely to reflect food levels remaining rather than daily patterns of foraging activity. We accounted for this in abundance models by including the "food remaining" parameter, with minutes after sunrise also included as a fixed effect to account for expected variation in foraging activity over the course of the day. Similarly, for visitation rate models we calculated the duration that food was available each day, and included it as a fixed effect to account for its likely influence on daily visitation rates. We included property ID in all models as a random effect, to account for variation between sites. In initial models we also included day length (this was correlated with season ID and so was removed), as well as observation day in the random effects structure (the effect was negligible so data were pooled across observation days for final models).

Prior to model fitting we assessed the distribution of each response variable using the *fitdistrplus* package (Delignette-Muller and Dutang, 2015) in R; the best-fitting distributions were used in the corresponding models. We estimated the parameters using negative binomial GLMMs, fitted using a Laplace approximation of maximum likelihood in the *glmmADMB* package (Skaug et al., 2015), in all cases [with the exception of the Eurasian blackbird (*Turdus merula*) abundance model where a Poisson error structure was the better fit]. For initial analyses, both ordinary and zero-inflated models were fitted. We used Pearson residual plots and Akaike's Information Criterion (AIC) to compare model fits, retaining the best fitting model for interpretation of effects. Additionally, we checked for overdispersion by dividing the sum of squared Pearson residuals by the residual degrees of freedom and comparing this to a χ^2 distribution (Venables and Ripley, 2002). Zero-inflation improved model fit for the house sparrow abundance model only. In initial model fitting, the blackbird, common starling (*Sturnus vulgaris)*, and silvereye (*Zosterops lateralis*) abundance models all had large SEs for the "seed" level of the "food remaining" parameter, as did the silvereye abundance and visitation rate models for the "summer 2013" level of the "season ID" parameter. This was due to separation in the data due to cells with zero frequencies within the contingency table of response variable × food available (and × season for silvereye). To yield sensible parameter SEs, we added a dummy row to the dataset that added a small non-negative constant (a count of 1) to the cells with zero counts (Agresti, 2002; Jones et al., 2012) with other cells containing mean values. We then refitted these models and checked parameter SEs. For the final models we assessed the significance of whole model terms using likelihood ratio tests (LRTs) implemented with the "drop1" function in R. *Post hoc* pairwise comparisons among levels of season ID were conducted using the *multcomp* package (Hothorn et al., 2008).

Individual Visitation (PIT-Tag Data)

We used GLMMs implemented in R to explore feeder visitation patterns of individuals (PIT-tag data). Two feeder-use parameters, daily presence at feeders (binomial; present = 1, absent = 0) and daily visitation rate (count data; total reads per day active at feeder), were modelled as the response variables. Daily presence data were modelled with a binomial error structure (logit link) using the *lme4* package (Bates et al., 2014). Daily visitation rate data were modelled using a negative binomial error structure using the *glmmADMB* package, after assessing the distribution using the *fitdistrplus* package. For both response variables we fitted initial models that included species as a fixed effect and individual ID as a random effect, to confirm differences among species in feeder use. This also allowed us to estimate the relative contribution of among-individual variability (i.e., individual heterogeneity) and among-species variability to the overall variation in feeder visitation data. We calculated the marginal R^2 (proportion of variance explained by the fixed effects) and the conditional R^2 (proportion of variance explained by fixed and random effects combined) using the "r.squaredGLMM" function in the R package *MuMIn* (Bartoń, 2015), which implements the methods of Nakagawa and Schielzeth (2013). $R^2_{\text{(CONDITIONAL)}}$ -$R^2_{\text{(MARGINAL)}}$ gives the random effect component of the variance, $R^2_{\text{(RANDOM)}}$. $R^2_{\text{(RANDOM)}}$ is analogous to repeatability (Nakagawa and Schielzeth, 2010; Crates et al., 2016). Note, estimating the variance components of fixed vs. random effects for negative binomial models required refitting models using the "glmmPQL" function of the *MASS* package (which uses penalized quasilikelihood for parameter estimation; Venables and Ripley, 2002), as *glmmADMB* objects cannot be passed to "r.squaredGLMM." Parameter estimates were comparable between "glmmPQL" and "glmmadmb" fitted models (see Supplementary Tables 4, 5); coefficients of the latter are presented in the results for consistency.

We then fitted separate GLMMs for each species for each response variable to examine the effects of season on feeder-use parameters. For both daily presence and daily visitation rate models, we included season (winter, spring, summer, and autumn), minimum daily temperature, rainfall, and day (number of days since feeding started) as fixed effects. Individual ID was included in the models as a random effect to account for repeated measures from the same individuals and to determine the contribution of individual heterogeneity to model variance. For all species except house sparrow, there were too few individuals at each property to adequately estimate among-property variation, thus data were pooled across properties. For house sparrow data, larger sample sizes at each property enabled initial models to be fitted with property ID (Feeder 1, 2, and 3) included as a fixed effect. As property ID did not contribute significantly to model fit for either response variable (see Supplementary Table 6), we pooled data across properties for final analyses as per the other species. Significance of whole model terms was assessed using LRTs.

RESULTS

Camera Trap Visitation Events

A total of 3066 visitation events were captured by the camera traps over 132 trap days. We recorded 725 visitation events in winter 2012, 1006 in spring 2012, 581 in summer 2013, and 754 in winter 2013. There were 723 visitation events made when bread and seed were both available, representing the time period immediately following food provision. There were fewest observations when only seed remained ($n = 31$); typically this food type was depleted fastest, leaving only bread remaining ($n = 839$ observations). The other visitation events occurred when there was no food remaining ($n = 1473$), although a small amount may have still been available on the table.

Structure of Bird Assemblage at Feeding Stations

Eleven bird species were recorded visiting the feeding stations (Supplementary Table 3). House sparrows and spotted doves were the most frequently observed species over all visitation events, present in 64.9 and 58.0% of recordings, respectively. Silvereyes were the only native species, present in only 4.6% of visitation events. Most visitation events had only a single species present (57.3%), with two species present in 33.5% of visitation events. Only 9.2% of visitation events had three or more species present. Mean total abundance per visitation event was 4.9 ± 1.53 birds, with a mean species richness of 1.5 ± 0.01 species.

A maximum of five species were observed together on the feeding station in one visitation event. Mean conspecific group size (number of same-species individuals feeding simultaneously) was highest in house sparrow with 5.2 ± 0.12 individuals per visitation event, and a maximum of 45 sparrows recorded in one visitation event. In contrast, Eurasian blackbirds, song thrushes (*Turdus philomelos*), and chaffinches (*Fringilla coelebs*) were typically observed without conspecifics present (Supplementary Table 3).

Species Visitation Patterns

House sparrows and spotted doves had the highest daily visitation rates (number of visitation events recorded by camera traps) to feeding stations (mean no. visitation events day^{-1} = 15.08 ± 0.71 and 13.46 ± 0.63, respectively), well above that for any other species (Supplementary Table 3, **Figure 1A**). Abundances of all six focal species (the most frequent feeder-visitors) at feeding stations were significantly influenced by food levels remaining (GLMM analyses; **Table 1**). In particular, abundances tended to be lowest when no food remained in comparison to when bread and seed remained (Wald-Z < -3.01, $p < 0.003$ in all cases). Furthermore, the speed at which food was depleted varied significantly among seasons (GLMM LRT: $\chi^2 = 28.2$, d.f. = 3, $p < 0.001$); food was available for longer in winter 2012 (mean min available day^{-1} = 247 min ± 27) and spring 2012 (290 min ± 30) compared to summer 2013 (134 min ± 17), when food was depleted fastest, and winter 2013 (170 min ± 18). This duration of food availability significantly affected the number of daily visitation events by Eurasian blackbird and common myna, and marginally improved model fit for spotted dove and silvereye daily visitation rate models (**Table 2**). Nevertheless, after accounting for this variation in food availability, abundances at feeding stations and daily visitation rates for all species varied significantly among seasonal periods (except the spotted dove abundance model; **Tables 1**, **2** and **Figures 1A,B**). This seasonality was most striking for silvereyes, with no feeder visitations recorded for the summer period at all (**Figure 1**). House sparrow abundance at feeders was higher in winter 2013 compared to winter 2012, whereas both daily visitation rate and abundance were lower in the second winter for Eurasian blackbirds (**Figure 1**, Supplementary Table 7). The remaining species showed no significant difference between winters for either visitation parameter (**Figure 1**, Supplementary Table 7).

Species Associations

A number of significant associations in overall species co-occurrence patterns at feeding stations were detected with the probabilistic modelling (**Figure 2**). Of the 36 species pairs, three were positive (8.3%) with the two species co-occurring at feeders significantly more frequently than expected, 10 were negative (27.8%) with species co-occurring significantly less frequently than expected, and 23 were random (63.9%). House sparrow had the highest number of significant associations with other species ($n = 6$), and the highest proportion of positive associations (37.5% of pairings). Eurasian blackbirds had five significant associations with other species, while spotted doves had four, with all of these being negative.

Analysis at the community level also indicated there were patterns in the assemblages of birds feeding concurrently at the feeding stations. Species composition was dominated by three species, house sparrows, spotted doves, and Eurasian blackbirds, with strong negative correlations between the abundances of these species (**Figure 3**); blackbirds were present at feeding stations when there were less spotted doves and house sparrows. Species composition at feeding stations varied significantly with seasonal period and food availability, and was influenced by property ID (PERMANOVA: $F = 2.38$, d.f. = 54, $P = 0.001$).

GLMM analyses provided further evidence of species associations, with the abundance of all focal species during visitation events influenced by the abundance of at least one other top feeder-visitor (**Table 1**). Significant effects were generally negative, with the exception of the house sparrow and silvereye models. With all other factors held constant, house sparrow abundance increased with common starling, silvereye, and common myna abundances, and silvereye abundance increased with house sparrow abundance. Effect sizes for season ID and food remaining were typically larger than for co-occurring species predictors though.

Individual Visitation Patterns

We PIT-tagged a total of 110 individuals from five feeder-visiting species (Supplementary Table 1). Numbers tagged were equivalent at each feeding station (Feeder 1: $n = 37$; Feeder 2: $n = 37$; Feeder 3: $n = 36$). The redetection rates varied among species (Supplementary Table 1), with 70 individuals (63.6%) overall redetected by RFID readers at feeding stations on at least one occasion after initial capture. In total 83265 reads were recorded (with duplicate reads removed) for these 70 individuals.

There were obvious differences among individuals in the consistency of feeder visitation over time, with some individuals returning almost daily while others only visited sporadically (Supplementary Figure 3). Daily presence at feeding stations differed significantly among species (i.e., the proportion of days birds returned to the feeder out of the total days monitored; GLMM LRT: $\chi^2 = 18.9$, d.f. = 4, $P < 0.001$; **Figure 4**; Supplementary Table 4). The variation among individuals explained a greater proportion of the variation in daily presence data than among-species variation (48.4 vs. 14.8%, respectively). Considering each species separately (except for common starling for which there were too few individuals for valid comparisons), GLMM analyses indicated that the daily likelihood of individuals visiting feeders varied seasonally for all species except house sparrow (**Table 3**). House sparrows showed the greatest variability among individuals, with individual heterogeneity accounting for 63.2% of the variance in the model [Eurasian blackbirds 52.1%, spotted doves 38.4%, Barbary doves (*Streptopelia roseogrisea*) 14.3%; **Table 3**].

Mean daily visitation rates varied significantly between species (GLMM LRT: $\chi^2 = 17.6$, d.f. = 4, $P < 0.01$; Supplementary Table 4). However, heterogeneity among individuals explained a greater proportion of the variance in daily visitation rate than among-species variation (19.6 vs. 4.9%, respectively). Three

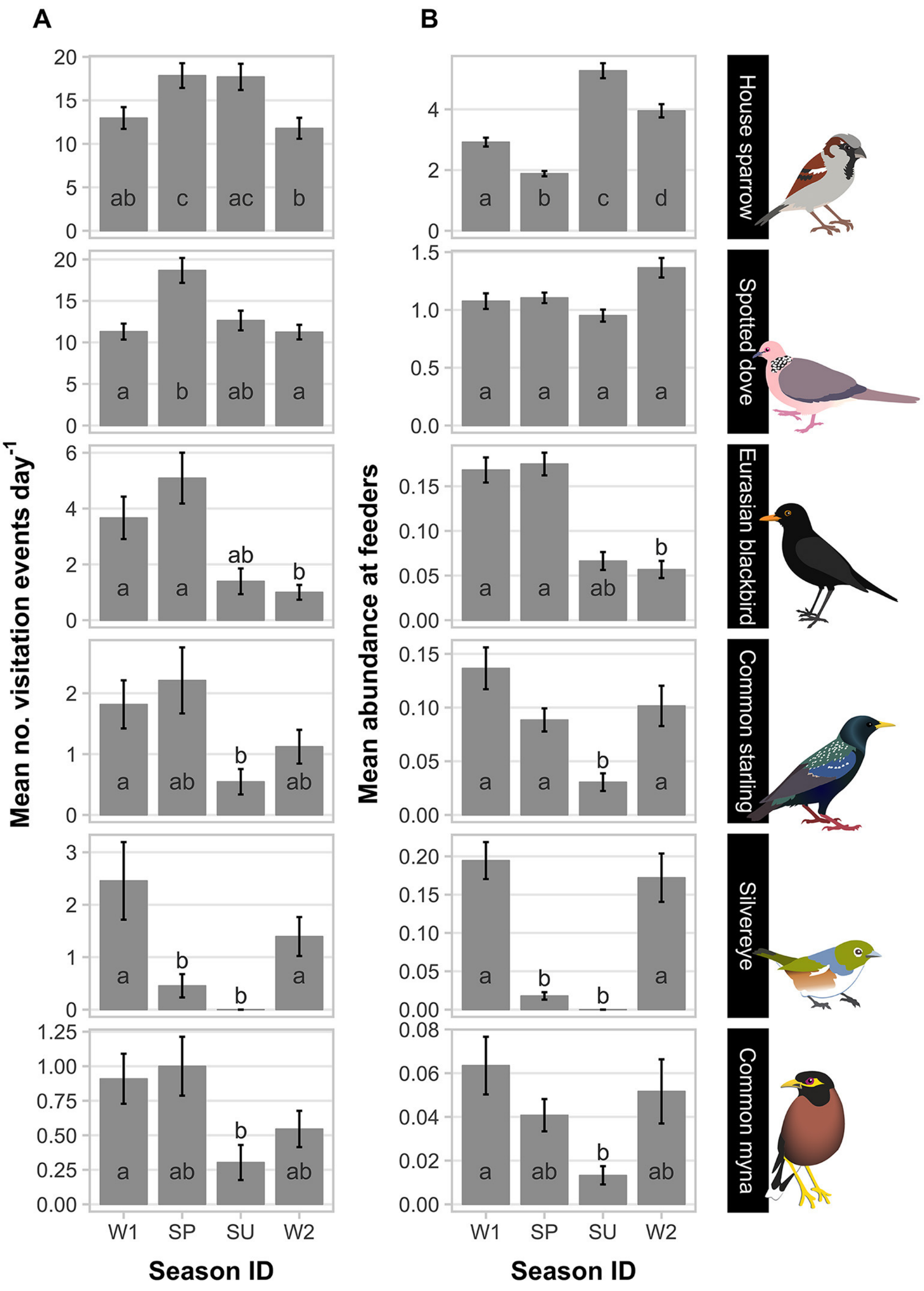

FIGURE 1 | Patterns of feeder visitation of the top six avian visitors captured in camera traps at experimental feeding stations at urban study properties in northern Auckland, New Zealand. Mean count data (± SEM) are presented for two visitation parameters scored from camera trap data: **(A)** daily visitation rate (number of daily visitation events recorded); and **(B)** abundance (number of individuals in a visitation event). In each panel, means not sharing the same letter are significantly different in pairwise comparisons (Tukey's HSD; $p < 0.05$; Supplementary Table 7). Sampling season ID: W1, winter 2012; SP, spring 2012; SU, summer 2013; W2, winter 2013. Note y-axis scale varies with species.

house sparrow individuals had the highest visitation rates per active day, with means of 32.7 (± 14.5) to 42.8 (± 8.2) reads per day (**Figure 5**). Analysed separately (again except for common starling), daily visitation rates varied significantly with season for all species (**Table 4**). Individual heterogeneity in daily visitation rate was highest for house sparrows and blackbirds, accounting for 31.1 and 28.4% of explained variance in the models, respectively (**Table 4**).

TABLE 1 | Results of Generalized Linear Mixed Models (GLMMs) examining factors affecting the abundance of garden birds captured in camera traps at experimental feeding stations in northern Auckland, New Zealand.

Explanatory variables	House sparrow	Spotted dove	Eurasian blackbird	Common starling	Silvereye	Common myna
Intercept	1.806 (0.143)	0.957 (0.188)	−1.179 (0.275)	−1.713 (0.433)	−1.439 (0.644)	−1.811 (0.449)
Season ID (reference: Winter 2012)	***	•	***	***	***	**
Spring 2012	−0.255 (0.073)	0.064 (0.063)	0.001 (0.143)	−0.116 (0.257)	−2.014 (0.300)	−0.278 (0.330)
Summer 2013	0.726 (0.103)	−0.062 (0.099)	−0.579 (0.258)	−1.829 (0.430)	−3.975 (1.084)	−1.993 (0.588)
Winter 2013	0.262 (0.074)	0.103 (0.067)	−0.805 (0.216)	−0.415 (0.263)	0.375 (0.277)	−0.383 (0.346)
Food remaining (reference: Bread and Seed)	***	***	***	***	***	***
Seed only	0.668 (0.177)	−0.712 (0.262)	−0.198 (1.034)	−1.402 (1.094)	−1.768 (1.158)	−1.081 (0.909)
Bread only	−0.340 (0.064)	−0.909 (0.053)	0.038 (0.162)	0.216 (0.207)	−0.489 (0.211)	−0.604 (0.306)
None	−1.094 (0.067)	−1.223 (0.059)	−0.721 (0.175)	−0.807 (0.268)	−1.776 (0.289)	−2.397 (0.450)
House sparrow abundance	–	−0.008 (0.005) •	−0.064 (0.018)***	0.073 (0.018)	0.040 (0.018)*	0.038 (0.025)
Spotted dove abundance	−0.018 (0.015)	–	−0.590 (0.076)***	−0.060 (0.049)	−0.292 (0.071)***	−0.088 (0.063)
Eurasian blackbird abundance	−0.353 (0.072)***	−0.835 (0.081)***	–	−0.458 (0.295)	−0.821 (0.331)**	−2.135 (0.749)***
Common starling abundance	0.101 (0.050)*	0.020 (0.048)	−0.373 (0.206)*	–	−0.740 (0.410)*	−0.366 (0.276)
Silvereye abundance	0.117 (0.048)*	−0.420 (0.063)***	−0.615 (0.228)***	−0.677 (0.402)*	–	−0.324 (0.253)
Common myna abundance	0.179 (0.078)*	−0.074 (0.056)	−1.345 (0.531)***	−0.488 (0.304) •	−0.055 (0.287)	–
Time of day (min after sunrise)	−0.001 (0.000)***	−0.001 (0.000)***	0.0003 (0.000)	−0.004 (0.001)***	−0.001 (0.001)	−0.003 (0.001)**
Minimum daily temperature (°C)[#]	0.001 (0.009)	0.007 (0.008)	−0.009 (0.019)	0.0004 (0.033)	−0.082 (0.034)*	0.059 (0.046)
Rainfall (mm)[#]	0.009 (0.006)	0.012 (0.004)**	0.034 (0.017) •	−0.060 (0.035) •	−0.003 (0.015)	−0.004 (0.030)
Property ID (random effect)[†]	0.132 (0.364)	0.341 (0.583)	0.410 (0.641)	1.119 (1.058)	3.136 (1.771)	0.297 (0.545)

*Parameter estimates and their standard error (± SE) are presented for each model term at the reference levels stated. Whole effects were tested with likelihood ratio tests (LRTs); significant chi-square test statistics from LRTs are indicated with: •$p < 0.10$, *$p < 0.05$, **$p < 0.01$, and ***, $p < 0.001$.*

Models were fitted with negative binomial error structures, except for house sparrow (zero-inflated negative binomial) and Eurasian blackbird (Poisson).

[#] *Data from NIWA National Climate Database.*

[†] *Variance (standard deviation) presented.*

Models were fitted for the six most frequent feeder-visiting species (presented here in descending order of frequency). n observations = 3066.

TABLE 2 | Results of Generalized Linear Mixed Models (GLMMs) testing whether the number of visitation events per day captured by camera traps varied among seasons.

Explanatory variables	House sparrow	Spotted dove	Eurasian blackbird	Common starling	Silvereye	Common myna
Intercept	2.512 (0.161)	2.204 (0.146)	0.595 (0.405)	0.387 (0.481)	−0.065 (0.666)	0.239 (0.393)
Season ID (reference: Winter 2012)	***	***	***	**	***	**
Spring 2012	0.338 (0.088)	0.425 (0.108)	0.390 (0.278)	0.206 (0.335)	−1.743 (0.413)	0.115 (0.319)
Summer 2013	0.290 (0.130)	0.043 (0.161)	−0.526 (0.457)	−1.349 (0.528)	−3.585 (1.167)	−1.426 (0.564)
Winter 2013	−0.130 (0.092)	0.023 (0.114)	−1.119 (0.331)	−0.520 (0.336)	0.058 (0.421)	−0.734 (0.356)
Duration food available (min day^{-1})	−0.0002 (0.0002)	0.0005 (0.0003) •	0.002 (0.0008)**	−0.0008 (0.0009)	0.002 (0.001) •	−0.002 (0.001)*
Minimum daily temperature (°C)[#]	0.0003 (0.011)	0.012 (0.014)	−0.028 (0.037)	−0.002 (0.044)	−0.033 (0.050)	0.014 (0.045)
Rainfall (mm)[#]	0.005 (0.007)	−0.001 (0.008)	0.018 (0.028)	−0.057 (0.041)	−0.008 (0.022)	0.010 (0.027)
Property ID (random effect)[†]	0.189 (0.434)	0.077 (0.279)	0.640 (0.800)	1.275 (1.129)	2.341 (1.530)	0.098 (0.027)

*Parameter estimates and their standard error (± SE) are presented for each model term at the reference levels stated. Whole effects were tested with likelihood ratio tests (LRTs); significant chi-square test statistics from LRTs are indicated with: •$p < 0.10$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Models were fitted with negative binomial error structures.*

[#] *Data from NIWA National Climate Database.*

[†] *Variance (standard deviation) presented.*

Models were fitted for the six most frequent feeder-visiting species (presented here in descending order of frequency). n observations = 132.

DISCUSSION

Summary of Findings

Our results demonstrate considerable among- and within-species variation in the use of supplementary food resources by birds *in situ* in urban habitats, confirming that feeder-visiting birds do not exploit supplementary food resources equally. The species using feeding stations in our study were predominantly introduced; this was predicted because of the grain-based feeding regime used by householders (in our study and more generally; Galbraith et al., 2014), and the division in dietary preferences of native vs. introduced birds in our study area (Galbraith et al., 2015). Two species in particular dominated resource use: house sparrow and spotted doves. Our study corroborates the findings of two other recent experimental studies, one focusing on great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*)

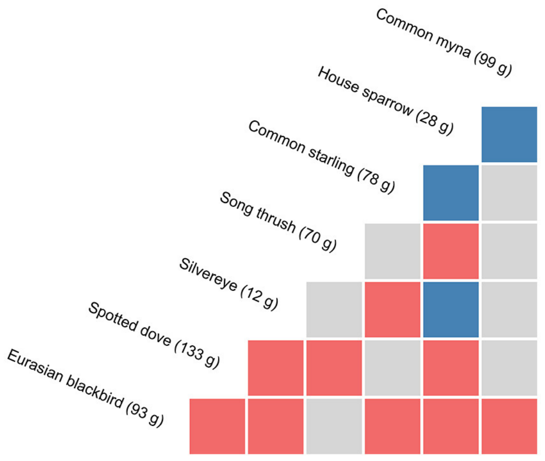

FIGURE 2 | Pairwise associations between species at experimental feeding stations at urban study properties in northern Auckland, New Zealand, determined through probabilistic modelling of species co-occurrences (Veech, 2013). Species that co-occur more frequently than by chance (α threshold = 0.05) are considered to have a positive association (in blue), those that co-occur less frequently than by chance are considered to have a negative association (in red), with random associations shown in grey. Note chaffinch and greenfinch pairings are not shown; no significant associations were found for these species. Species masses are means from birds captured in this study.

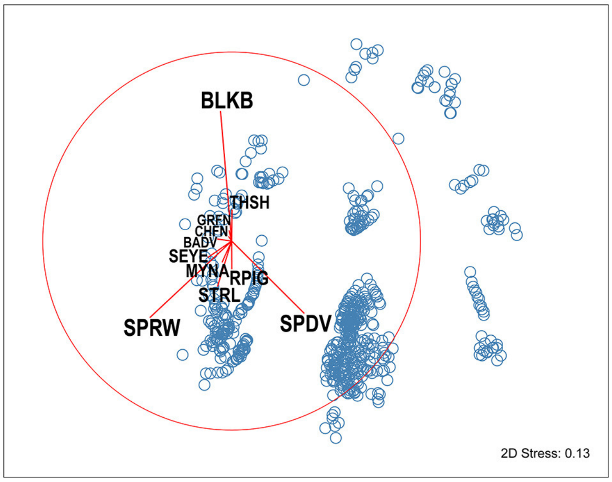

FIGURE 3 | NMDS ordination of avian community composition at experimental feeding stations at urban study properties in northern Auckland, New Zealand. Each point (blue circles) represents the avian assemblage recorded in a single visitation event. The overlaid species vectors (red lines) illustrate that three bird species, Eurasian blackbirds (BLKB), house sparrows (SPRW), and spotted doves (SPDV) dominate community composition (as they have the longest vectors), and that there are strong negative correlations between the abundances of these species. BADV, Barbary dove; CHFN, chaffinch; GRFN, greenfinch; MYNA, common myna; RPIG, rock pigeon; SEYE, silvereye; STRL, common starling; THSH, song thrush.

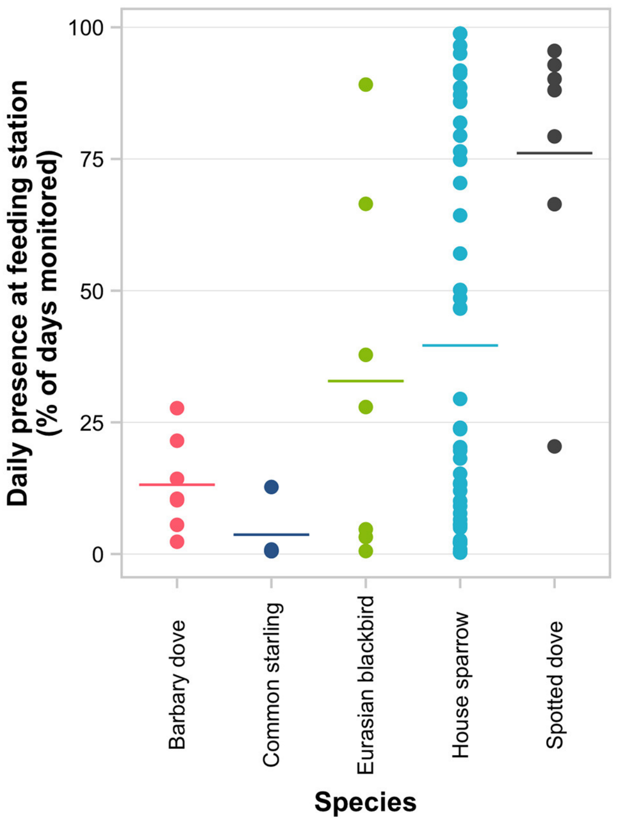

FIGURE 4 | Consistency of feeder visitation of 70 individually PIT-tagged birds at experimental feeding stations in northern Auckland, New Zealand. Points represent the percentage of days an individual was active at a feeding station out of all days monitored for that individual. Horizontal lines represent the group means for each species.

in woodland habitat in Oxford, UK, during winter (Crates et al., 2016) and the other examining visitation patterns of six species at a university campus in Cornwall, UK (Jack, 2016). These studies both reported individual and interspecific variation in use of supplementary food at experimental feeders over a comparatively shorter timeframe (c. 3 months). The findings from our longer-term study also demonstrate that season has an important influence on a number of visitation parameters both at the individual and species levels. Relatedly, seasonal differences in movement of birds among networks of urban feeders have been found in great tits and blue tits (Cox et al., 2016). We acknowledge, though, that in our study there are limitations to interpreting the results from those PIT-tagged species with small sample sizes. Nevertheless, significant seasonal variation in feeder use was evident across most species and visitation parameters we assessed.

Visitation Patterns

The interspecific differences in feeder use observed in our study reinforce the concern that typical feeding practices in New Zealand do not benefit native species, but instead support introduced species (Galbraith et al., 2015). Feeding grain-based food types (e.g., bread, seed) is the prevalent practice in New Zealand (Galbraith et al., 2014), yet most native garden birds here are frugivores, nectarivores, or insectivores (Heather and

TABLE 3 | Results of Generalized Linear Mixed Models (GLMMs) testing the effect of season on daily visitation (presence/absence) of individually PIT-tagged birds to experimental feeding stations.

	House sparrow	Spotted dove	Eurasian blackbird	Barbary dove
n observations	13367	1453	2088	2479
n individuals	45	7	7	7
Intercept	0.435 (0.423)	4.969 (0.834)	0.168 (0.999)	−0.836 (0.841)
Season (reference: Winter)		***	**	***
Spring	−0.058 (0.109)	−0.156 (0.259)	0.675 (0.280)	1.599 (0.488)
Summer	0.143 (0.112)	−1.050 (0.337)	1.043 (0.280)	1.403 (0.451)
Autumn	0.090 (0.082)	−0.994 (0.218)	0.345 (0.213)	0.165 (0.424)
Minimum daily temperature (°C)#	0.003 (0.009)	−0.031 (0.026)	0.028 (0.022)	−0.004 (0.026)
Rainfall (mm)#	−0.009 (0.004)*	0.001 (0.009)	−0.006 (0.009)	−0.026 (0.018)
Day (numerical from start of feeding)	−0.004 (0.000)***	−0.007 (0.001)***	−0.007 (0.001)***	−0.008 (0.001)***
Individual ID (random effect)†	5.992 (2.448)	2.388 (1.545)	4.767 (2.183)	0.906 (0.952)
Marginal R^2	0.021	0.088	0.119	0.337
Conditional R^2	0.653	0.471	0.640	0.480

*Parameter estimates and their standard error (± SE) are presented for each model term at the reference levels stated. Whole effects were tested with likelihood ratio tests (LRTs); significant chi-square test statistics from LRTs are indicated with: •p < 0.10, *p < 0.05, **p < 0.01, and ***p < 0.001. Models were fitted with binomial error structures.*

#Data from NIWA National Climate Database.

†Variance (standard deviation) presented.

Species ordered by frequency of occurrence from Supplementary Table 3.

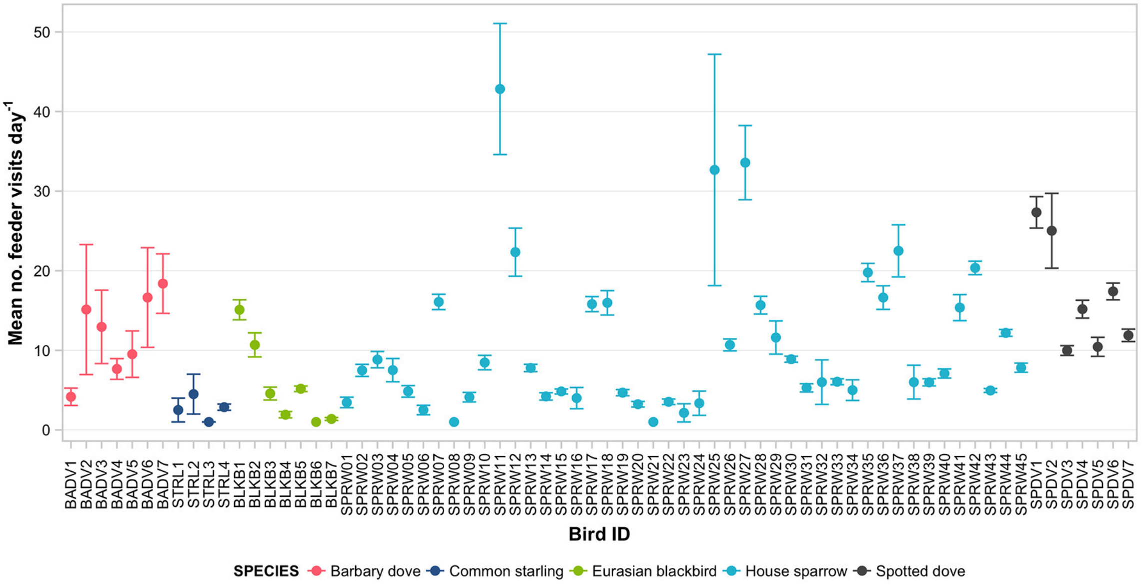

FIGURE 5 | Daily rates of feeder visitation (number of visitation events per day actively using the feeder) of 70 individually PIT-tagged birds at experimental feeding stations in northern Auckland, New Zealand. Data are raw means ± SEM.

Robertson, 1996). Thus, unsurprisingly, native species were almost entirely absent from our experimental feeding stations, with the exception of the silvereye which primarily visited in winter. Of the 11 recorded feeder-visiting species, two largely granivorous introduced species—house sparrow and spotted dove—dominated the majority of visitation events. They were also the two most abundant species at feeders, both in terms of mean and maximum conspecific group size using the feeder simultaneously. These results reflect the findings of bird counts conducted at study properties over the duration of the experiment, in which house sparrow and spotted dove showed dramatic increases in abundance at feeding locales in response to the feeding treatment, contributing to a shift in community composition (Galbraith et al., 2015). This is concerning for urban ecosystems in New Zealand, where introduced birds are already the dominant component of avifaunal assemblages, and increasingly relevant globally, as supplementary feeding is implicated as a contributing factor in the spread of other invasive bird species, such as monk parakeets (*Myiopsitta monachus*) in the northern USA (Davis et al., 2014) and ring-necked parakeets

TABLE 4 | Results of Generalized Linear Mixed Models (GLMMs) testing the effect of season on daily visitation rate (no. visits per active day) of individually PIT-tagged birds at experimental feeding stations.

	House sparrow	Spotted dove	Eurasian blackbird	Barbary dove
n observations	4695	990	787	316
n individuals	45	7	7	7
Intercept	3.423 (0.144)	4.831 (0.205)	4.145 (0.395)	5.735 (0.812)
Season (reference: Winter)	***	***	**	***
Spring	−0.052 (0.054)	0.242 (0.086)	−0.221 (0.119)	−0.740 (0.558)
Summer	−0.469 (0.056)	−0.403 (0.117)	−1.052 (0.124)	−1.746 (0.508)
Autumn	−0.054 (0.039)	−0.055 (0.072)	−0.913 (0.105)	−1.049 (0.511)
Minimum daily temperature (°C)#	−0.012 (0.004)**	−0.036 (0.008)***	−0.042 (0.010)	−0.000 (0.025)
Rainfall (mm)#	0.001 (0.002)	0.001 (0.003)	0.008 (0.005)	−0.022 (0.018)
Day (numerical from start of feeding)	−0.003 (0.000)***	−0.005 (0.000)***	−0.005 (0.000)***	−0.011 (0.001)***
Individual ID (random effect)†	0.428 (0.654)	0.067 (0.259)	0.672 (0.820)	0.037 (0.193)
Marginal R^2	0.065	0.197	0.226	0.505
Conditional R^2	0.376	0.247	0.510	0.522

*Parameter estimates and their standard error (± SE) are presented for each model term at the reference levels stated. Whole effects were tested with likelihood ratio tests (LRTs); significant chi-square test statistics from LRTs are indicated with: •p < 0.10, *p < 0.05, **p < 0.01, and ***p < 0.001.*

Models were fitted with negative binomial error structures.

#Data from NIWA National Climate Database.

†Variance (standard deviation) presented.

Species ordered by frequency of occurrence from Supplementary Table 3.

(*Psittacula krameri*) in the UK and mainland Europe (Strubbe and Matthysen, 2007; Peck et al., 2014; Orros and Fellowes, 2015b). This concern is not limited to introduced birds, however, as native species may also become numerically dominant as a result of food subsidies and displace other birds.

The prevalence of house sparrows and spotted doves in visitation events and their distinct positive numerical responses to the feeding treatment (Galbraith et al., 2015) suggests both species were successfully competing for access to the food resource. However, there were differences between house sparrows and spotted doves in visitation patterns which may be indicative of distinct, but equally successful, competitive behaviours. Spotted dove feeder visitors appear to consist of a core-group of individuals that are highly consistent in their feeder use (i.e., show high fidelity to the resource once they discover it), whereas house sparrow feeder visitors are highly numerous but also highly variable in their feeder use.

Our results provide multiple lines of evidence (both camera trap and PIT-tag data), that seasonality has an important effect on feeder visitation patterns. Unsurprisingly, the level of food remaining had a significant impact on feeder visitation parameters–when there was more food left, bird abundance at feeders was higher. Nevertheless, after accounting for the effect of food availability, species abundance and species daily visitation rate varied significantly with seasonal period for most focal species. We also found that the daily presence and visitation rates of individuals varied significantly among seasons for all focal species (though we could not account for food availability here). There were, however, two exceptions: first, the number of spotted doves feeding at the same time was consistent among seasonal periods. This may well reflect the maximum capacity of the feeding station structure itself—ultimately the number of birds, particularly large-bodied species, such as spotted doves, feeding simultaneously will be restricted by physical space at feeders. Second, no seasonal effect on the likelihood of individuals being present each day was evident for house sparrow—individual heterogeneity was more influential compared to season for this species.

The physiological demands on birds vary seasonally with changes in environmental conditions, natural food availability, and behavioural activity, thereby influencing the demand for supplementary food. Here our proxy for demand was the speed of food depletion, which indicated that demand for supplementary food resources did fluctuate seasonally in our study. Demand was highest in summer rather than winter, which is contrary to the common public perception that birds should be fed in the winter when their struggle for survival is greatest (Jones, 2017). However, in our study region winter conditions are fairly mild, so the physiological demand is likely less compared to many regions. Instead, the energetic costs of breeding activities (e.g., increased energy required for reproduction, and/or reduced foraging time due to incubation and chick provisioning activities; Martin, 1987) may drive the increased demand for readily available supplementary food in summer. Additionally, it is crucial to note that our study period encompassed a pronounced, New-Zealand-wide drought event over the summer of 2012–2013 (Porteous and Mullan, 2013). This event would probably have had significant impacts on natural food availability, and may well have contributed to food demand. The differences observed between winters may also reflect follow-on impacts of the drought event, if, for instance, natural food resource availability had not recovered. However, visitation patterns of the only native feeder visitor provide a notable contrast to the overall seasonal pattern of demand. Silvereyes were scarce at feeders in spring and entirely absent in summer, suggesting that natural food resources exploited by this species were more abundant (or more profitable) during these seasons and that supplementary food was unnecessary to meet energy requirements.

Species Associations

We found evidence that access to supplementary food was influenced by species associations at feeding stations. Feeder activity typically peaked in the morning following food provisioning. Although multiple species were typically present in the vicinity of feeders, single-species visitation events were more common than those with multiple species at the feeder simultaneously. It is likely, then, that some species excluded others from access to feeders and some avoided using feeders when heterospecifics were present. Multi-species visitation events, however, still comprised a substantial proportion of our observations (42.7% of visitation events).

A number of significant interspecific associations were identified from the probabilistic modelling of the likelihood of co-occurrence between species pairs, which generally agreed with findings from GLMM analyses of predictors of species abundance at feeders. Body size frequently determines, or contributes to, the development of dominance hierarchies, outcomes of agonistic interactions, and subsequent resource access (Forrester, 1991; Wojczulanis-Jakubas et al., 2015; Miller et al., 2017). However, there were no obvious patterns in the species co-occurrence matrix to indicate that negative associations were more common between species closest in body size, or that positive associations were more common between species of greater size disparity. Thus, potentially, behavioural differences among species (i.e., in foraging strategies, territoriality, and sociality), rather than morphological differences alone, are driving co-occurrence patterns in this system.

The known ecology of the focal species provides some insight here. For instance, house sparrow are a highly gregarious species, typically foraging, roosting, and nesting in conspecific flocks, and frequently foraging with other species (Higgins et al., 2006a). Consistent with this apparent tolerance of foraging with heterospecifics, house sparrow had the highest number of positive associations with other feeder-visitors. In contrast, Eurasian blackbirds are highly territorial, typically forage singly or in pairs, with agonistic interspecific interactions occurring where they have been observed foraging with other species (Higgins et al., 2006a). This was reflected in our observed mean conspecific group size for blackbirds at feeders (1.04 birds) and the predominantly negative associations we found between blackbirds and other feeder-visiting species. Thus, blackbirds were apparently intolerant of using feeders in the presence of other species or could not gain access to them. They may have avoided using the feeder when other birds were present, actively excluded other species, been excluded by other species, or a combination of these. Detailed behavioural analysis of camera trap videos would help elucidate the direction of these interspecific interactions.

Underlying Foraging Mechanisms

Although we did not explicitly investigate which competitive mechanisms controlled feeder access, it seems probable that both interference and exploitative competition were at play. Agonistic interactions at feeding stations were regularly observed in this study (between species but more frequently within species; JAG pers. obs.) and are frequent among feeder-visiting birds in other systems (e.g., Wojczulanis-Jakubas et al., 2015), indicating that interference competition certainly influences feeder access to some degree. However, in our study system exploitative competition is also likely to be a critical mechanism. For example, house sparrows and spotted doves typically arrived at feeding stations in the greatest numbers and consumed food quickly, which are important indicators of exploitative ability (Holway, 1999; Bertelsmeier et al., 2015). Additionally, house sparrows forage in localised areas, typically moving through slowly (Higgins et al., 2006b); spending longer in a particular patch adds to efficiency in resource exploitation (Holway, 1999; Shochat et al., 2004). The exploitative ability of the spotted dove may also be enhanced by the reduced food handling time and food intake rates associated with consuming seeds whole (Shochat et al., 2004).

Individual Variability

Individual variation or "specialisation" is widespread in many taxa, yet conspecific individuals are treated as ecologically equivalent in the majority of studies concerning resource use (Bolnick et al., 2003; Araújo et al., 2011). Here we found convincing evidence that, within species, individuals varied considerably in their feeder use. Individual heterogeneity was highest in the house sparrow, in terms of both feeder visitation rates per day and likelihood of daily presence at feeders, with these individual differences highly repeatable. This high level of individual variation may result from a number of behavioural mechanisms. For instance, it may be indicative of an intraspecific dominance hierarchy dictated by the competitive ability of individuals (Richner, 1989; Rat et al., 2015). Alternatively, individual variation may reflect behavioural flexibility, a trait which has been linked to successful urban exploiter and invasive species (Sol et al., 2002; Peck et al., 2014), especially in regard to flexible foraging strategies. Distinctive individual foraging strategies have been found, for example, within white storks (*Ciconia ciconia*) in southwestern Spain which show both consistent (i.e., specialist) and flexible (i.e., generalist) use of landfills vs. rice fields (Sanz-Aguilar et al., 2015). These differences in foraging strategy within a species may arise from phenotypic, physiological, or competitive discrepancies among individuals (Araújo et al., 2011; Sanz-Aguilar et al., 2015). Individual-level variation in feeder use may also be indicative of differences in personality (Aplin et al., 2014). Among Eurasian blackbird individuals the high variation in feeder use may reflect territorial behaviour. Expectedly, territory owners would use feeders more frequently and with greater consistency, whereas territory intruders would face a greater challenge accessing feeders due to territorial defence behaviour of the territory owner, and so be less frequent and/or less consistent feeder visitors. Studies in territorial calliope hummingbirds (*Selasphorus calliope*; Tamm, 1985) and great tits (Ydenberg, 1984) have found that individual birds that have been provided with supplementary food show increased display and/or defence behaviours, thus making it more difficult for others to access the resource.

Individual heterogeneity in feeder use has important implications at both the individual-and population-level. Among other impacts, individuals that heavily rely on supplementary food may have altered body condition, reproductive success, home range size, and/or survival likelihood (Annett and Pierotti, 1999; Oro et al., 2013). The collective impacts of supplementary feeding on individuals determines the overall effect on population dynamics, including changes in population size, distribution, and migratory patterns (Robb et al., 2008; Oro et al., 2013; Amrhein, 2014), and will depend on both the proportion of the population using feeders and individual heterogeneity in feeder use, particularly where feeder users are a nonrandom subset of the population (Sanz-Aguilar et al., 2015). Furthermore, individuals which follow a high-use, consistent foraging strategy at feeders are more likely to pose a disease risk than sporadic users, as exposure to pathogens and parasites increases as feeder visitation increases (Adelman et al., 2015).

CONCLUSIONS

Our study highlights that individual and species-specific differences in feeder use are present within feeder-visiting bird communities, importantly demonstrating this across seasons within an urban system. These intraspecific and interspecific asymmetries support the likelihood of competitive interactions operating to regulate access to food, and suggest that the effects of supplementary feeding are unlikely to be equivalent across all birds within communities of feeder visitors. In New Zealand resource dominance by introduced species is particularly important, with negative outcomes for native species conservation in cities possible. Individual differences in feeder use observed here are likely to affect the population-level impacts of bird feeding, and consequently should be considered in future studies of garden bird feeding.

AUTHOR CONTRIBUTIONS

JG was the lead author and investigator on the study, leading the experimental design, data collection, and data analysis. DJ, JB, and MS helped develop the experimental design, assisted with logistics and data collection, and reviewed the manuscript. KP assisted with statistical analyses and reviewed the manuscript. All authors reviewed and approved the final version of the manuscript.

FUNDING

This study was funded by the University of Auckland and the Auckland Council.

ACKNOWLEDGMENTS

Our thanks to the volunteer householders involved in the study and our many field volunteers, including the Galbraith Family, Jo Peace, Ellery McNaughton, Cheryl Krull, Megan Young, and Auckland Zoo staff. Particular thanks to Oliver Hannaford for statistical support, and Doug Black from Microchips Australia Pty Ltd for his technical support setting up RFID readers. EcoStock donated bread for the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00081/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 11 April 2017
doi: 10.3389/fevo.2017.00029

Does Urbanization Affect Predation of Bird Nests? A Meta-Analysis

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Urbanization can affect interspecific interactions such as predator-prey relationships. Several hypotheses have been postulated to predict how predation on bird nests changes along urbanization gradients; some predict increased and others decreased predation pressures in urban compared to rural habitats. Using a formal meta-analytical approach, we carried out a systematic literature review to test whether predation on natural and artificial bird nests increased or decreased with urbanization. We found that the effect was highly heterogeneous among studies, due to contrasting results between studies that used artificial nests and those that used natural nests. For artificial nests, survival rate tended to decrease with increasing urbanization, with higher predation in more urbanized study sites. For natural nests, survival tended to increase with the level of urbanization. The latter finding supports predictions of the "urban habitats as predation-safe zones" and "urban nest predator paradox" hypotheses, but the effect may be confounded by many studies not distinguishing between predation and other sources of mortality. None of the other considered methodological and ecological variables explained the variation in a robust way. The discrepancy between the results of artificial and natural nest studies may be due to differences in experimental design (e.g., cavity nests have been more commonly studied in natural nest studies), intrinsic differences between the two nest types (e.g., lack of parental nest defense in artificial nests), or sampling bias. We conclude that the direction of the relationship between urbanization and nest predation is likely to depend on the methodology of the study. Therefore, results from studies using different methodologies, particularly natural or artificial nests, should be generalized with caution to avoid over-interpretations.

Keywords: urban ecology, interspecific interactions, avian nest mortality, predation pressure, survival rate, top-down control, meta-regression

Edited by:
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Specialty section:
This article was submitted to
Urban Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *02 February 2017*
Accepted: *24 March 2017*
Published: *11 April 2017*

Citation:
Vincze E, Seress G, Lagisz M, Nakagawa S, Dingemanse NJ and Sprau P (2017) Does Urbanization Affect Predation of Bird Nests? A Meta-Analysis. Front. Ecol. Evol. 5:29. doi: 10.3389/fevo.2017.00029

INTRODUCTION

Urbanization, i.e., the expansion and development of cities, suburban and exurban areas, creates novel, and often challenging, environments for wild animals. Compared to natural areas, urban habitats are characterized by many altered environmental factors such as elevated levels of chemical, noise and light pollution, transformed landscapes or various disturbances resulting from the increased human population (Marzluff et al., 2001b; Sol et al., 2013; Sprau et al., 2016). These altered environmental factors impact ecological factors that affect population dynamics and persistence, such as food availability and predation pressure (Seress and Liker, 2015). As a consequence, interspecific interactions such as predator-prey relationships can differ qualitatively between urban and non-urban habitats (Faeth et al., 2005; Chace and Walsh, 2006; Fischer et al., 2012).

Although avian species are frequently used model organisms in urban ecological studies on predation, it is unclear how urbanization affects predation on birds and their nests. Different mechanisms have been postulated that predict either increased or decreased rates of predation in cities (Chamberlain et al., 2009)While some hypotheses, as detailed below, suggest that prey species thrive in cities because those habitats are "predator-safe zones" (Gering and Blair, 1999; Ryder et al., 2010; Møller, 2012), others predict an opposite relationship, with predators thriving in cities and imposing increased predation pressure on their prey (Jokimäki and Huhta, 2000; Haskell et al., 2001). Notably, the suggested mechanisms are non-exclusive, and thus any difference in predation rates between urban and rural habitats, or lack of it, may also be a net result of their joint effects. It is also possible that the net effect is zero, and empirical evidence in either direction is just the upper and lower extremes of a distribution around it.

For example, larger species are often less tolerant toward high human density; predators, usually larger than their prey, are therefore expected to be present in lower abundances in cities compared to natural habitats, resulting in lower predation rates (Møller, 2012). However, many opportunistic, medium-sized predator species, such as crows (*Corvus* sp.) (Marzluff and Neatherlin, 2006; Kövér et al., 2015), magpies (*Pica pica*) (Jerzak, 1997), or raccoons (*Procyon lotor*) (Haskell et al., 2001; Prange and Gehrt, 2004), are known to reach higher densities in urban compared to rural habitats. Such patterns suggest that these species also pose higher predation pressures on their prey species in urban habitats. Similarly, domesticated predators, particularly cats (*Felis silvestris catus*), are often found in extremely high abundances in urban and suburban areas (Sims et al., 2008), and increase the risk of predation for avian species (Baker et al., 2008; Balogh et al., 2011; Stracey, 2011). Other authors, by contrast, suggest that, despite this high abundance of certain predator species, urban predation rates can still be low if urban prey populations are also relatively large, which may explain the "urban predation paradox" (Rodewald et al., 2011; Fischer et al., 2012).

Environmental characteristics of urban habitats, such as noise (Slabbekoorn and Halfwerk, 2009), light (Navara and Nelson, 2007), and vegetation (Chace and Walsh, 2006) can also affect predation rates, again either positively or negatively. For example, high noise levels in cities may increase the difficulty for prey animals to detect predation risk and to respond to it, resulting in increased predation rates (Templeton et al., 2016). However, urban noise can also have an opposite effect, as it may disturb predators and make it more difficult for them to detect their prey, thus resulting in decreased predation rates (Francis et al., 2009), For example, noise is known to influence begging calls of nestlings (Leonard and Horn, 2008), and it might be possible that it conceals the vocalizing chicks from predators. Similarly, artificial lighting can make prey more conspicuous to predators, increasing predation rates (Clarke, 1983), but also make it easier for prey to detect predators (Gorenzel and Salmon, 1995). Furthermore, vegetation is more fragmented in urban habitats, and fragmentation can increase predation risk (Hartley and Hunter, 1998), possibly because there is less shelter for prey animals.

Weaker anti-predator responses (e.g., shorter flight initiation distances, lower vigilance behavior, shorter recovery rates) of urban animals (compared to non-urban conspecifics) have also been described as indirect evidence for decreased predation risk in cities (Møller and Ibáñez-Álamo, 2012). However, apart from a handful of experiments with predator dummies (Seress et al., 2011) or playbacks of alarm calls (Myers and Hyman, 2016), the majority of empirical studies comparing anti-predator behavior of urban and rural populations have been conducted using humans as potential predators (reviewed by Samia et al., 2015). While humans might be perceived as potential predators, they usually do not represent a direct predation threat (Beale and Monaghan, 2004). Therefore, these results are difficult to generalize to non-human predators. In fact, tolerance of humans can also lead to decreased vigilance in prey, which can lead to higher predation rates in urban areas (Geffroy et al., 2015).

Studies investigating mechanisms postulated above have used a diverse array of methods. Some studies compare the diet composition of urban and rural predators, e.g., using remains found in pellets of birds of prey (e.g., Brack et al., 1985; Kristan et al., 2004; Lesiński et al., 2009). However, while pellet analysis can be informative regarding the relative predation pressure on various prey species, it tells very little about the overall predation pressure. Another approach is the direct monitoring of prey mortality. Studies on post-fledging and adult mortality are uncommon, because population censuses and capture-recapture methods (Chiron and Julliard, 2007; Brown and Graham, 2015) might estimate survival inaccurately, or because tracing individual prey animals (e.g., by radio-telemetry; McCleery et al., 2008; Ausprey and Rodewald, 2011; Balogh et al., 2011; Shipley et al., 2013) is often costly and based on small sample sizes. Bird nests, in contrast, are immobile, easy to monitor, and are often preyed upon by various predator species. Thus, natural bird nests, as well as artificial nests baited with real or fake eggs, are most often used as study system when comparing predation rates between differently urbanized habitats. However, some factors may increase heterogeneity in effects reported in the literature, hindering interpretation and generalization of results, such as differences in characteristics between artificial and natural nests (Moore and Robinson, 2004; Robinson et al., 2005), nesting characteristics specific to the study species (e.g., nest height from the ground, nest openness), and inconsistent, study-specific definitions of urbanization (Marzluff et al., 2001a).

In this paper, we investigate how predation rates on bird nests change with habitat urbanization by conducting a formal meta-analysis. To our knowledge, this paper is the first formal meta-analysis that attempts to quantitatively synthetize published results concerning this question. We hypothesize that if the mechanisms that increase predation rate with urbanization, as detailed above, outweigh those that decrease predation rate, we will find higher predation rates in urban habitats, and vice versa. Furthermore, we also conducted meta-regressions (meta-analyses with additional explanatory variables, henceforth referred to as "moderators"), to explore whether variation in effect size between studies can be explained by differences in study design (using natural vs. artificial nests), bird species, or definitions of predation rate or urbanization.

METHODS

Literature Screening and Data Collection

We followed the PRISMA protocol for collecting data from the published literature (Moher et al., 2009; Nakagawa and Poulin, 2012). We first performed literature searches using the online search engines Scopus (http://www.scopus.com/) and Web of Science (http://wokinfo.com/) with the following keyword string: "*nest∗ AND (*urban* OR anthropogenic OR rural) AND predat**." We then screened the titles and abstracts of the papers found by the search, and decided whether the following criteria were met:

- The abstract indicated that study was conducted on bird nests, either by monitoring natural bird populations, or by performing experimental studies on artificial nests baited with real or fake eggs.
- The abstract indicated that the survival (or mortality) rates of these nests were reported in the main text as a function of an urbanization gradient.

One of us (EV) screened all the abstracts found by Scopus, while another (GS) screened all the abstracts found by searching the Web of Science (WoS), and coded whether the papers met the selection criteria or not. Papers that were found by both search engines were scored by both observers and used for testing between-observer repeatability (see Table S1). We also performed backward searches, i.e., screened reference lists of relevant papers and reviews, and visited author websites, to find additional eligible papers that might have been missed during the systematic database searches. We did not limit our search to English-language papers, but also included papers written in Spanish, French, German, Russian, or Polish.

Papers considered to meet the selection criteria by at least one observer were taken forward for full-text screening. Each paper was screened by a single person. During full-text screening we excluded the papers that:

- Did not define an urbanization gradient (i.e., because all studied sites had the same level of urbanization, or because the information was not provided);
- Addressed other forms of anthropogenic disturbance (i.e., fragmentation of natural or semi-natural habitats, agricultural practices) without using any urban study sites;
- Did not report any nest survival (or mortality) data, only presence/absence of species, adult survival or individual offspring survival;
- Had overlapping data with another paper (i.e., when the two papers tested different hypotheses using the same dataset). In these cases we included the paper which contained the most information (i.e., reported more complete data or used a larger data set);
- Did not report their nest survival data in relation to urbanization, despite conducting the study in differently urbanized habitats;
- Reported the data in a format that did not allow extraction of effect sizes (e.g., multivariate regressions, mixed-effects models, daily survival rates from logistic exposure models—see further justification in Section Data Extraction).

In the latter two cases, we contacted the original authors provided that their e-mail addresses were available. We got feedback from 19 out of 28 contacted authors. For four studies, we obtained raw data from the authors from which we managed to extract effect sizes ourselves; we also included these papers in the analysis.

Scoring Urbanization

Studies varied widely in methods used to quantify the levels of urbanization of their study sites. To make studies comparable, two observers (EV and GS) independently scored the level of urbanization of each study on a five-level urbanization scale (using a modified version of the scale proposed by Marzluff et al., 2001a):

1. Wildland/natural area: Interior of a large (>200 ha) forest or meadow, with little anthropogenic effects.
2. Rural area: Landscape dominated by anthropogenic effects, such as agriculture (pasture, crop field, orchard, farmland), or very intensive forestry (clear-cuts), with little housing (<2.5/ha); or a small forest patch (<200 ha)/forest edge within these types of landscapes.
3. Suburban edge/exurban area: Landscape in the proximity of urban or developed industrial areas, with a low housing density (<2.5/ha) or a low proportion of built/developed surface (<20%, or >80% vegetation), e.g., brownfields, golf courses, areas with detached houses; or a small forest patch (<200 ha)/forest edge within a suburban matrix.
4. Suburban area: Landscape with medium housing density (2.5–10/ha) or medium proportion of built/developed surface (20–50%; or 50–80% vegetation), e.g., most urban parks, residential areas with single-family houses, lawns and gardens, university campuses.
5. Urban area: Landscape with high housing density (>10/ha, 1,000/km^2) or high proportion of built/developed surface (>50%; or <50% vegetation), e.g., residential areas with multi-story buildings and blocks of flat, commercial, service, and industrial buildings.

Information on numbers of sites scored by the two observers, and on between-observer repeatability, is provided in Table S2. Sites that got different scores from the two observers were assigned one of the five scores via subsequent discussion. In most studies ($N = 40$; 78%), the order of the study sites based on these urbanization scores matched the order indicated by the original authors. However, for some papers ($N = 11$), sites that represented different urbanization levels according to the authors were given equal scores by us.

Data Extraction

We used correlation coefficients between nest survival (i.e., the probability of nests not failing) and urbanization as our effect size. We calculated these correlation coefficients from the numbers of surviving and failed nests at each study site unless already provided in the paper. The numbers of surviving and failed nests were obtained in several ways:

(i) The exact numbers of surviving and failed nests in an experiment were directly reported ($N = 22$) or given to us by the original authors ($N = 4$);
(ii) The numbers could be calculated by multiplying the reported percentage of survived nests sample sizes ($N = 20$);
(iii) If the survival rates were reported in figures as column diagrams ($N = 3$), we measured the height of the columns and calculated the number of surviving and failed nests from these;
(iv) One paper reported daily nest survival rates calculated by Mayfield's method (Mayfield, 1961, 1975) along with the numbers of exposure days; for this paper we calculated the number of failed nests by multiplying daily predation rate by the number of exposure days. Other papers that reported daily nest survival rates ($N = 7$, Table S3) were excluded, as we did not find a reliable way to back-calculate the number of survived and failed nests.

There was a single paper that reported only a correlation coefficient (Lumpkin et al., 2012), which we used as our effect size. For all other papers we coded each nest as failed (0) or survived (1), ranked urbanization around the nest according to the five levels on an ordinal scale from the least to the most urbanized based on the urbanization scores assigned by us, and ran a Spearman rank correlation between these two variables for each study. We used the Fisher's Z-transformed correlation (Zr) value as our effect size (see Section Statistical Analyses below for more explanation for this choice). We excluded studies where only test statistics from regression models other than correlation tests were available for which we did not obtain data from the authors ($N = 18$, Table S3). We excluded these studies because the conversion of multivariate regression estimates to correlation coefficients often does not accurately approximate correlation coefficients when calculated from raw data (Aloe, 2015). Sampling error variance of each effect size (Fisher's Zr) was calculated as the reciprocal of the total number of nests minus three.

Some papers ($N = 16$ natural nest studies and $N = 20$ artificial nest studies) distinguished between predation and other forms of nest failure (e.g., abandonment, weather destroying the nest, vandalism by humans). Since we were primarily interested in predation, whenever it was possible, we omitted nests that failed due to causes other than predation, and used only nests that survived and nests that failed due to predation. For papers that did not distinguish between sources of nest failure ($N = 15$ studies, all on natural nests), we simply used numbers of survived and failed nests to calculate effect sizes, regardless of the actual source of nest failure. Similarly, partially predated nests (i.e., where some eggs or offspring disappeared, but at least one of them survived) were considered as predated in some studies ($N = 19$) and as survived in others ($N = 28$), while some studies were conducted on nests with only one egg ($N = 4$). To test whether these two methodological differences affected our effect sizes, we applied the moderator "source of mortality" as a binary factor to describe whether our effect size was calculated from predation-only mortality or from total mortality, and the moderator "partial predation," also as a binary factor, to code whether failed nests included the partially predated nests or not.

For studies spanning multiple years, we extracted separate effect sizes for each year, with "study year" as a moderator. However, in some papers only pooled data from multiple years were available. In these cases we used the median value of all study years as a measure of "study year." We also recorded the following moderators: whether the study reported observational data collected in a natural population ($N = 28$) vs. experimental data using artificial nests ($N = 20$); the number of eggs per nest (average number for natural nests, exact number for artificial nests); nest position (ground nest or elevated, e.g., on a tree or building); nest height above ground in meters; nest openness (cup nest—a hemispheric, open nest on the ground or a branch; orb nest—a spherical nest closed on top; or cavity nest—one inside a den or a box); the number of days the nest was exposed to predators (the length of the experiment for artificial nest, the length of the nesting cycle for natural nests); and, in case of natural nests, the study species. We also recorded the year and journal in which the paper was published.

Statistical Analyses

Before conducting analyses, we converted correlation coefficients r (Spearman's r) into a standard normal metric (using Fisher's z-transformation), Zr. This was done because r is bounded between –1 and 1, and thus does not follow a normal distribution (Hedges and Olkin, 1985). All analyses were performed using the transformed values (Zr), but the results were transformed back to correlations (r) for visual presentations. Correlational effect of $r = 0.1$, 0.3, and 0.5, can be considered as small, medium and large effect sizes, respectively, following Cohen's (1988) tentative benchmarks. Point estimates from statistical models were considered significantly different from zero when their 95% Confidence Intervals (CI) did not overlap zero.

We performed all statistical analyses within R statistical software v3.2.4 (R Core Team, 2016). For meta-analysis and meta-regression we used the *metafor* package (Viechtbauer, 2010). The phylogenetic tree for the bird species included in the dataset was created based on the global phylogeny of birds (Jetz et al., 2012) and visualized using the *ape* package (Paradis et al., 2004). We used multilevel meta-analyses, representing a type of linear mixed model (Viechtbauer, 2010; Nakagawa and Santos, 2012), to control for various sources of non-independence in the data. Non-independence can be present when multiple effect sizes are extracted from the same study, multiple effect sizes are available for the same species, and/or due to phylogenetic relationships among species. To take such non-independences into account, we included random intercepts for study identity, species identity (for natural nests), and phylogeny (for natural nests; using phylogenetic meta-analysis, Hadfield and Nakagawa, 2010; Nakagawa and Santos, 2012) in our models.

Meta-Analyses and Meta-Regressions

We first ran a simple meta-analytical model (an intercept-only model without any moderators) on all available data to examine the overall effect, which tested whether the meta-analytic mean was different from zero. This meta-analytic mean represents the overall relationship between urbanization level and nest predation rate over all data. We quantified total heterogeneity in the dataset by computing I^2 statistic (Higgins et al., 2003; Supplementary Methods S2.1). We also assessed separate meta-analytical models (and quantified heterogeneity) for the two data subsets composed of effect sizes from studies using either artificial or natural nests, respectively. To interpret the difference between the predation rates in differently urbanized habitats, we converted our meta-analytical means from correlation coefficients to odds ratios (OR; Borenstein et al., 2009); both numbers are reported in the Section Results.

We conducted meta-regression analyses to identify variables accounting for heterogeneity across studies. Continuous moderators were standardized prior to the analyses, so that they had a mean of 0 and *SD* of 1. Nest height above ground was log-transformed due to skewedness. We first quantified whether the relationship between urbanization and predation differed on average between the two major study approaches, i.e., artificial vs. natural nest studies. To do so, we added nest type (artificial vs. natural) as a moderator. Because the data subsets for artificial and natural nests had different combinations of applicable moderators, we then performed separate meta-regression analyses on these two data subsets. We did not construct models with multiple moderators, because the sample sizes of the subsets were inadequate and/or information on key moderators was missing for many data points. For artificial nests we considered the following moderators: nest openness (cup/hole), nest position (elevated/ground/mix), average egg number per nest, study duration, median study year, study publication year, minimum (min) urbanization score of the site gradient (1/2/3/4), maximum (max) urbanization score of the site gradient (3/4/5). For the natural nests, we considered the following moderators: source of mortality (i.e., whether failures due to other causes than predation were excluded from calculating the effect sizes or not: yes/no), nest openness (cup/hole/orb), nest position (elevated/ground), nest height above ground in meters, average egg number per nest, study duration, median study year, study publication year, minimum urbanization score of the site gradient (1/2/4), maximum urbanization score of the site gradient (3/4/5).

Sensitivity Analyses and Publication Bias

To test whether our results were sensitive to the way we scored urbanization, we repeated all analyses with effect sizes calculated from alternative urbanization scores based on the authors' scores.

We also assessed evidence for publication bias (Supplementary Methods S2.2), which can affect conclusions of a meta-analytic studies when published studies are biased toward significant findings (Rothstein et al., 2005).

RESULTS

General Results

We screened the abstracts of 412 papers, 138 of which were taken forward for full-text screening (Figure S1). We excluded 87 papers based on our inclusion criteria (fully detailed in Table S3). Our final dataset thus comprised 117 effect sizes from 51 papers published between 1985 and 2015 (**Table 1**). The median number of nests monitored within a publication was 104 (mean = 177.6, *SD* = 246.5); only in eight cases were effect sizes calculated based on sample sizes of more than 500 nests. Almost half of the effect sizes came from studies that scored mortality in natural nests (58, vs. 59 effect sizes for studies using artificial nests). Effect sizes from natural nest observations represented 32 different species from 21 Families within 6 Orders (with 25 species from 16 families belonging to the Order *Passeriformes*). Most species were represented in our meta-analysis by only one study; only data for the House wren *Troglodytes aedon*, European magpie *Pica pica* and Common blackbird *Turdus merula* were available from 2, 3, and 3 studies, respectively (**Table 1**). All continents (except for Antarctica) were represented in the meta-analysis, with most data from North America (46 effect sizes from 23 studies) and Europe (46 effect sizes from 15 studies; **Table 1**).

Studies using artificial nests differed from those with natural nests in several aspects of their study design (Table S4). First, artificial (vs. natural) nests were, on average, followed for much shorter periods of time (Mean ± *SD* = 12.2 ± 5.9 vs. 41.2 ± 21.1 days). Second, studies using artificial nests were more often performed in less urbanized locations. Third, artificial nests were located on the ground in about half of the studies (28 out of 59 effect sizes), whereas natural nests were usually located at least 2 m above ground level, with only 6 out of 58 effect sizes coming from studies on ground nests. Fourth, artificial nests were usually open, i.e., cup-shaped (90%; 53 out of 59 effect sizes) and 10% were hole-like (nesting box/cavity). In contrast, natural nests were open in 62% of our data points (36 out of 58 effect sizes), and 34.5% were hole-like. Fifth, failures of artificial nests were always assumed to result from predation (100%), whereas most studies on natural nests reported overall survival rates that did not distinguish between predation and other sources of mortality (66%; 38 out of 58 effect sizes). Finally, all artificial nests were considered as predated when at least one egg/offspring died, while in natural nests usually only complete brood loss was counted as a predation event (97%; 56 out of 58 effect sizes).

Meta-Analyses and Meta-Regressions

We found no overall relationship between nest survival and level of urbanization: the meta-analytic mean for the slope between urbanization gradient and nest survival was not distinguishable from, and was centered around, zero [meta-analytical mean (β) = −0.003; 95% Confidence Intervals (CI) = −0.080 to 0.074; OR = 0.99; **Figure 1**, Table S5]. Effect sizes were, at the same time, highly heterogeneous ($I^2 = 92.7\%$). High heterogeneity suggests effects of moderators, and this finding thereby justified our subsequent meta-regression analyses (Higgins and Thompson, 2002).

When meta-analytic means were estimated separately for the studies using artificial vs. natural nests, we found weak evidence for an effect of urbanization on nest survival for both cases. The meta-analytic mean for artificial studies was small and negative, with confidence intervals marginally overlapping with zero, suggesting a decreasing nest survival with increasing urbanization ($\beta = -0.118$; 95% CI = −0.238 to 0.006; OR =

TABLE 1 | Summary of the papers included in the meta-analysis.

References	Species latin name	Nest type	*k*	Data source	Continent
Lin et al., 2015	*Accipiter trivirgatus*	Natural	1	Numbers (reported)	Asia
Kuranov, 2008	*Acrocephalus dumetorum*	Natural	1	Numbers (reported)	Europe
Grandmaison and Niemi, 2007	*Agelaius phoeniceus*	Natural	1	Numbers (reported)	North America
Bowman and Woolfenden, 2001	*Aphelocoma coerulescens*	Natural	6	Percentage (reported)	North America
Vennesland and Butler, 2004	*Ardea herodias*	Natural	2	Percentage (from author)	North America
England et al., 1995	*Buteo swainsoni*	Natural	5	Numbers (reported)	North America
Kosiński, 2001	*Carduelis chloris*	Natural	1	Percentage (reported)	Europe
Patten and Bolger, 2003	*Aimophila ruficeps, Chamaea fasciata, Pipillio crissalis, Pipillio maculatus*	Natural	4	Numbers (from author)	North America
Beck and Heinsohn, 2006	*Corcorax melanorhamphos*	Natural	1	Numbers (reported)	Australia
Brahmia et al., 2013	*Cyanistes caeruleus*	Natural	3	Numbers (from author)	Africa
Vigallon and Marzluff, 2005	*Cyanocitta stelleri*	Natural	1	Numbers (reported)	North America
Reidy et al., 2008	*Dendroica chrysoparia*	Natural	1	Numbers (reported)	North America
Cempulik, 1993	*Gallinula chloropus*	Natural	1	Numbers (from author)	Europe
Newell and Kostalos, 2007	*Hylocichla mustelina*	Natural	1	Daily predation rates	North America
Pretelli et al., 2015	*Hymenops perspicillatus, Pseudoliestes virescens*	Natural	2	Numbers (reported)	South America
Mazumdar and Kumar, 2014	*Nectarinia asiatica*	Natural	1	Numbers (reported)	Asia
Eden, 1985	*Pica pica*	Natural	1	Numbers (reported)	Europe
Antonov and Atanasova, 2003	*Pica pica*	Natural	1	Numbers (reported)	Europe
Sachteleben et al., 1992	*Pica pica*	Natural	1	Percentage (reported)	Europe
Morimoto et al., 2012	*Seiurus aurocapilla*	Natural	1	Percentage (reported)	North America
Misztal et al., 2008	*Sitta europaea*	Natural	1	Numbers (reported)	Europe
Solonen and Ursin, 2008	*Strix aluco*	Natural	10	Numbers (from author)	Europe
Kuranov, 2009	*Ficedula hypoleuca, Sturnus vulgaris, Parus major, Phoenicurus phoenicurus*	Natural	4	Percentage (reported)	Asia
Newhouse et al., 2008	*Troglodytes aedon*	Natural	1	Numbers (reported)	North America
Hofer et al., 2010	*Troglodytes aedon*	Natural	1	Numbers (reported)	North America
Ibáñez-Álamo and Soler, 2010	*Turdus merula*	Natural	1	Percentage (reported)	Europe
Vogrin, 2000	*Turdus merula*	Natural	1	Numbers (reported)	Australia
Kentish et al., 1995	*Turdus merula*	Natural	1	Percentage (reported)	Europe
Cardilini et al., 2013	*Vanellus miles*	Natural	1	Numbers (reported)	Australia
Kamp et al., 2014	*Vanellus vanellus*	Natural	1	Numbers (reported)	Europe
Thorington and Bowman, 2003	NA	Artificial	1	Percentage (reported)	North America
Gering and Blair, 1999	NA	Artificial	4	Figure	North America
Wilcove, 1985	NA	Artificial	1	Percentage (reported)	North America
van Heezik et al., 2008	NA	Artificial	1	Percentage (reported)	New Zealand
López-Flores et al., 2009	NA	Artificial	1	Numbers (reported)	Central America
Ryder et al., 2010	NA	Artificial	1	Percentage (reported)	North America
Jokimäki and Huhta, 2000	NA	Artificial	4	Percentage (reported)	Europe
Melampy et al., 1999	NA	Artificial	2	Percentage (reported)	North America
Danielson et al., 1997	NA	Artificial	1	Percentage (reported)	North America
Jobin and Picman, 1997	NA	Artificial	2	Percentage (reported)	North America
Jobin and Picman, 2002	NA	Artificial	2	Percentage (reported)	North America
Jokimäki et al., 2005	NA	Artificial	3	Numbers (reported)	Europe
Latta et al., 2012	NA	Artificial	1	Numbers (reported)	North America
Sasvári et al., 1995	NA	Artificial	18	Figure	Europe
Piper and Catterall, 2004	NA	Artificial	6	Percentage (reported)	Australia
Piper et al., 2002	NA	Artificial	1	Percentage (reported)	Australia
De Santo and Willson, 2001	NA	Artificial	2	Figure	North America
Keyser, 2002	NA	Artificial	4	Numbers (reported)	North America
Matthews et al., 1999	NA	Artificial	2	Percentage (reported)	Australia
Lumpkin et al., 2012	NA	Artificial	1	Coefficient	North America
Czyzowski et al., 2006	NA	Artificial	1	Numbers (reported)	Europe

K, Number of effect sizes per study; NA, Not applicable due to artificial nests being used in the study.

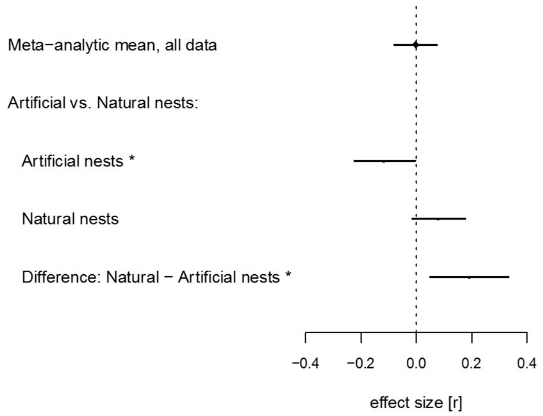

FIGURE 1 | Forest plot representing results of analyses performed on the full data set. The meta-analytic mean indicates overall effect when data from studies using artificial and natural nests are combined. Meta-regression models estimated intercepts independently for studies using artificial and natural nests, respectively, and the difference between overall effect sizes for these two data subsets. Points represent mean estimates from the models, lines represent 95% Confidence Intervals. Stars indicate estimates that are significantly different from zero (95% Confidence Intervals not crossing zero).

0.65; **Figure 1**; Table S5). In contrast, the meta-analytic mean for natural nest studies was small and positive; again, confidence intervals were marginally overlapping zero, suggesting that nest survival increased (instead of decreased, see above) with urbanization ($\beta = 0.079$; 95% CI $= -0.007$ to 0.165; OR $=$ 1.33). The latter effect was lessened when phylogeny was taken into account ($\beta = 0.034$; 95% CI $= -0.163$ to 0.228, **Figure 2**, Table S7; we note that phylogenetic effects were not applicable for artificial nest studies, as those did not have associated species). Total heterogeneity was above 90% in all meta-analytic models (Tables S5–S7).

When we included nest type (artificial vs. natural nests) as a moderator in the meta-regression model on the full data set, the difference between average effect sizes for artificial and natural nests was small but statistically significant (difference between β artificial and β natural $= 0.195$; 95% CI $= 0.050$ to 0.332; **Figure 1**; Table S5), implying that the slopes of the urbanization-mortality relationship differ between the two nest types. Moderators considered affected the relationship between urbanization and nest predation neither in artificial nest (**Figure 2A**; Table S6) nor in natural nest studies (**Figure 2B**; Table S7). When we used species identity as a moderator, we found high variation among species (Figure S2, Table S8); note, however, that most species were represented with only one study in the meta-analysis, making further interpretations of this latter result difficult.

Sensitivity Analyses and Publication Bias

All analyses using alternative urbanization scores (based on the quantification of urbanization levels by the original study authors) had results qualitatively identical to these from the main analyses (Tables S9–S12), indicating that our results are robust to the way urbanization was scored.

We found no evidence for publication bias. Visual inspection of funnel plots and Egger's regression test revealed no indication of funnel shape asymmetry [**Figure 3**; $t_{(115)} = 0.716$, $p = 0.476$]. A trim-and-fill method also implied that there were no missing effect sizes, consistent with the absence of publication bias.

DISCUSSION

Overall, our meta-analysis did not detect any strong relationship between urbanization and nest mortality. However, predation rates of artificial nests and of natural nests showed opposite trends in their relationships with urbanization: namely, the chance of natural nests to fail tended to decrease (i.e., the odds of survival of natural nests increased by 33%) with increasing urbanization, whereas the trend in artificial nests was significantly different, and in the opposite direction (i.e., the odds of survival was about 35% lower in more urbanized habitats). None of our considered moderators, ecological or methodological, explained the variation in effect sizes consistently in either study type, although our sensitivity analyses suggest nest openness may have an effect (Table S11, Supplementary Discussion S3.1).

The weak positive trend for a correlation between nest survival and urbanization in natural nests is in line with the "predator-safe zone hypothesis" (Gering and Blair, 1999; Ryder et al., 2010), which assigns the lower predation risk in cities as a principal reason of why certain prey species can thrive in urban habitats. Lower predation rates can be the result of low abundance of nest predators (Møller, 2012). However, many potential nest predator species are found in higher abundances in cities than in the surrounding natural habitats (Jokimäki and Huhta, 2000; Haskell et al., 2001). This apparent contradiction is called the "nest predation paradox" (Fischer et al., 2012). This paradox might be resolved in several ways. First, some prey species are often extremely abundant and/or found in higher densities in more urban habitats (McKinney, 2006). Thus, despite the high absolute numbers of nest predators, their relative abundance to prey can still be low compared to natural habitats (Fischer et al., 2012). Second, urbanization changes predator species composition (Rodewald and Kearns, 2011). This shift can mean a decrease in specialized ("strong") nest predators and an increase in opportunistic ("weak") nest predators (Stracey, 2011). For example, snakes, which are often specialized nest predators, are less abundant in cities than in rural areas (Patten and Bolger, 2003). In contrast, house cats, which are both fed by humans and hunt for live prey, and thus are mostly opportunistic nest predators, become more abundant with housing density (Sims et al., 2008), although they might be less common (or less often outdoors) in city centers compared to suburbs. Third, potential predators may specialize on different prey in urban than in rural areas as the most abundant prey species might differ between them, which can relax predation pressure on less abundant species (Fischer et al., 2012). Alternatively, in the case of omnivorous predator species, the shift can be toward anthropogenic food sources that are easier to access, which can relax the actual predation pressure on all prey species (Rodewald

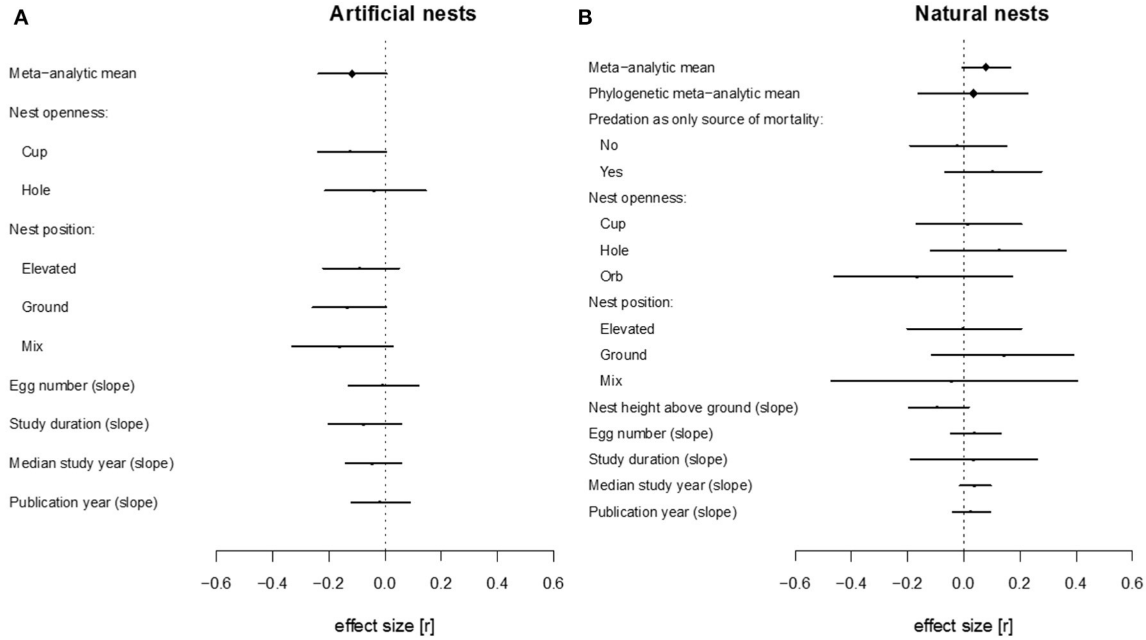

FIGURE 2 | Forest plots of effects size estimates for the studies using artificial nests (A) and natural nests (B). Positive estimate value for meta-analytic mean indicates higher survival in more urbanized habitats. Positive estimate values for moderators can be interpreted as increased likelihood positive effect sizes (higher survival in more urbanized habitats) with increasing values of a continuous moderator, or for a given level of a categorical moderator.

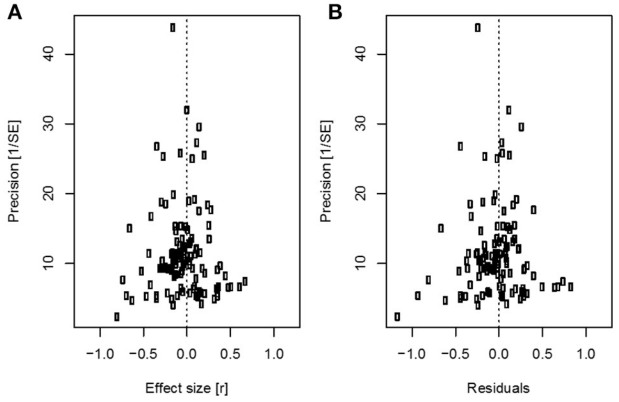

FIGURE 3 | Funnel plots used to estimate publication bias in the data set. (A) Effect size estimates from plotted against their precision; **(B)** Residual effect sizes from the model with nest type (artificial nest/natural nests) used as a moderator. The dashed line indicates zero, i.e., no relationship between nest mortality and urbanization scores.

et al., 2011). Finally, higher abundance of nest predators in urban habitats can facilitate local adaptation in the behavior of their prey. As high predation pressure in cities should eliminate those individuals that could not effectively defend their nests from predators (either by hiding their nests or actively mobbing predators), the current urban prey population is better at nest defense and thus has higher survival rates than rural prey (Stracey, 2011).

In contrast to natural nests, we found a decreasing trend in the survival of artificial nests with increasing urbanization. This result is in line with a meta-analysis which revealed more predation on artificial nests with increasing fragmentation of forest habitats (Hartley and Hunter, 1998). Also, similar to our findings, a number of studies showed that predation rates in artificial nests often do not reflect those observed in natural nests (Haskell, 1995; Weidinger, 2001; Moore and Robinson, 2004; Robinson et al., 2005), although one study that used artificial and natural nests in the same conditions did not find significant difference between the two (Blair, 2004). The discrepancy between artificial and natural nests may be explained by several different mechanisms. First, our sensitivity analyses on natural nests indicated that predation rates of cavity nests and open nests change differently with urbanization (Table S11). Namely, cavity nests are predated significantly less in urban than in rural habitats, while open nests show no such habitat difference. Since the majority of artificial nest studies were conducted on open nests, their results are more comparable to those from natural open nests than to those from hole-like nests (which are also likely to be located high above the ground and exposed to different conditions). Second, as mentioned above, it is hypothesized that local adaptation may make urban bird parents better at nest defense behavior than their non-urban conspecifics (Stracey, 2011). As artificial nests are not defended by parents, they may be more likely to be depredated in urban habitats than natural nests. Third, the local adaptation hypothesis also suggests that urban birds are better at hiding their nests from predators (Stracey, 2011). As the locations of artificial nests are chosen by the experimenters, rather than the birds themselves, nests may be placed in more conspicuous places in urban habitats, thus predators can find them more easily, resulting in a higher predation rate. Fourth, although artificial nests try to emulate natural nests as much as possible, they might still be perceived by predators as novel compared to natural nests. It has been hypothesized that urban animals show less food neophobia (i.e., are more likely to accept novel food sources) than their rural conspecifics (Sol et al., 2011), and thus non-urban predators may be aversive toward artificial nests. Fifth, some discrepancy between the results of natural vs. artificial nest studies may come from the fact that partially predated nests were counted as survived in most natural nest studies, whereas they were counted as predated in artificial nest studies. Partial predation may be more common in cities where the predator is more likely to be interrupted by human disturbance while feeding on a nest. Finally, we cannot exclude the possibility that the real predation pressure on natural nests is higher in cities than in rural habitats, as studies on artificial nests suggest, but the effect is masked by sampling bias. Urban natural nests that were not concealed or defended well-enough may already have been predated before the researchers found them, and thus not included in the sample, while this might not be the case in rural habitats. The resulting sampling bias may lead to a seemingly higher predation rate in rural compared to urban habitats.

In conclusion, our results show that natural nests tend to be less predated in urban habitats than in rural habitats, but this trend is not reflected by studies on artificial nests. We have several recommendations for future studies addressing the relationship of urbanization and nest predation. First, we suggest that the cause of nest failure should be identified as precisely as possible, because both predation and other forms of mortality (weather, nest abandonment, vandalism by humans) can vary with urbanization, affecting the overall nest survival. Second, to identify whether variation is due to differences between species, multiple populations of the same species should be studied, and studies should preferably include data from multiple species. Third, data from the currently available literature is not sufficient for more sophisticated analyses, such as testing multiple biological and ecological factors in the same model and possible interactions between them, therefore more studies, with more balanced design for ecological characteristics such as nest height and nest openness, are required. Finally, as patterns derived from studies using artificial nests often do not qualitatively reflect those derived from natural nests, researchers should perform studies where artificial and natural nests with similar characteristics are monitored within the same area (e.g., Blair, 2004), and investigate sampling bias. Such validation is important to draw firm conclusions regarding the level of predation in urban vs. rural areas in future studies.

CODE AND DATA AVAILABILITY

All data used in this manuscript are available in the Supplementary Material of this paper (Supplementary Data). The R scripts for all analyses are available at GitHub (https://github.com/mlagisz/nest_predation_MA).

AUTHOR CONTRIBUTIONS

EV, PS, ND, and SN conceived and designed the study, EV and GS collected and proofed data, ML and SN run the analyses and created the figures. The first version of the manuscript was written by EV (Introduction, Methods: Data collection, Discussion) and ML (Methods: Statistical analyses, Results); all authors contributed to revisions.

FUNDING

EV was supported by Deutsche Bundesstiftung Umwelt's international scholarship grant (30015/588) during data collection and by the ÚNKP-2016-3-IV "New National Excellence Program" of the Ministry of Human Capacities of Hungary during data analysis and preparation of the manuscript. GS was supported by the Hungarian National Research, Development and Innovation Office (NKFIH K112838 and PD 120998) grants. SN was supported by an ARC Future Fellowship (FT130100268). Publication fees were taken from a grant provided by the Deutsche Forschungsgemeinschaft (SP 1450/3-1) granted to PS.

ACKNOWLEDGMENTS

We thank the following authors for answering questions about their studies and providing additional information and/or raw data: C. C. Catterall, P. Cempulik, T. Csörgö, J. C. Gering, C. Holzapfel, C. C. Hofer, J. D. Ibáñez-Alamo, L. Jerzak, M. A. Patten, J. L. Reidy, A. D. Rodewald, R. Scheifler, T. Solonen, C. M. Stracey, R. G. Vennesland and D.S. Wilcove. We also thank A. Rutten for her help with programming, and M. Moiron and J. Limonet for helping with the translation of two foreign-language papers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00029/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 11 July 2017
doi: 10.3389/fevo.2017.00072

Switch to a Novel Breeding Resource Influences Coexistence of Two Passerine Birds

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A crucial step in adapting to urban habitat is switching to novel, often man-made, resources. Switching to a novel resource can influence the dynamics of species coexistence, particularly if it alters trade-offs in performance. While such switches are frequently documented, their influence on species coexistence has been difficult to assess because it requires knowledge of performance trade-offs in the context of both historical and novel resource use. Western and mountain bluebirds provide a unique system in which to investigate the effects of a resource switch on species coexistence because both depend on secondary nest cavities to breed and, across a large part of their range, have switched to using man-made nest boxes. Western bluebirds are less dispersive, but more aggressive, than mountain bluebirds leading to a successional pattern of species replacement in many nest box populations; however, there is evidence of continued coexistence in natural post-fire habitat. Nest boxes differ from natural cavities in both distribution, which may affect the dynamics of interference competition between the species, and thermal conductance, which may impact competition by altering survival of ectothermic young. Here, we use a combination of experimental manipulations of nest box density and more than a decade of fitness and incubation temperature data to investigate whether altered resource distribution or thermal environment best explain patterns of species replacement in nest box populations. In both species, we found that females breeding in nest boxes were unable to maintain normal incubation temperatures during inclement weather and experienced similar offspring mortality patterns. Moreover, climatic variation across populations did not predict species' relative abundance. Instead, experimental manipulation of nest box density showed that mountain bluebirds persisted longer when nest boxes were distributed farther apart, suggesting that nest box distribution may be a key factor in understanding how human-created habitat impacts coexistence of bluebird species. These results emphasize that knowledge of species interactions in the historical habitat is crucial to understanding population dynamics as species transition to novel, man-made habitat.

Keywords: successional dynamics, species replacement, interference competition, competitive dominance, colonization, resource heterogeneity

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *22 February 2017*
Accepted: *19 June 2017*
Published: *11 July 2017*

Citation:
Duckworth RA, Hallinger KK, Hall N and Potticary AL (2017) Switch to a Novel Breeding Resource Influences Coexistence of Two Passerine Birds. Front. Ecol. Evol. 5:72. doi: 10.3389/fevo.2017.00072

INTRODUCTION

Urbanization has eliminated habitat and resources that many species require, leading to their disappearance from areas where they were once common (Marzluff, 2001; Shochat et al., 2006). Although many species are lost as a consequence of urbanization, changes in habitat structure and resource availability often provide a boon to a small subset of species making community composition in urban areas distinct from more natural environments (Devictor et al., 2007; Chamberlain et al., 2009). Yet, the processes underlying shifts in community composition are poorly understood (Shochat et al., 2010). In particular, it is unclear why some species excel in human-altered landscapes and other species rapidly decline.

One explanation for such differential success is that the changes in resource quality and availability in urban and agricultural areas may affect species' relative competitive ability. For example, superior interference competitors, which dominate in direct interactions for scarce resources (Schoener, 1983), may be able to monopolize resources more efficiently in resource-rich habitat patches and this can alter competitive dominance across patches that vary in resource density (Tessier and Woodruff, 2002; Palmer, 2003). Interference ability may also interact with variation in resource quality to influence patterns of coexistence (Amarasekare, 2002). Strong interference competitors can monopolize the highest quality resources, but may ignore lower quality resources, providing the opportunity for a subdominant competitor to coexist locally (Alatalo and Moreno, 1987). Moreover, interference ability often comes at the expense of investment in other traits such as dispersal, abiotic tolerances, reproductive investment or efficiency of resource use (Tilman, 1994; Hughes et al., 2003; Pfennig and Pfennig, 2005; Cadotte et al., 2006). Such trade-offs may allow species that are poor interference competitors to persist because the costs of strong interference ability may not be supported on low quality resources. Thus, differences in both the distribution and quality of resources in man-made vs. natural habitat can alter species composition in novel environments (Chesson, 2000; Amarasekare and Nisbet, 2001; Kneitel and Chase, 2004).

While a switch to a novel resource is frequently documented in species using human-altered habitat (Lancaster and Rees, 1979; Blair, 1996; Robb et al., 2008), its influence on species coexistence has been difficult to assess because it requires a detailed knowledge of performance trade-offs, how they may change in a novel environment and species' relative interference abilities. Cavity nesting birds provide a unique opportunity to examine how such a switch influences species coexistence. Urban development, such as the clearing of a forest, typically eliminates natural nesting holes on which many cavity nesting species depend and as a consequence can cause rapid population declines in cavity nesting species (Newton, 1994). Such declines have led to widespread implementation of nest box programs where people provide man-made nesting cavities in urban parks and agricultural areas (Semel and Sherman, 1995; Rodríguez et al., 2011; Brazill-Boast et al., 2013). These programs have been quite successful and nest boxes have largely replaced natural cavities as the main breeding resource for cavity nesting birds in many agricultural and highly populated areas (Griffith et al., 2008; Davies et al., 2009). However, man-made nest boxes differ from natural cavities in their distribution and quality in ways that can influence species' relative competitive ability (Duckworth, 2014).

In this study, we investigate mechanisms underlying variable patterns of species coexistence in two species of *Sialia* bluebirds. Mountain and western bluebirds (*S. currucoides* and *S. mexicana*) compete for nest cavities and hold interspecific territories in areas where their ranges overlap in the Northwestern United States. Before the widespread placement of nest boxes, new bluebird habitat in this region was largely created by forest fire, which generates suitable habitat by opening up understory vegetation and creating dead snags. Primary cavity nesters, such as woodpeckers, excavate cavities in these snags that are eventually used by secondary cavity nesters. This habitat lasts for up to 30 years until regrowth of the forest eliminates the open meadows bluebirds depend on to forage for insect prey (Power and Lombardo, 1996; Guinan et al., 2000). The successional nature of post-fire habitat means that historically, the persistence of bluebirds depended on their ability to continually recolonize new habitat patches (Schieck and Song, 2006). Mountain bluebirds are more dispersive than western bluebirds and are among the earliest colonizers following forest fires (Hutto, 1995; Schieck and Song, 2006), whereas western bluebirds often show delayed patterns of colonization (Kotliar et al., 2007; Saab et al., 2007; Hutto and Patterson, 2016), yet are better interference competitors (Duckworth and Badyaev, 2007; Duckworth et al., 2015).

The two species show similar patterns of colonization in both natural and nest box populations with mountain bluebirds arriving first and western bluebirds arriving later (Saab et al., 2007; Duckworth, 2014), however, the ability of these species to coexist differs among populations. Studies of nest box populations of bluebirds have documented several instances of the complete and rapid replacement of mountain bluebirds by western bluebirds (Duckworth and Badyaev, 2007), yet studies in post-fire habitat found that mountain bluebirds persist at high numbers for at least 10 years after western bluebirds' arrival (Saab et al., 2007) suggesting that complete species replacement may be less common in natural habitat. Moreover, rapid species replacement does not always occur in nest box populations—in some populations the two species have been co-occurring stably for over a decade (Duckworth, 2014).

Given that nest cavities are the main limiting resource for these species (Brawn and Balda, 1988; Power and Lombardo, 1996), differences in nest cavity quality and distribution between natural post-fire forest and nest box populations may play an important role in explaining variable patterns of species replacement. In the United States, many conservation organizations encourage people to set up "nest box trails" by placing nest boxes along roads in suburban and agricultural areas. Typically, these trails are in highly open parks and ranchland where a lack of natural cavities limits the number of bluebirds breeding in these areas. Therefore, people essentially create new bluebird habitat when they set up "nest box trails." While the distribution of natural nest cavities in post-fire forest is often highly variable, nest boxes in these human-modified suburban and agricultural landscapes are often distributed evenly in a grid or linear transect (Lehmkuhl et al., 2003; Remm and Lõhmus, 2011; Duckworth, 2014). Moreover, in post-fire forests, nest cavity quality is highly variable depending on how it was created (e.g., by rot or various woodpecker species; (Robles and Martin, 2013); whereas, in human-created nest box trails, nest boxes are typically the same shape, size, and thickness. Thus, human-created bluebird habitat differs in both the distribution and extent of variation in quality of nest sites compared to natural populations.

To test the idea that variable patterns of species replacement across populations are due to changes in resource quality (**Table 1**), we first test experimentally whether natural cavities used by bluebirds are more insulated than nest boxes. We consider more insulated nest cavities to be of higher quality because the main source of offspring mortality in nest box populations is inclement weather during early spring cold snaps (Duckworth, 2006b). The difference in insulation properties of natural and man-made cavities might influence coexistence of mountain and western bluebirds because mountain bluebirds breed at both higher elevations and latitudes than western bluebirds (Power and Lombardo, 1996; Guinan et al., 2000), making them more likely to be adapted to cold weather conditions. If so, this might explain their ability to persist in some populations. We first test the assumption that mountain bluebirds are less impacted by cold snaps in general and then compare offspring survival of both species during cold snaps. Finally, we use elevational variation as a proxy for climatic conditions across populations and examine whether it is associated with changes in species abundances over time (see **Table 1** for predictions).

Alternatively, the dynamics of species replacement may be most strongly influenced by differences in the distribution of nest boxes across populations. In an observational study, a strong dependency of species settlement patterns on the density at which nest boxes were placed suggested that mountain bluebirds were more likely to persist in habitat patches where nest box density is low compared to patches where it is high (Duckworth, 2014). Mountain bluebirds are larger and require larger territories than western bluebirds (Pinkowski, 1979; Duckworth, 2014). If nest cavity density is low, the distance between nest sites exceeds the maximum territory size of both species, which limits the importance of interference competition. However, in areas where nest cavity density is high, western bluebirds breed close to mountain bluebirds limiting their territory size to below the species optimal size, essentially "crowding them out" (Duckworth, 2008, 2014). Here, we conducted a long-term experiment manipulating local variation in nest box density to determine whether altered resource distribution explains mountain bluebirds' ability to persist in some areas (**Table 1**).

METHODS

Data were collected from multiple nest box populations of western and mountain bluebirds in western Montana, including four sites surrounding Missoula, MT, one site near St. Regis, MT, two sites in the Blackfoot Valley and one natural post-fire site outside of Missoula (see **Table 2** for site names, locations, and sampling details). Three of the nest box sites near Missoula were suburban recreation areas comprised of open space with housing developments on at least one side. The rest of the nest box sites were agricultural with nest boxes placed along roadways bordering open ranchland. Thus, there were very few natural cavities in the nest box populations. All nest boxes in this study were of similar design and size—they were cut from 2 cm thick wood with interior dimensions of 14.5 × 12 cm and a 4 cm hole diameter. Both species of bluebirds show high breeding site fidelity and although their breeding phenology is very similar, western bluebirds generally lay larger clutches than mountain bluebirds (Power and Lombardo, 1996; Guinan et al., 2000).

Comparing Insulation Properties of Natural Cavities and Nest Boxes

To determine whether the insulation properties of natural and man-made nest cavities differed, in 2015 ($N = 4$) and 2016 ($N = 15$), we used a paired design to directly compare the thermal microclimate of nest boxes and natural cavities relative to outside ambient temperature in the same location. Natural cavities were found primarily at the post-fire site but a few natural cavities found near the Blackfoot Valley sites were also included (**Table 2**).

TABLE 1 | Summary of hypotheses, tests, and predictions.

Hypothesis	Test	Prediction
Variation in resource quality best explains variable patterns of species coexistence	Paired natural and nest box temperature experiment	• Nest boxes are less insulated than natural cavities resulting in more similar temperature fluctuations to ambient compared to natural cavity temperatures
	Species response to cold snaps	• Mountain bluebirds have less variable incubation temperatures during cold snaps than western bluebirds • Mountain bluebirds have higher offspring survival during cold snaps compared to western bluebirds
	Coexistence dynamics across populations	• Mountain bluebirds will be able to persist longer in higher elevation populations
Variation in resource distribution best explains variable patterns of species coexistence	Nest box density experiment	• Mountain bluebirds will be more prevalent in lower density transects • Mountain bluebirds will be more prevalent in exterior transect positions

TABLE 2 | General characteristics of sites and summary of data collected.

Site	Latitude, Longitude	Nearest town	Habitat type	Mean elevation (m)§	# Nest boxes	Nest box density (#/ha)§	Cavity insulation (replicates)	Incubation temp. data (nests)	Offspring survival nestlings (nests)	Nest box density expt. transects (pairs)
BMT	46° 55′ N, 114°05′ W	Missoula	Suburban recreation area		65			71	2,349 (440)	
MTJ	46° 53′ N, 113°57′ W	Missoula	Suburban recreation area	1,238	21	0.079		11	208 (37)	
OVD	47°00′ N, 113°06′ W	Ovando	Agricultural	1,262	72	0.073	5	41	807 (159)	18 (239)
PAU	46°54′ N, 113°25′ W	Potomac	Agricultural	1,151	40	0.068	2	32	610 (112)	10 (151)
STR	47° 14′ N, 115°01′ W	St. Regis	Agricultural		39			47	147 (26)	
UMC	46° 46′ N, 114°00′ W	Missoula	Agricultural	1,231	31	0.145		8	479 (89)	
WWH	46° 53′ N, 113°59′ W	Missoula	Suburban recreation area	1,110	36	0.076		15	523 (96)	
BKMT	46° 50′ N, 114° 07′ W	Missoula	Post-fire		0*		12			
Total					304		19	225	5,123 (959)	28 (390)

*§Listed only for populations with both species present. *Nest boxes added at this site were used for temperature measurements only and were not accessible to birds.*

Briefly, we found active nests in natural cavities, determined species identity of the pair and monitored nest progress. Once the natural cavity was vacated, we placed a nest box directly below the natural cavity with the box entrance in the same orientation as that of the natural cavity. Holes of the nest boxes were covered by a wire screen to prevent their use during the experiment (**Figure S1**). As this experiment was conducted after nesting attempts by bluebirds, the majority of the paired comparisons were established late in the breeding season (late June and July). Old nests were placed in nest boxes to standardize for possible temperature effects of nest material between natural cavities and nest boxes. We placed a thermistor in the nest box and natural cavity, ∼2 inches above the nest, and an additional thermistor outside the nest box to gather ambient temperature data. We connected all three thermistor wires to a single Hobo Stowaway multi-port datalogger (Onset Computer, Bourne, MA), which allowed for concurrent recording of temperatures for both cavity types relative to each other and ambient temperature. Thermistor readings were taken every 30 s for 7–21 days to capture the range of variation that may occur in a typical bluebird nesting attempt (∼5–6 weeks from egg laying to fledge for a successful nest). We excluded days where temperature spikes indicated the presence of an animal using the natural cavity or readings where the temperature fluctuated due to thermistor failure. All Hobo datalogger temperature data were reviewed in HOBOware (Version 3.7.2, Onset Computer, Bourne, MA).

Testing for Species-Specific Effects of Cold Snaps

We monitored female incubation patterns by placing iButton (Dallas Semiconductor, Sunnyvale, CA) thermocouples directly under the eggs in a subset of nests at five sites (**Table 2**). These thermocouples were programmed to record temperatures every 5 min for the duration of the incubation period, allowing for detection of the presence or absence of the incubating female through the associated degree of temperature change of the incubated eggs. For both western and mountain bluebirds, we assessed variability of incubation temperatures (measured as standard deviation) for females that experienced a cold snap vs. females that did not. We defined cold snaps as periods in which the daily maximum temperature was <15°C for at least 2 days over which at least 10 mm of rain fell. These criteria were chosen as prior work has shown them to be meaningful thresholds at which nests begin to fail due to inclement weather (Duckworth, 2006b).

To assess whether inclement weather causes differential reproductive success among species, we compared reproductive success of nests that had experienced a broad range of weather conditions (across years and populations). For this analysis, we included nests initiated between 2003 and 2015. We excluded any nests that were subjected to an experimental treatment that might reasonably have impacted reproductive success (e.g., egg swap, clutch size manipulation, yolk sampling), and also required that nests had been checked with sufficient frequency to enable an approximation of nest stage. When nest stage was not precisely known, we used knowledge of a single reproductive event to approximate the others. For example, nestling age was used to estimate hatch date, hatching was assumed to occur 14 days after the onset of incubation, and incubation onset was assumed to occur on the day of clutch completion. Because females typically lay one egg each day until their clutch is complete, any observation of a nest during laying allowed us to estimate first egg date.

To measure reproductive success, we determined the percent of offspring that fledged from each nest. We used percent fledged because the species differ in average clutch size with western bluebirds typically having slightly larger clutches than mountain bluebirds (Power and Lombardo, 1996; Guinan et al., 2000) and we were interested in understanding the extent to which cold snaps affected reproductive success while controlling for baseline differences in clutch size among the species. Nestlings were assumed to have fledged if they were present in the nest during the last active nest check (typically on day 15) and the nest was empty during the next check (day 21). The nestling period is typically 21 days for these two species. Nests were considered to have experienced a cold snap if any portion of incubation or the nestling period overlapped with any portion of a defined cold snap (see above). We excluded nests that were initiated after June 10th because there were no cold snaps after that time in any of our populations.

Influence of Elevation on Rate of Species Replacement across Populations

To examine whether variable climatic conditions across populations influenced the rate at which western bluebirds replace mountain bluebirds in nest box populations, we used variation in elevation as a proxy for variable climatic conditions. **Table S1** shows weather station data for our lowest and highest elevation site, showing that the highest elevation site has consistently lower temperatures across the breeding season and across all years compared to the lowest elevation site. We compared change in species abundances across five sites (**Table 2**) which varied in elevation and for which we had at least 5 years of nest box occupancy data on both western and mountain bluebirds. BMT and STR sites were excluded from this analysis because mountain bluebirds were largely absent from these sites during the study period.

For each site, we calculated the percent of the western bluebirds out of all bluebirds breeding there and used this to generate standardized regression coefficients from a linear regression analysis with percent western bluebird breeders as the dependent variable and year as independent variable. We then used these standardized regression coefficients as a measure of the rate of species replacement. We were unable to do a similar meaningful comparison with nest box density as only one site (UMC) differed substantially in nest box density (**Table 2**). Moreover, bluebirds breeding in natural cavities were rare at all sites due to the placement of nest boxes in open meadows or ranchland.

Experimental Test of Nest Box Density on Dynamics of Species Replacement

In 2008, to examine the influence of nest cavity density on patterns of species replacement, we created replicated linear transects of high and low density (**Table 2**; **Figure S2**) at two sites (PAU, OVD). High density transects had four nest boxes placed 75–100 m apart and low density transects had four nest boxes placed 150–200 m apart. These distances were chosen based on observed average territory sizes for each species (Duckworth, 2014). Adjacent transects were at least 300 m apart and high and low density transects were interspersed throughout the study sites (**Figure S2**). We recorded the identity of species breeding in each box for every year of the study (2008–2016), with the exception of 2010 and 2011. Because birds on the edges of transects might be subject to less crowding compared to birds breeding in middle boxes, position in each transect was recorded as exterior if a pair was breeding in the first or last box and interior if they were in either of the middle boxes. Within each year, we only used data from the first nesting attempt because interference competition for nest boxes and territories is most relevant early in the breeding season when all individuals are simultaneously acquiring territories.

Statistical Analysis

All analysis was completed in SAS® software V9.4. To determine whether insulation of natural cavities differed from nest boxes, we used paired t-tests to compare the average maximum and minimum daily temperatures recorded in each. We used linear mixed models (PROC MIXED in SAS) to determine the effect of cold snaps on variability of female incubation temperatures with standard deviation of incubation temperatures as the dependent variable, cold snap presence/absence, species identity and their interaction as fixed factors. We included population as a random factor. To determine the influence of cold snaps and species identity on nest success, we used only first nests as these are the most likely ones to overlap cold snaps. We excluded nests that failed due to predation. Because cold snaps may influence nest success differently during distinct stages of the nesting cycle, we categorized cold snaps that occurred during incubation and nestling periods separately. We used mixed models with percent of offspring fledged as the dependent variable and incubation and nestling period cold snap occurrence and species identity as fixed factors. Brood size differed between the species [$F_{(1,\,434)} = 41.95$, $P < 0.001$] and we included it as a covariate. We included population and year as random factors. To determine whether the species were affected differentially by cold snaps, we also included the interactions between nestling and incubation period cold snap occurrence and species identity. Initially, we included nest initiation date as a covariate, but as the two species did not differ in their mean nest initiation dates ($t = 0.56$, $P = 0.58$) and it was not significantly related to the percent of offspring fledged ($P > 0.15$), we excluded it from the model. We used a Spearman rank correlation to examine whether variable elevational variation across populations was related to the rate of species replacement (measured as standardized regression coefficients).

For analysis of the nest box density experiment, we used generalized linear mixed models (PROC GLIMMIX in SAS) fitting a binomial distribution with a logit-link function. The dependent variable was either mountain or western bluebird presence per number of boxes available (using events/trials syntax) with density treatment (high vs. low), position in transect (interior vs. exterior) and year as fixed effects. Site was included as a random effect. We analyzed mountain and western bluebird nest box occupancy separately as we expected that nest box density might affect their settlement patterns in different ways. We fitted year as a linear trend for western bluebirds and as a second order polynomial for mountain bluebirds. We excluded boxes from this analysis if they were not occupied by either species of bluebird over the 9 years of the study. Lack of occupancy of particular boxes likely reflected microhabitat preferences of the species (e.g., a few boxes adjacent to lakes or wetlands were never occupied by bluebirds) and thus were not informative about competitive dynamics and settlement patterns. In a subset of boxes, the bluebird species occupying them switched across years. We used a sign test to assess whether box occupancy was more likely to switch from mountain to western or western to mountain across years.

RESULTS

Testing the Assumption That Natural Cavities and Nest Boxes Differ In Insulation Properties

Natural cavities were more insulated than nest boxes (**Figure 1**) as they had a lower average daily maximum temperature (mean maximum temperature for natural nest cavities = 25.48°C ± 1.13 SE vs. for nest boxes = 26.75°C ± 1.15; $t = -2.13$, $P = 0.049$) and a higher average daily minimum temperature compared to nest boxes (mean minimum temperature for natural nest cavities = 11.96°C ± 0.87 SE vs. for nest boxes = 8.70°C ± 0.91; $t = 6.03$, $P < 0.0001$; **Figure 1**). The temperature difference of nest boxes and natural cavities did not differ in relation to which species used the cavity for either maximum temperature differences (mean ± SE nest box minus natural cavity temperatures for mountain vs. western bluebirds: 5.41°C ± 1.55 vs. 3.08°C ± 1.41, $t = 1.11$, $P = 0.28$) or minimum temperature differences (−7.05°C ± 1.05 vs. −5.46°C ± 0.86, $t = -1.19$, $P = 0.25$).

Influence of Cold Snaps on Incubation Patterns and Nest Success

During the study period, 71% of nests experienced a cold snap during either the incubation or nestling period. Females that experienced a cold snap during incubation had greater fluctuations in incubation temperatures compared to females that did not [$F_{(1, 149)} = 11.19$, $P = 0.001$] but the species did not differ in how variable their incubation temperatures were [$F_{(1, 149)} = 0.87$, $P = 0.35$]. Moreover, the species did not differ in how cold snaps affected their incubation patterns as there was no significant interaction between presence of a cold snap and species identity on variability of incubation temperatures [$F_{(1, 149)} = 0.43$, $P = 0.51$]. Population affiliation did not affect female incubation temperatures ($Z = 0.97$, $P = 0.16$).

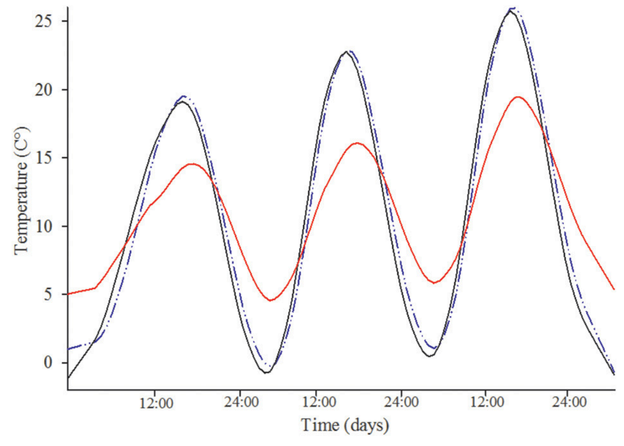

FIGURE 1 | Example showing that natural nest cavities are more insulated than man-made nest boxes. Shown are daily fluctuations for ambient temperature (black solid line), unoccupied man-made nest box interior temperature (blue dashed line) and unoccupied natural nest cavity interior temperature (red solid line). Ambient temperature was recorded directly outside the natural nest cavity. Nest box was placed directly below the natural nest cavity.

Cold snaps had a negative impact on offspring survival, but only when they occurred during the nestling stage [$F_{(1, 428)} = 8.65$, $P = 0.003$]. Nests that experienced a cold snap only during incubation did not differ in survival compared to nests that did not experience a cold snap during incubation [$F_{(1, 428)} = 0.80$, $P = 0.37$]. There was no difference among the species in percent of offspring fledged for pairs that did and did not experience either incubation or nestling period cold snaps [interaction between incubation stage cold snap presence and species identity: $F_{(1, 428)} = 0.41$, $P = 0.52$; interaction between nestling stage cold snap presence and species identity: $F_{(1, 428)} = 2.31$, $P = 0.13$] indicating that the two species were affected by cold snaps similarly. Overall, mountain bluebirds fledged a higher percentage of offspring compared to western bluebirds [$F_{(1, 428)} = 5.10$, $P = 0.024$] despite having lower brood size (mean ± SE brood size, mountain = 4.99 ± 0.09 vs. western = 5.06 ± 0.08). Year explained a significant amount of the variance in percent offspring fledged ($Z = 1.97$, $P = 0.024$), but population did not ($Z = 1.23$, $P = 0.11$).

Influence of Elevation on Dynamics of Species Replacement

The five nest box populations varied in the percent of western bluebirds breeding in the population over time [interaction between population and year: $F_{(9, 30)} = 8.88$, $P = 0.0002$; **Figures 2A–E**]. PAU had the greatest increase of western bluebirds relative to mountain bluebirds [$F_{(1, 5)} = 28.80$, $P = 0.006$, $B_{ST} = 0.937$], followed by OVD [$F_{(1, 7)} = 7.66$, $P = 0.04$, $B_{ST} = 0.778$], UMC [$F_{(1, 7)} = 7.86$, $P = 0.031$, $B_{ST} = 0.753$], and WWH [$F_{(1, 4)} = 0.18$, $P = 0.696$, $B_{ST} = 0.241$]. At the MTJ site, the percent of breeding western bluebird pairs decreased over time [$F_{(1, 4)} = 13.53$, $P = 0.035$, $B_{ST} = -0.905$]. However, elevation, our proxy for variation in climatic conditions, was not correlated to the strength of change over time in the percent of western bluebirds breeding in these populations ($r = -0.14$, $P = 0.82$; **Figure 2F**).

Influence of Experimental Manipulation of Nest Box Density on Settlement Patterns

At sites where we carried out the nest box density experiment, western bluebirds' box occupancy strongly increased over time [$F_{(1, 73)} = 42.04$, $P < 0.0001$], while mountain bluebird numbers were lower in the first and the last year of the study compared with all other years [$F_{(1, 72)} = 20.44$, $P < 0.001$; **Figure 3A**]. Across years, there were 41 changes in box occupancy from one bluebird species to the other. Western bluebirds were more likely to replace mountain bluebirds ($N = 30$) than vice versa ($N = 11$; $P = 0.004$). Western bluebird nest box occupancy was not influenced by either transect density [$F_{(1, 73)} = 0.01$, $P = 0.925$], box location within a transect [$F_{(1, 73)} = 0.15$, $P = 0.697$] or their interaction [$F_{(1, 73)} = 1.57$, $P = 0.214$]. There was a significant interaction between transect density and box location

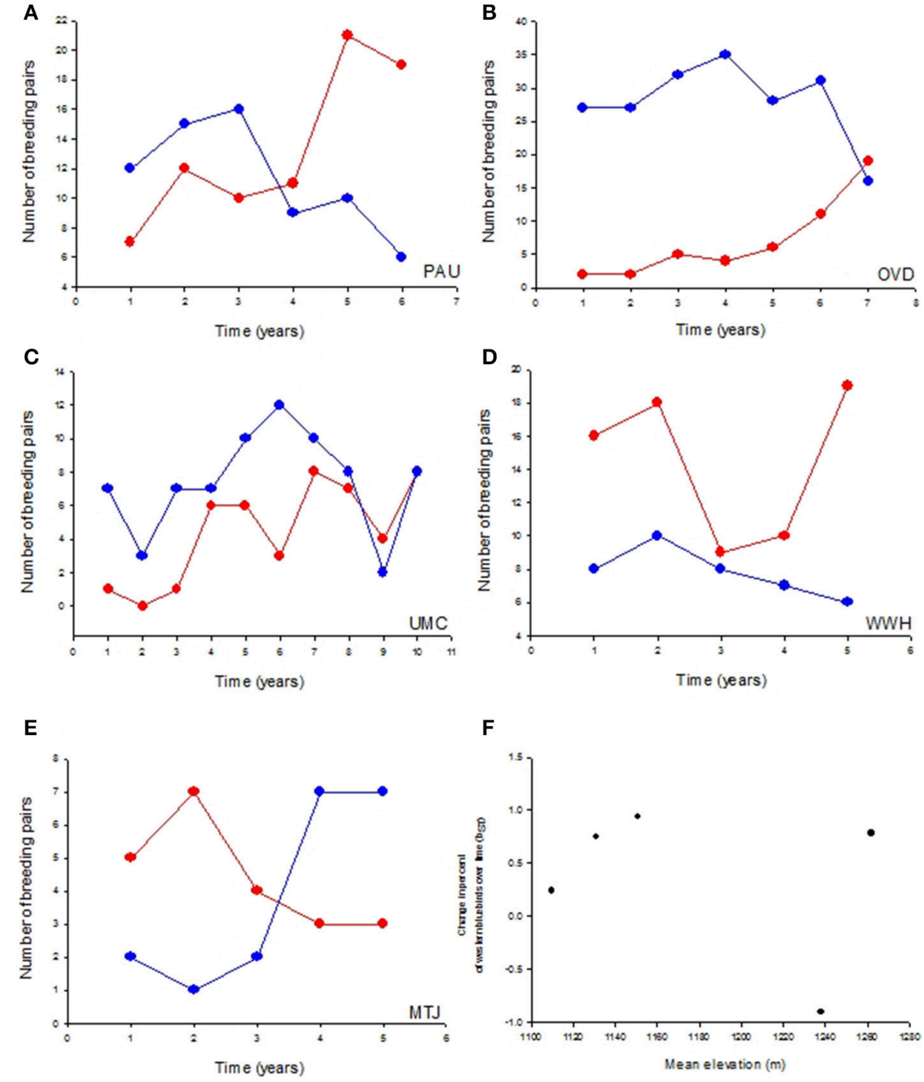

FIGURE 2 | Dynamics of species replacement were not related to variable climatic conditions across populations. **(A)** The number of breeding pairs of western bluebirds (red lines) increased over time compared to mountain bluebirds (blue lines) in four nest box populations **(A–D)** but declined **(E)** in one population. **(F)** The relative change in western bluebird numbers over time was unrelated to population elevation, a proxy for variable climatic conditions.

on mountain bluebird box occupancy [$F_{(1,\,72)} = 5.47, P = 0.022$] such that mountain bluebirds were more common in low density transects but only for nest boxes located in the middle of transects (**Figure 3B**). Moreover, after accounting for the interaction effect, there was an independent influence of nest box location within transects on mountain bluebird settlement patterns with a higher prevalence of mountain bluebirds in middle transect positions [$F_{(1,\,72)} = 6.65, P = 0.012$].

DISCUSSION

A long-standing paradox of human-modified habitats is that they are characterized by simultaneously high population densities and low biodiversity compared to more natural populations (Shochat et al., 2010). While high population densities suggest that resources are plentiful for some species in human-altered landscapes, one potential explanation for low species diversity is a lack of heterogeneity in resource distribution and quality compared to natural ecosystems (Shochat et al., 2010). Resource heterogeneity is a main mechanism for the maintenance of species diversity (Hutchinson, 1959; Tilman and Pacala, 1993; Rosenzweig, 1995) and studies of urban populations often show a link between complexity of urban habitat and avian species diversity (Lancaster and Rees, 1979; Melles et al., 2003; Devictor et al., 2007; Evans et al., 2009). Yet, the mechanisms underlying these patterns are poorly understood.

This study of the mechanisms underlying variable species replacement across populations of two cavity nesting birds breeding in human-altered environments clearly supported a

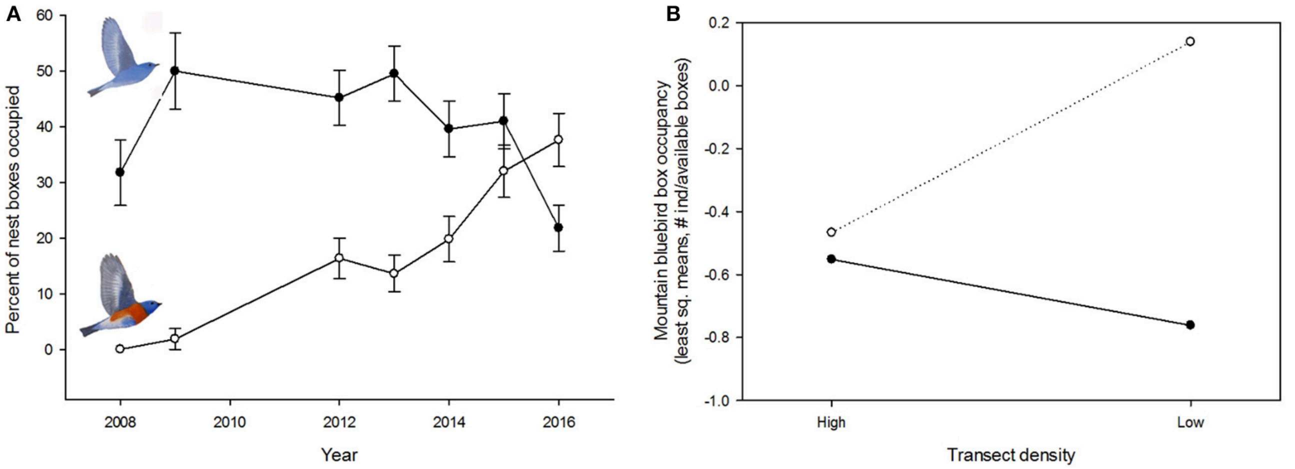

FIGURE 3 | Colonization dynamics of mountain and western bluebirds in a nest box population where nest box density was experimentally manipulated. **(A)** Western bluebirds' (open circles) rapid increase in box occupancy in later years of the experiment was accompanied by a decrease in mountain bluebird (closed circles) box occupancy. **(B)** Mountain bluebirds were more common in low density transects but only in boxes that were in interior positions (dotted line) of transects. In high density transects, box occupancy by mountain bluebirds did not differ between interior and exterior (solid line) positions. Shown are the least square means from a generalized linear mixed model (see Section Methods for details).

role of variable resource distribution impacting coexistence dynamics, but did not support a role of variable resource quality. Specifically, we found that, in a multi-year experiment manipulating nest box density, mountain bluebirds were more common in the presence of western bluebirds when resource density was low (**Figure 3B**). However, the effects of nest box distribution on mountain bluebird settlement patterns cannot be fully explained by competition with western bluebirds. We had predicted that mountain bluebirds would persist longer in exterior positions of transects because they would be less likely to be crowded out by western bluebirds in these positions. However, we found the opposite pattern, suggesting that colonization dynamics are influenced by both competition among species and nest site selection preferences within each species. Second, we did not find evidence that differences in nest cavity insulation between natural and nest box populations explained variable patterns of species coexistence. In contrast to our prediction (**Table 1**), mountain bluebirds breeding in nest boxes were just as likely to show highly variable incubation temperatures and high offspring mortality during cold snaps as western bluebirds. Finally, the rate of change in species' abundances was not related to variation in elevation of populations.

Given that mountain bluebirds occur across a much wider elevational and latitudinal range compared to western bluebirds, our finding that they show similar offspring mortality patterns during cold snaps is surprising (Power and Lombardo, 1996; Guinan et al., 2000). One potential explanation for this finding is that the insulation properties of natural nest cavities are so superior to nest boxes that, even at high elevations, mountain bluebirds breeding in natural cavities rarely experience strong selection for high cold tolerance of eggs and young nestlings. Multiple studies (Coombs et al., 2010; Amat-Valera et al., 2014), including this study (**Figure 1**), have found that natural cavities are well-insulated while internal temperatures of nest boxes do not differ from ambient temperatures. Thus, it will be interesting in future studies to investigate whether selection on offspring survival due to cold snaps differs between nest box and natural post-fire populations. In fact, in contrast to nest box populations, several studies have found nest predation rates in natural cavity-nesting populations to be the dominant source of nest mortality (see Johnson and Kermott, 1994 for review), suggesting that selection pressures in natural vs. nest box populations differ. Alternatively, cold snaps may primarily influence offspring survival by making it difficult for parents to find enough insects to feed offspring, making insulation properties of nest cavities less important than availability of food resources. If this is true, it may explain the differential influence of cold snaps during the incubation and nestling periods. Cold snaps may have a stronger impact during the nestling period because of the added difficulty of keeping offspring fed in addition to keeping them warm. Moreover, we detected a difference in offspring survival when a nest experienced a cold snap, but were not able in this study to test for any differential effects of cold snap length. Therefore, it will be important in future studies to determine whether there are also more nuanced influences of cold snaps on offspring survival that vary with cold snap severity.

Interestingly, even though cold snaps did not differentially affect reproductive success of the species, mountain bluebirds overall fledged a higher percentage of offspring than western bluebirds. This differential reproductive success may reflect a trade-off between interference ability and reproductive investment (Case and Gilpin, 1974; Vance, 1984; Holway, 1999). Such a trade-off has been documented within western bluebirds where less aggressive males invest more in offspring care compared to more aggressive males (Duckworth, 2006b). It will be interesting to see whether this trade-off also holds at the interspecific level for these species.

The joint influence of nest box density and location within a transect suggests that interference competition and microhabitat preferences may interact to determine mountain bluebird settlement patterns. If mountain bluebirds prefer territories with multiple nest cavities, as has been shown in both western and eastern bluebirds (*S. sialis*; Plissner and Gowaty, 1995; Duckworth, 2006a), this may explain their propensity to select interior transect positions. Interior positions have the potential to provide access to three nest cavities, assuming a bird's territory is large enough to encompass neighboring nest boxes. Alternatively, mountain bluebirds' overrepresentation in interior transect positions may reflect the dominant western bluebirds' preference for exterior transect positions. The earliest arriving western bluebirds benefit from acquiring large territories in recently colonized populations (Duckworth, 2008). By choosing nest boxes at the edges of transects, western bluebirds are able to expand their territory boundary well beyond the average territory size before encountering a neighbor. However, if this latter explanation were correct, we would expect western bluebirds to be overrepresented in these positions, but we did not find any effect of either transect density or nest box position on western bluebird settlement patterns. Thus, given these patterns, we suggest that mountain bluebirds' settlement patterns reflect a combination of their own microhabitat preferences and western bluebirds' ability to crowd them out of areas where resource density is high.

In three out of the five nest box populations, western bluebird population density increased linearly over time and was accompanied by a concomitant decrease in mountain bluebird numbers (**Figure 2**). The nest box density experiment provided strong evidence for a causal link between increases in western bluebird numbers and the decline of mountain bluebirds. In cases where species occupancy of a nest box changed across consecutive years, western bluebirds were three times more likely to replace a mountain bluebird than vice versa. However, despite evidence for the importance of variable resource density, it cannot be the sole explanation for the patterns that we observe across populations. In two of our nest box populations, mountain bluebirds either remained steady or increased over time despite similar densities of nest boxes across sites (**Table 2**), patterns that are similar to what has been observed in natural post-fire habitat (Saab et al., 2007).

What other factors might influence species coexistence in this system to produce such variable patterns of species replacement in both nest box and post-fire populations? In post-fire habitat, there is immense variation in nest cavities along multiple axes of quality, including cavity height, depth, and hole size (Robles and Martin, 2013). Among secondary cavity nesters, dominant species often occupy the highest quality cavities pushing subdominant species into low quality cavities (Martin et al., 2004). Thus, it is possible that, in post-fire habitat, western bluebirds occupy the highest quality nest cavities and mountain bluebirds' ability to persist is enabled by their occupancy of lower quality nest cavities that western bluebirds ignore. Even though nest boxes are poorer in quality in terms of their insulation properties, they may be perceived by bluebirds as high quality given their small hole size, an attribute that is important protection against nest predators (Newton, 1994).

These findings have important implications for our understanding of patterns of species coexistence in both natural and human-influenced environments. It has been suggested that lower species diversity in human-modified environments is due to a combination of low predation pressure and a stable and abundant resource base (Anderies et al., 2007). Our results suggest that resource distribution and heterogeneity may be additional important factors influencing patterns of diversity in wildland vs. human-modified habitats. Moreover, we suggest that, given our ability to manipulate a key resource, comparisons of cavity-nesting birds in natural vs. man-made habitat have the potential to provide particularly novel insight into the mechanisms by which resource heterogeneity can influence patterns of species diversity.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations and guidelines of the University of Arizona IACUC committee. The protocol was approved by the University of Arizona IACUC committee.

AUTHOR CONTRIBUTIONS

RD conceived of and designed the study, wrote the manuscript, analyzed, and collected the data. AP designed the nest box microclimate experiment, wrote the manuscript, analyzed, and collected the data. KH wrote the manuscript and analyzed data. NH analyzed and extracted data on incubation patterns and offspring survival.

ACKNOWLEDGMENTS

We thank Erin Morrison, Katie Chenard, Georgy Semenov, and Alex Badyaev for discussion and comments which improved this manuscript. We are also grateful to members of Mountain Bluebird Trails for allowing us to work on their nest box trails and Jay Kolbe, Paws Up staff and landowners in the Blackfoot Valley for permission to set up experimental transects on both the Blackfoot-Clearwater Game Range and private property. We thank the National Forest Service for permission to conduct part of this work on Forest Service property. This work was supported by US National Science Foundation grants (DEB-0918095 and DEB-1350107) to RD and grant from the American Museum of Natural History and Rosemary Grant, and the US National Science Foundation Graduate Research Fellowship Program (NSF DGE-1143953) to AP.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00072/full#supplementary-material

Figure S1 | An example of the paired nest box/natural nest cavity temperature measurement experiment. This particular set-up was located at the BKMT post-fire natural habitat site. The nest box hole is covered to prevent its use by cavity nesting species.

Figure S2 | Aerial photo showing example of two experimental nest box trails at the OVD site with high (red lines) and low (blue lines) density transects. White circles in the two bottommost transects indicate locations of nest boxes which are 75–100 m apart in the high density treatment and 150–200 m apart in the low density treatment. Adjacent transects were separated by at least 300 m. High and low transects were interspersed evenly in a linear fashion.

Table S1 | Temperature and rainfall data from weather stations in Missoula and Blackfoot Valley during the study period. The Missoula weather station is located closest to WWH, the lowest elevation site and the Blackfoot Valley station is located closest to OVD, the highest elevation site. In all time periods, at the lower elevation site, the average daily maximum temperature was higher and the average daily minimum temperature was lower than at the higher elevation site.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 24 February 2017
doi: 10.3389/fevo.2017.00006

Multi-Element Analysis of Blood Samples in a Passerine Species: Excesses and Deficiencies of Trace Elements in an Urbanization Study

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Urbanization is a growing phenomenon characterized by a complete restructuring of natural areas. In urban bird populations, a reduced offspring survival and body condition and an overall lower breeding success are often observed compared to populations inhabiting more natural habitats. Higher pollution levels and poorer quality of natural resources in cities are two environmental factors frequently mentioned in the literature to explain the differences between urban and non-urban populations. Pollution and poor quality of food may lead to an excess of non-essential elements such as Pb or Cd or to deficiencies of essential elements such as Cu or Zn, which may explain some of the impacts, notably on immunity, observed in urbanization studies. The present study compared the breeding parameters, brood body mass and condition, and haptoglobin levels, a marker of inflammatory immunity, in two urban and two forest populations of Great tits in Eastern France, together with a multi-element analysis (25 non-essential and essential trace elements) of blood samples from 13-day-old nestlings from the four populations. The concentration of NO_2, a gaseous pollutant typical of urban pollution, was also measured. The NO_2 concentrations were significantly higher in the urban areas, but no association with biological variables was detected. Non-essential metals were undetectable in the plasma of the birds from both habitats, except Pb, whose concentrations, however, did not differ between the urban and forest birds. A positive relationship was found between the plasmatic richness in essential elements (as assessed from the coordinates of the first axis of a PCA including 12 elements) and the average brood body mass and condition. We suggest that lower quality resources or/and a higher metabolic demand may be a causal mechanism for the reduced body condition often observed in urban bird nestlings. Finally, our exploratory study could promote more mechanistic experiments (e.g., supplementation) to explain the negative effect of urban conditions on bird populations.

Keywords: urban ecology, pollution, *Parus major*, body condition, NO_2

Edited by:
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Specialty section:
This article was submitted to
Urban Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *22 October 2016*
Accepted: *03 February 2017*
Published: *24 February 2017*

Citation:
Bailly J, Faivre B, Bernard N, Sage M, Crini N, Driget V, Garnier S, Rieffel D and Scheifler R (2017) Multi-Element Analysis of Blood Samples in a Passerine Species: Excesses and Deficiencies of Trace Elements in an Urbanization Study.
Front. Ecol. Evol. 5:6.
doi: 10.3389/fevo.2017.00006

INTRODUCTION

Urbanization is increasing, and 54% of the world population now lives in urban areas (United Nations, 2014). Urban habitats have been colonized by many wild species, including birds (McKinney and Lockwood, 1999), which can reach high densities (Chace and Walsh, 2006). Many studies have suggested that urban conditions exert strong constraints on bird fitness (Chamberlain et al., 2009; Vaugoyeau et al., 2016). A number of breeding parameters are affected, with the most consistent patterns being earlier laying dates, lower clutch size, lower nestling weight or condition, and an overall lower productivity per nesting attempt in urban areas (Chamberlain et al., 2009) even if inter specific variations between those life history traits and the intensity of urbanization have recently been demonstrated (Vaugoyeau et al., 2016). Other individual parameters can be modified in urban bird populations compared to rural ones. For example, urban birds are often—but not always—smaller and in poorer condition (Liker et al., 2008; Evans et al., 2009a) and show higher oxidative stress and antioxidant activity than birds from more natural habitats (Isaksson et al., 2005).

Urban and natural environments differ strongly in many factors (Grimm et al., 2008). Temperature, predator and parasite communities, artificial noise and light, availability and/or quality of resources, and pollution are among important features that contrast between cities and natural habitat (Collier, 2006; Evans et al., 2009b; Slabbekoorn, 2013). Cities are frequently described as resource-poor habitats for birds during the breeding season, and several works have shown that prey required for chicks are less abundant, smaller and poorer in key nutrients for growth and survival, such as carotenoids (Isaksson and Andersson, 2007; Seress et al., 2012). Those descriptive studies provided some data that allowed the design of experimental supplementation aimed at testing whether the quantity (energy intake) or the quality of resources (e.g., carotenoids) could explain the contrast of breeding performance between urban and non-urban populations (Peach et al., 2014; Giraudeau et al., 2015). Several other key resources, such as essential elements, could be involved in the differences of growth, body condition, and/or breeding performance between rural and urban nestlings. For instance, calcium (Ca) is an essential micronutrient during egg formation and skeletal development of nestlings (Patten, 2007; Reynolds and Perrins, 2010). Supplementation experiments in wild populations of Great tits (*Parus major*) reported that Ca-supplemented birds produced larger brood and raised more nestlings of better body condition compared to control groups (Tilgar and Reynolds, 2005; Espín et al., 2016a). Other essential elements such as zinc (Zn) or copper (Cu) are also of great interest in the bird physiology and behavior. They affect enzymatic activity and antioxidant defenses (Powell, 2000; Sahin et al., 2005) and play an important role in embryo development and nestling growth (Park et al., 2004). Their concentration can exceptionally raise toxicity thresholds (Sundaresan et al., 2008), but, more likely for vertebrates that are very tolerant to these elements, they may impact physiological performances if their concentrations do not meet homeostatic requirements (deficiencies) (Keen et al., 1998). Despite the crucial role of essential elements in nestling growth, and in bird physiology more generally, very few studies have focused on the potential deficiencies in these elements in urbanization studies. Therefore, quantifying these elements in birds might allow the discrimination of urban and rural populations and the identification of deficiencies in urban individuals.

Similarly, the impact of pollution has received surprisingly little attention in urbanization studies, although cities are characterized by the circulation of many different pollutants in air, water, and soils. The air pollutants that draw the most concerns for ecosystem and human health include particulate matter (PM), ozone (O_3), carbon monoxide (CO), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), lead (Pb), volatile organic compounds (VOC), and polycyclic aromatic hydrocarbons (PAHs) (Mayer, 1999; Berback et al., 2001; Marr et al., 2004). Very few prior studies were dedicated to the effects of air pollutants on birds in the context of urbanization, while studies in (generally heavily) industrially contaminated sites are numerous. To our knowledge, only one study found that reduced reproductive success of House sparrows (*Passer domesticus*) along an urban gradient was associated with air pollution from traffic as assessed by NO_2 concentrations in the air (Peach et al., 2008). Studies dealing with pollution by non-essential metal elements are more numerous, but most of them only report biomonitoring data. For instance, studies found higher concentrations of Pb in urban birds than in their rural counterparts with individuals exhibiting blood concentrations higher than the benchmark value related to subclinical and physiological effects in birds (Scheifler et al., 2006; Roux and Marra, 2007). Clearly, descriptive studies assessing elements in rural and urban birds may provide data about possible excesses of non-essential and potentially toxic elements such as Pb, arsenic (As), cadmium (Cd), or mercury (Hg) in urban birds compared to rural individuals, and may facilitate the design of more mechanistic experiments about the impacts of urbanization on birds.

Pollutants may interact with the immune system depending on the nature of the contamination (element or molecule involved, chronic vs. acute, high vs. low dose) with consequences on host immune profile. Components of inflammatory response showed both down-regulated or up-regulated activities in vertebrates exposed to contaminants (Tersago et al., 2004; Schiraldi and Monestier, 2009) with expected consequences on host-parasite interactions and host fitness (Sorci et al., 2013). However, how urban pollution may affect host immunity has been neglected, and the assessment of markers such as inflammatory markers can provide information on immune status of urban individuals. Recently, we observed contrasts in the production of haptoglobin, an acute protein associated to the inflammatory process, between urban and forest nestlings (Bailly et al., 2016a). Therefore, it could be of interest to explore how this inflammatory marker is affected by urban pollution.

In this study, we measured the concentrations of 25 non-essential and essential trace elements (TEs) in nestling plasma together with the breeding parameters, nestling body condition, and the baseline plasma levels of an inflammatory marker (haptoglobin) in Great tits from two medium-sized cities of Eastern France (Besançon and Dijon) and two forests from the surroundings of each city (Forêt de Chaux and Forêt d'Auxonne, respectively). The atmospheric concentrations of NO_2, one of the main traffic-related pollutants and precursor forming photochemical smog (Han and Naeher, 2006), were also measured with passive air samplers in the studied areas. We hypothesized (i) contrasts between urban and rural populations in circulating TEs (with lower levels of essential elements and higher levels of non-essential elements in urban birds), breeding parameters (clutch size, hatching success, nestling survival, brood body mass and condition) and immunological marker (baseline level of circulating haptoglobin) with harsher conditions for urban birds, and (ii) correlations suggesting negative effects of non-essential TEs and positive effects of essential TEs. Comparison with rural organisms may bring crucial insights about the mechanisms underlying the urbanization process and open the route for more mechanistic and experimental approaches to test them.

MATERIALS AND METHODS

Study Sites

Two cities (Besançon, 47°25 N, 6°03 E; Dijon, 47°32 N, 5°02 E) and two forests (Forêt de Chaux, 47°09 N, 5°68 E; Forêt d'Auxonne, 47°10 N, 5°26 E) were studied in two regions of Eastern France, Franche-Comté, and Burgundy. Besançon and Dijon are two middle-sized cities (176,764 and 244,577 inhabitants in 2008, respectively, and 432.3 and 219.3 km^2, respectively) whose economies are dominated by tertiary activities (http://www.insee.fr). None of those cities have important industrial sources of metals like metallurgic plants for instance. The Forêt d'Auxonne and the Forêt de Chaux are deciduous forests of 7,800 and 20,493 ha, respectively. The most abundant tree species are oaks (*Quercus petrae* and *Q. robur*), beechwood (*Fagus sylvatica*) and charm (*Carpinus* sp.). Nest boxes were installed in medium- (50 years old) to old- (100 years old) growth stands at least 1 km from the edge of the forests. Each site received approximatively 150 artificial nestboxes (Nest box 1B with protective front panel, 12 cm diameter and 20 cm height, Schwegler, Germany). The nest boxes were hung from 0.5 m long steel tubes to protect the nests from predation. The nest boxes in the cities were dispersed in parks, squares, and along streets, with each patch containing one to 25 nest boxes separated by at least 50 m from each other. The vegetation of most of the area of the parks has been formed artificially. The tree cover is patchy with tree-free areas mainly covered by grass. The tree patches are composed of deciduous and coniferous species of different stands, including both native and exotic species. Each study site covers an average area measuring 5.6 km long and 2.8 km wide. In the forests, the nest boxes were placed along pathways to facilitate access, with the same minimal distance between two nest boxes as in the cities.

Reproductive Parameters and Blood Sampling

The reproduction of the Great tits was monitored from March to June 2013. Only the first breeding attempts were included in the study, second broods were not studied due to logistical constraints. The nests were inspected once a week from March 20th to record the laying date of the first egg. When more than one egg was found in the nest, the laying date of the first egg was back calculated, assuming that one egg was laid per day. Incubation was assumed to begin 1 day before clutch completion, and we did not observe asynchronous hatching (except sometimes for one egg). The nests were not visited again until the estimated hatching date (13 days after the beginning of incubation), and any eggs that had not hatched were then checked every 2 days until hatching. The number of nestlings was noted on day 1 (D1), 7 (D7), and 13 (D13) post-hatching. The body mass and tarsus length of the 13-day-old nestlings were measured using a Pesola spring balance (±0.05 g), and an electronic caliper (±0.1 mm). We defined clutch size as the number of eggs laid and hatching success was the proportion of eggs that hatched in the clutches where at least one egg hatched. We defined nestling survival as the proportion of hatched nestlings that were still alive on D13 in nests where at least one nestling survived until D13. When nestlings were 7 days old, they were fitted with individually numbered metal leg rings (Museum National d'Histoire Naturelle, CRBPO, Paris, France). Blood was sampled (50–100 µl) from one brachial vein of 13-day-old nestlings using sterile needles and heparinized capillary tubes. The blood samples were stored in a cool box until their return to the laboratory within 4 h of collection. Then, the blood was centrifuged (10 min, 4,000 rpm, 4°C), and the plasma was separated from the pellet and stored at −80°C until analysis.

Haptoglobin Assay

Haptoglobin (Hp) belongs to the category of acute phase proteins and is used as a marker to measure the intensity of the inflammatory response in vertebrates, including birds (Matson et al., 2006, 2012). The production of this protein by the liver is stimulated by activated inflammatory cytokines (Dobryszycka, 1997), so as the amount of plasma Hp increases, the inflammatory reaction becomes stronger. Here, we quantified the baseline level of this protein to assess the inflammatory status of the nestlings. Hp was measured in the plasma of 13-day-old nestlings using a commercial assay kit (TP-801, Tridelta Development LTD, Ireland). First, 7.5 µl of each sample were randomly aliquoted on a flat-bottomed, 96-well plate. Samples were distributed randomly among the plates. Standard curves were included (in duplicate) on each plate ($n = 16$) containing the plasma samples, and the curves consisted of dilutions of an initial standard of 2.5 mg/ml diluted five times to 0 mg/ml. One hundred microliters of a stock solution of hemoglobin (catalyser) and 140 µl of a stock solution of chromogen (coloration) were added to all wells. The plates were agitated to ensure the mixing of the samples with hemoglobin and chromogen and left to incubate for 5 min at room temperature. The absorbance of each well was read at 630 nm, and the intra-assay and inter-plate variations were, respectively, 2.4 and 2.99%. The samples above the detection threshold were diluted and assayed again.

NO_2 Measurements

Passive NO_2 samplers were put on 6–58 nest boxes in the four study sites during the nestling stage (6 and 7 in the two forests, 38 in Dijon, and 58 in Besançon). Not all nestboxes were equipped for both financial and logistical reasons. The samplers were set up vertically on the middle of the tube from which the nest boxes were hung, allowing air to circulate freely around them. Passive samplers (Palmes et al., 1977) are calibrated tubes, 7 cm long, with an inside diameter of 1 cm, in which gases move only by molecular diffusion (Gradko International, Winchester, Great Britain). A triethanolamine solution, which was deposited on the grid at one end of the tube, fixed the NO_2. The other end of the tube remained open for gas diffusion during the sampling period. Each passive sampler allowed a measurement of the NO_2 pollution level over a period of 14 days, optimal duration of sampling (Bernard et al., 1997). At 21.1°C and at a pressure of 1 atmosphere, the diffusion coefficient for NO_2 is 0.154 cm^2/s, which means that the collection rate for NO_2 passive samplers could be calculated at 72 cm^3/h. Mean hourly concentration of NO_2 (expressed in $\mu g/m^3$; 1 ppb = 1.91 μg/m3 at 21.1°C) in the air sample was calculated on the basis of the amount of pollutant collected, exposure time, and gas collection rate in the passive sampler. Absorbed NO_2 was measured by spectrophotometry using a variant of the Griess-Saltzman method (Atkins, 1990). In an earlier study, NO_2 measurements with passive samplers were validated on chemiluminescence analysers, equipment used by the French network of air quality monitoring and advocated by European legislation (Bernard et al., 1998).

Multi-Element Analysis

The concentrations of 25 TEs [aluminum (Al), antimony (Sb), As, Cd, Ca, chromium (Cr), cobalt (Co), Cu, tin (Sn), iron (Fe), magnesium (Mg), manganese (Mn), Hg, molybdenum (Mo), phosphorus (P), Pb, potassium (K), nickel (Ni), selenium (Se), silicon (Si), sodium (Na), strontium (Sr), titanium (Ti), thallium (Tl), and Zn] were determined in 93 composite (one sample per family) plasma samples. Four samples had volumes lower than 50 μl and could not been analyzed. Eight samples had a volume ranging from 55 to 85 μl. For the other samples, 100 μl were used. The samples were acid digested in 700 μl of nitric acid (HNO_3, 65%, analytical quality, Optima) for half a day in open tubes under hood, and then for 60 h in closed tubes at 60°C in an oven. After digestion, samples were diluted to 30 ml by the addition of ultra-pure water (18.2 $M\Omega/cm^2$). Blanks (acid + ultra-pure water) and Certified Reference Materials (CRMs; lobster hepatopancreas, TORT-2, National Research Council Canada, and drinking water, ERM®-CA011b, European Reference Materials) were prepared and analyzed using the same methods as the samples. Elements were analyzed by Inductively Coupled Plasma—Atomic Emission Spectrometry (ICP-AES, ThermoFisher Scientific iCAP 6,000) or by Inductively Coupled Plasma—Mass Spectrometry (ICP-MS, ThermoFischer Scientific XSeries 2). Over the 25 elements measured, only 12 had a satisfactory proportion of values above quantification limits (Table S1), allowing for statistical analyses. For the values under quantification limits for these 12 elements, the value was replaced by the quantification limit divided by square root of two for statistical analysis (Helsel, 2010). Average recoveries of the CRMs ranged from 73 (for Fe) to 135% (for Pb) for the TORT-2, and from 78 (Fe) to 124% (for Se) for the ERM®-CA011b (Table S1). The concentrations of the elements in the plasma are expressed as μg/dl.

Statistical Analysis

Levels of atmospheric NO_2, plasmatic TEs concentrations, and biological parameters were compared among the four study sites using the non-parametric Kruskal–Wallis test because the distribution of most of the data were skewed. When a significant effect was detected, *post-hoc* Tukey HSD tests were applied to detect differences among sites. Urban vs. forest differences were analyzed using Wilcoxon Mann–Whitney test. All NO_2 data were used to compare NO_2 levels among the four study sites while only the data related to nestboxes hosting successful reproduction were used to study the relationships between NO_2 levels and biological parameters. Moreover, because NO_2 concentrations in the forests were, as expected, very low and exhibited low variations, only data from the two cities were analyzed to test the relationships between the NO_2 concentrations and biological parameters (clutch size, hatching success, nestling survival, brood body mass and condition, baseline level of circulating haptoglobin). Correlations among TEs concentrations and NO_2 levels were studied using Spearman's correlations due to skewed distribution. A Principal Component Analysis (PCA) was conducted to investigate the relationships among TEs concentrations. The first axis of the PCA (PC1) was used as a plasma synthetic index describing richness in the essential elements and was used for further analysis with trace elements (see Section Results). Because we have only one measure of NO_2 and TEs concentrations per brood, the average brood mass, tarsus length, and baseline levels of Hp per brood were used for further analysis. An average brood condition at D13 was estimated as the residuals of the linear regression between the log-transformed average brood mass and the log-transformed average brood tarsus. The correlations between richness in essential elements as assessed by PC1 and brood mass and condition were studied using Spearman's correlations because of the skewed distribution of the data.

Clutch size, hatching success, nestling survival, and brood mass, body condition, and Hp levels were analyzed using habitat and NO_2 levels or PC1 (as an estimate of the plasmatic richness in essential elements) as explanatory variables, and some co-variates when needed (see below). The variation in clutch size was analyzed using generalized linear models (GLMs) with Poisson error distributions (Zuur et al., 2009). For hatching success and nestling survival, GLMs with binomial distributions and logit link functions were used (Warton and Hui, 2011), and the first egg-laying date (standardized as the number of days between March 1st and the first egg-laying date) was added as a covariate (Cresswell and McCleery, 2003; Blondel, 2007). Because overdispersion was detected, the standard errors were corrected using a quasi-GLM model (Zuur et al., 2009). Linear models (LMs) were used for brood mass and body condition at D13 with the first egg-laying date and brood size included as covariates. Because body mass may affect nestling immunity (Alonso-Alvarez and Tella, 2001), it was included as a covariate for the Hp analysis.

Each analysis was performed using the full model with software R v.3.15.1 (R Development Core Team, 2014). Statistical significance was set at $p < 0.05$ for all results.

RESULTS

Reproduction Parameters and Immune Marker

Laying dates differed significantly among all groups except between Besançon and Dijon (KW $\text{Chi}^2 = 61.5$, df $= 3$, $p < 0.001$, Table S2) and urban Great tits laid earlier than forest individuals, in average of 4.3 days ($W = 6699$, $p < 0.001$). The number of eggs laid did not differ neither between the two cities nor between the two forests (KW $\text{Chi}^2 = 90.0$, df $= 3$, $p < 0.001$, Table S2). Urban Great tits, however, laid in average 2.7 eggs less than forest tits ($W = 8153$, $p < 0.001$). Nestlings were significantly heavier in the Forêt d'Auxonne than in the two cities but did not differ from the nestlings from the Forêt de Chaux (KW $\text{Chi}^2 = 43.3$, df $= 3$, $p < 0.001$, Table S2). Urban broods were significantly and in average of 1.8 g lighter than forest broods ($W = 2298$, $p < 0.001$). Nestling body condition was the highest in the Forêt d'Auxonne and did not differ between the two cities (KW $\text{Chi}^2 = 56.9$, df $= 3$, $p < 0.001$, Table S2). The condition was better for forest individuals than for urban ones ($W = 2312$, $p < 0.001$). Hatching success and nestling survival did not differ not among sites nor between the two habitats (Table S2). The baseline levels of circulating haptoglobin was higher in the Forêt de Chaux than in the other forest and in Dijon (KW $\text{Chi}^2 = 12.9$, df $= 3$, $p < 0.005$, Table S2). There was no difference of haptoglobin levels between urban and forest individuals ($W = 817$, $p = 0.3$).

Relationships between NO_2 and Biological Parameters

Nitrogen dioxide concentrations in those both middle-size urban areas were close to 20 $\mu g/m^3$ (mean ± standard deviation: 19.6 ± 4.6 and 23.4 ± 10.5 $\mu g/m^3$ in Dijon and Besançon, respectively, **Figure 1**) and those of forests were 7- to 10-fold lower (4.0 ± 0.9 and 1.8 ± 0.8 $\mu g/m^3$ in the Forêt d'Auxonne and the Forêt de Chaux, respectively). Nitrogen dioxide concentrations did not differ neither between the two cities nor between the two forests (KW $\text{Chi}^2 = 33.6$, df $= 3$, $p < 0.001$) while urban NO_2 concentrations were significantly higher than the ones measured in the forests ($W = 13$, $p < 0.001$). No significant correlation was detected between the NO_2 in urban areas and any of the biological parameters measured.

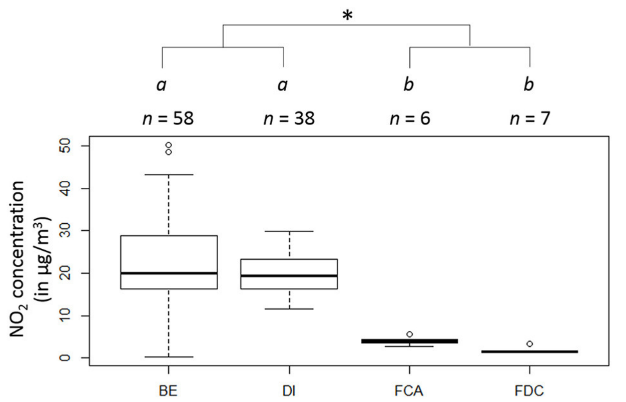

FIGURE 1 | Atmospheric concentrations of NO_2 ($\mu g/m^3$) in the different study sites: Besançon (BE), Dijon (DI), the Forêt d'Auxonne (FCA), and the Forêt de Chaux (FDC). Sites with different letters are significantly different. The asterisk indicates a difference between urban and forest levels.

Relationships between Element Concentrations and Biological Parameters

Over the 25 elements analyzed in this study, 13 were detected in very few or even no birds, including the non-essential As, Cd, and Hg. Over the 12 remaining elements, Spearman's correlation showed significant but not very strong correlations ($r < 0.5$) among metals (Figure S1). The plasma concentrations of Ca ($W = 1297$, $p = 0.002$), Cu ($W = 1416$, $p < 0.001$), Fe ($W = 1301$, $p = 0.018$), P ($W = 1332$, $p < 0.001$), Si ($W = 1355$, $p < 0.001$), and Zn ($W = 1173$, $p = 0.045$) were lower in urban nestlings than in forest individuals, whereas the Mo concentrations were higher ($W = 86$, $p < 0.001$) in urban nestlings than in forest ones (Table S3). K, Mg, Na, Pb, and Se concentrations did not differ between the urban and forest populations of Great tits ($ps >$ Table S3).

PCA analysis found that 74.4% of the variation was driven by five elements (Ca, Na, Mg, P, and Si, Figure S2). The first and second axes of the PCA explained 27 and 18% of the variation, respectively. Based on the results of the PCA, we used PC1 as an index of the richness in essential elements, with negative values indicating high concentrations of essential elements, and positive values indicating low concentrations. The richness of the essential elements of Great tits did not differ neither between the two cities nor between the two forest sites, but was significantly higher in the forests than in the cities ($W = 265.5$, $p < 0.001$, **Figure 2**). Brood body mass and body condition were negatively correlated with PC1 (**Figure 3**, **Table 1**), therefore positively correlated to the richness in essential elements.

DISCUSSION

Our results showed that several components of Great tit reproductive output were negatively affected in the urban habitats. The clutch size, nestling body mass, and nestling body condition were lower in urban sites than in forests. However, we observed no difference in the circulating haptoglobin. The essential elements richness was lower in urban sites and, interestingly, it appeared to be positively correlated with the nestling mass and body condition. Our results are consistent with those reported in previous studies (Hõrak, 1993; Chamberlain et al., 2009; Bailly et al., 2016b) on the components of reproductive output, and suggest mechanisms to explain the negative effect of urbanization on the reproductive performance of Great tits.

Among ecological factors that might explain the observed contrast in clutch size, brood mass and condition, food availability has frequently been cited in the literature (see for instance Chamberlain et al., 2009). The quantity and the quality

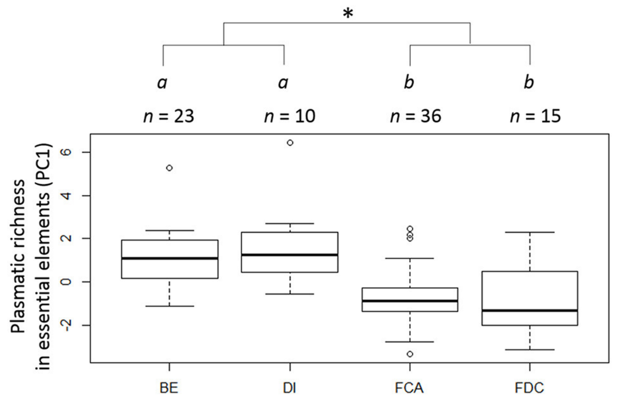

FIGURE 2 | Richness in essential elements (as assessed by the coordinates on the first axis of a PCA: PC1, with negative values indicating high concentrations of essential elements, and positive values indicating low concentrations) in the plasma of Great tit 13-day old nestlings from the four study sites: Besançon (BE), Dijon (DI), the Forêt d'Auxonne (FCA), and the Forêt de Chaux (FDC). Sites with different letters are significantly different. The asterisk indicates a difference between urban and forest individuals.

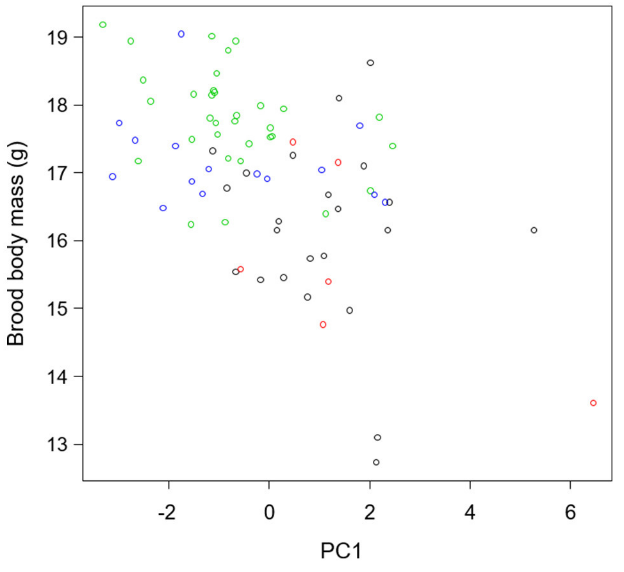

FIGURE 3 | Relationship between richness in essential elements (PC1) and brood body mass of 13-day old nestlings of Great tit. Black circles: Besançon, red circles: Dijon, green circles: Forêt d'Auxonne, blue circles: Forêt de Chaux.

TABLE 1 | Relationship between richness in essential elements (as assessed by the coordinates on the first axis of a PCA: PC1) and average brood mass and body condition of 13-day old nestlings of Great tits according to their habitats (urban vs. forest).

	Brood mass			Brood condition		
	β	SE	*p*	β	SE	*p*
PC1	−0.18	0.07	0.012	−0.13	0.07	0.053
Habitat	−1.54	0.30	< 0.001	−1.65	0.29	< 0.001
First egg date	−0.03	0.02	0.11	−0.03	0.02	0.09
Brood size	−0.10	0.05	0.06	−0.13	0.05	0.01
R^2	0.43			0.45		
p	<0.001			<0.001		

Covariates, R^2 and significance of full models are also presented.

of trophic resources strongly influence female investment in the clutch through its own condition (Marzal et al., 2005; Sofaer et al., 2012) as well as nestling growth and survival (Naef-Daenzer and Keller, 1999). The energetic requirement during growth is very high for tissue formation (for instance muscle, bone marrow) and the maintenance of those already established (Starck and Ricklefs, 1998). Several nutrients and elements are also essential for nestling development, and organisms have to obtain some of them from their food because they cannot synthetize these elements. For instance, Isaksson and Andersson (2007) showed that carotenoids, crucial compounds for several functions, are less abundant in prey consumed by urban than by forest Great tits. Similarly, in the surroundings of a metal-processing factory in Harjavalta, SW Finland, both the intensity of the yellow color in the plumage of *P. major* nestlings and caterpillar abundance increased with increasing distant from the pollution source, suggesting a deficiency of carotenoids in birds breeding close to the factory (Eeva et al., 1998). Here, the richness of essential elements was positively correlated with average brood mass and body condition, suggesting that deficiency in essential elements may be one of the mechanisms underlying the lower body condition often observed in urban nestlings compared to forest ones. According to the PCA, the variability of the dataset is mainly driven by the concentrations of 5 elements, namely Ca, Na, Mg, P, and Si. Ca is particularly important for the structural strength of the avian skeleton, plays vital roles in many biochemical reactions and allows birds to lay large megalecithal eggs (Dacke, 2000). Because of its fundamental role in bird physiology, Ca has received relatively higher attention in environmental studies than the other elements studied here. Indeed, among the abundant literature dealing with the effects of acidification or naturally base-poor habitats on bird reproduction (see for instance Graveland et al., 1994; Graveland, 1996). Mänd et al. (2000) found a positive effect of Ca supplementation on the tarsus length and body mass in fledgling Great tits growing in base-poor pine forests. This positive effect was observed only in the most unfavorable year (in terms of weather conditions) over the 3 years studied (Mänd et al., 2000). A similar positive effect of Ca supplementation was observed in *P. major* nestlings in a Ca-poor area associated with metal pollution in Harjavalta, SW Finland (Espín et al., 2016a). Eeva and Lehikoinen (2004) found reduced eggshell thickness, egg size, and hatchability, and delayed ossification of nestling leg and wing bones in Pied flycatchers (*Ficedula hypoleuca*) living close to a copper smelter in Finland where Ca availability is poor (Eeva and Lehikoinen, 2004). However, this effect was not found in the control Great tits studied in the same area. In our study, Ca was one the main essential elements explaining the variability of the TEs dataset and circulated at lower levels in urban nestling plasma, suggesting that urban birds suffer from reduced Ca-rich resources compared to the forest populations. Phosphorus deficiencies can lead to poor bone mineralization or even abnormal bone development such as chondrodystrophy (deformity of the leg bones) (Driver et al., 2006), and Na is an essential nutrient known to influence several aspects of normal animal growth. Sodium deficiency has been shown to reduce growth and food consumption, impair feed conversion water intake, acid-base balance, and basal metabolism (Vieira et al., 2003). Magnesium and Si are also considered as bone minerals even if the role of Si in bone health is still unclear (Jugdaohsingh, 2007). We are not aware of any study reporting Mg, Na, P, or Si deficiency effects on wild birds, but our results showed their lower levels in urban nestling plasma (two of them, P and Si being significantly less concentrated in urban nestling plasma).

Taken together, these results concerning so-called bone minerals, and the positive relationship between essential elements richness and body mass and condition, could explain the reduced growth found in urban nestlings. This may be linked to a lower quality, in terms of essential elements, of the resources that birds can forage in the cities compared to what is available in forest areas. An alternative hypothesis is that whatever the availability of these elements in urban habitat, the metabolic requirement of urban nestlings might be higher because of higher element consumption by organs. Indeed, stressful environmental conditions, as that prevailing in towns, are known to increase the organism demand for energetic and non-energetic resources.

Our study also shows that non-essential elements that are often considered as potentially toxic in urban or industrial areas are almost undetectable in the plasma of Great tits from the two middle size cities studied here. This may be linked to the fact that several metals have been shown to be mainly associated to erythrocytes rather than to plasma, as analyzed in the present work (Coeurdassier et al., 2012), but the lack of differences of the concentrations in the plasma of urban and forest birds suggests a low exposure to those elements in the two habitats. Pb, one of the non-essential elements for which there has been a great concern for wildlife conservation in urban and industrial areas, was detected in 78% of the broods studied but its concentrations did not differ between urban and forest birds. This suggests that this element, which has been banned from gasoline in most industrialized countries, tends to be less at risk for wildlife than it has been in the past for this category of middle-size and tertiary towns (see for instance Scheifler et al., 2006).

Atmospheric NO_2 is a pollutant that is particularly relevant in the context of urbanization as it is emitted from the burning of fossil fuels for traffic and heating. All the average NO_2 concentrations in the urban and forest study sites are below the thresholds fixed by French legislation (Code de l'environnement—Titre R22, 2013). They are also below the European hourly and annual average for urban background concentrations (European Environmental Agency, 2016), and below the values advised for health protection by the World Health Organization (2006). NO_2 concentrations in Besançon and Dijon are in accordance with atmospheric levels of NO_2 measured and modeled in middle-sized cities by Tenailleau et al. (2015). NO_2 levels in both of these middle-sized urban areas are low as compared to those observed in large cities (Lebret et al., 2000). Therefore, these values indicate that Besançon and Dijon are moderately polluted area.

Urban populations of Great tits were exposed to higher levels of atmospheric NO_2 than forest ones, but these concentrations were not linked with any component of the reproductive success measured in this study. This is not in agreement with the work of Peach et al. (2008) on the House sparrow, showing (for quite similar NO_2 concentrations ranging from 15 to 35 $\mu g/m^3$ in their study compared to 10–50 $\mu g/m^3$ in the present work) that the brood body condition of 2- to 6-day-old nestlings and the brood tarsus length and body mass of 10- to 12-day-old nestlings were strongly and negatively correlated to local summer NO_2 concentrations. The discrepancy between our study and this previous work may be due to a number of factors linked with environmental conditions, such as weather, and food availability, or with species-specific sensitivity to NO_2.

CONCLUSION

Overall, our study brings new data about plasmatic concentrations of various essential elements in birds from urban and forest habitats and their potential effects on nestling body mass and condition. In our opinion, this is a supplementary clue that suggests food availability and/or quality may indeed be a causal mechanism for reduced growth of nestling birds in urban areas, an issue that needs further investigation. More specifically, the simultaneous assessment of circulating essential and non-essential elements allows for (i) design supplementation experiments to test how the availability of specific essential elements or blends of elements explains the reduced performances of birds in urban habitat or/and (ii) identify non-essential elements that may alter biological functions in urban birds. Our case study suggests that supplementation experiments with Ca, Cu, Fe, P, or Si should be relevant as a next step to analyze the negative consequences of urban habitat on the reproduction of Great tits. Several Ca-supplementation experiments have already been performed, in particular in the surroundings of the Cu smelter of Harjavalta, SW Finland (Espín et al., 2016a,b), but to our knowledge not in a context of urbanization without heavy industrial pollution sources. However, even though the present study did not show an effect of low concentrations of NO_2 on any of the parameters studied and the concentrations of non-essential elements did not differ in the plasma of birds from the two habitats, the ecotoxicological dimension of the impacts of urbanization on birds deserves further attention because many common pollutants, and their interactions, have not yet been studied.

ETHICS STATEMENT

This study conforms to the legal requirements of France. The experiment has received the agreement of the Animal Care and Ethical Committee of the Université de Bourgogne, Dijon (protocol # 8112) and of the *Préfectures* from Côte d'Or, Doubs, and Jura (Arrêté # 448).

AUTHOR CONTRIBUTIONS

BF, RS, and JB conceived the design of the study. JB, BF, MS, VD, SG, DR, and RS conducted the field work. JB, NB, and NC conducted the lab analyses. JB, BF, and RS analyzed the data and wrote the manuscript.

ACKNOWLEDGMENTS

The Regional Council of Burgundy and the Center for Research and Higher Education provided financial support for this work. This study was also funded by the Universities of Burgundy and Franche-Comté ("2012 BQR-PRES project n°38") and the Ville de Dijon, de Besançon, d'Auxonne and the *Office National des Forêts* provided permissions to install nest boxes. The study site in Franche-Comté belongs to the CNRS long-term ecological research site "Zone Atelier Arc Jurassien" (http://zaaj.univ-fcomte.fr/?lang=en). We thank M. Coeurdassier who helped with the fieldwork.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00006/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 03 February 2017
doi: 10.3389/fevo.2017.00004

Anthropogenic Nest Materials May Increase Breeding Costs for Urban Birds

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Even in socially monogamous species, sexual conflict is one reason that often promotes differences in the roles of sexes during reproduction, which may lead to one sex making a disproportionate contribution, and thus incurring disproportionate costs, at particular moments of the breeding process. In Mexico City, a number of songbird species line their nests with fibers from discarded cigarette butts, which reduce ectoparasite load but are genotoxic. As male *Passer domesticus* make substantial contributions to nest building whereas male *Carpodacus mexicanus* do not contribute to nest building, we hypothesized that the toxic effects of exposure to cigarette butts should be greater for females *C. mexicanus* than for conspecific males, but that there should be little or no difference in *P. domesticus*. As expected there was more exogenous genotoxic damage in the red-blood cells of incubating female *C. mexicanus* the more cigarette butts were found in their nest, and much more than in their conspecific males. Damage in males was not associated to cigarette butts; it was initially lower than in females, but it increased near fledging, together with their breeding effort. In both male and female *P. domesticus*, however, genotoxic damage was equally apparent and greater the more cigarette butts were in the nest. The novel use of a toxic, anthropogenic parasite repellent by urban birds may be thus asymmetrically increasing the breeding costs paid by the member of the pair most involved in nest building and incubation.

Keywords: cigarette butts, genotoxicity, *Carpodacus mexicanus*, *Passer domesticus*, parental care, sex roles

Edited by:
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Specialty section:
This article was submitted to
Urban Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 28 September 2016
Accepted: 19 January 2017
Published: 03 February 2017

Citation:
Suárez-Rodríguez M,
Montero-Montoya RD and Macías
Garcia C (2017) Anthropogenic Nest
Materials May Increase Breeding
Costs for Urban Birds.
Front. Ecol. Evol. 5:4.
doi: 10.3389/fevo.2017.00004

INTRODUCTION

Human activities impact the environment in a variety of ways, often with negative consequences for the local biota (Miller and Hobbs, 2002). This is clearly the case with urbanization, where organisms are exposed to a new array of stressors that impose substantial constraints on their biology (Ditchkoff et al., 2006). Behavioral modifications are amongst the first responses of animals to life in the cities (Baldwin, 1896; Price et al., 2003). This is because behavior can be speedily adjusted through learning, thus providing a rapid means to adapt to new conditions (Ditchkoff et al., 2006; Sih et al., 2011; Sih, 2013). For instance, the plasticity of Passeriformes' song production (Slabbekoorn, 2013) allows them to make instantaneous adjustments of frequency in response to sudden increases of anthropogenic noise, as has been experimentally shown by Bermúdez-Cuamatzin et al. (2010) and Gross et al. (2010). Other examples include short-term increases in competitive behavior of invasive crabs (Tanner et al., 2011) and changes of time budgets in birds near airports (Gil et al., 2014).

Animals may modify behaviorally to an array of human disturbances. Thus the negative effects of habitat fragmentation may be contended with by adjusting dispersal and foraging patterns (Rathcke and Jules, 1993; Hovland et al., 1999; Tscharntke and Brandl, 2004; Schtickzelle et al., 2006). For instance, the bog fritillary butterfly (*Proclossiana eunomia*) show a dispersal depression in fragmented landscapes, as dispersal between landscape fragments is linked to high mortality (Schtickzelle et al., 2006), whereas root voles (*Microtus oeconomus*) forage preferentially on the edges of fragmented landscape patches (Hovland et al., 1999). Human alteration of the environment often involves the translocation of organisms. For instance, urban biota is increasingly comprised of non-native species, which may negatively interact with native organisms. Consequently, prey species often have to adapt their behavior to the presence of invading predators, whereas competing local species may need to escalate competition to reduce the negative effects of such introductions (Sih et al., 2010). Equally insidious is the effect of invasive species on the local species mating systems (e.g., Valero et al., 2008).

Humans also modify the environment by polluting it. In particular, anthropogenic chemicals may interfere with the expression of sexual traits such as coloration, courtship or singing performance (Gorissen et al., 2005; Markman et al., 2008) partly because they often act as hormonal disruptors, which may provoke changes in the operational sex-ratio, thus interfering with mating behavior (Fry, 1995). A complex form of pollution is that caused by solid waste. This is composed of discarded traces of products used by humans. A sometimes large fraction of the consumables that we use is discarded; it is mostly unfriendly with the environment and may take a long time to degrade. Several types of solid waste are harmful to humans, animals and the ecosystems (Hamer, 2003). Human-generated waste is ubiquitous in cities and elsewhere, and, as it often resembles natural materials, it is frequently used by mammals (Cavia et al., 2009) and birds (Wang et al., 2009; Votier et al., 2011; Townsend and Barker, 2014) in the construction of their nests. Since the distribution and physical properties of waste products is not the same as those of natural materials, the use of waste products requires behavioral adjustments, such as the ripping-of and unweaving of cigarette butts to line nests (Suárez-Rodríguez et al., 2013). Given the paramount influence of the nest materials on the microclimate and the biota to which developing offspring are exposed (Collias, 1964; Hilton et al., 2004), incorporating anthropogenic materials on the nests is likely to have a major influence on the natural history of those using them (Hartwig et al., 2007; Wang et al., 2009; Antczak et al., 2010; Suárez-Rodríguez et al., 2013).

Eggs and chicks are directly in contact with the nest lining; a thin soft structure of feathers, fur and the like. Often, it also includes green plant materials that serve to repel ectoparasites (e.g., in the common starling *Sturnus vulgaris*; Clark and Mason, 1987, 1988) as they contain secondary metabolites evolved to prevent herbivory (Wink, 1988; Wittstock and Gershenzon, 2002). In other species such as blue tits (*Cyanistes caerules*), green materials seem to act against bacteria in the skin of chicks (Mennerat et al., 2009a) which is linked with faster mass gain (Mennerat et al., 2009b). Note, however, that the same materials may be added for reasons unrelated to egg/chick wellbeing. For instance, in spotless starlings (*S. unicolor*) the addition of green plant material to the nest serves mainly as a sexually selected signal that stimulates the production of testosterone in females, and may also signal male status (Veiga et al., 2006; Polo et al., 2010). Some bird species use this strategy to repel arthropods from their nests (Wimberger, 1984; Gwinner and Berger, 2006; Ontiveros et al., 2007) thus it has been considered a way of actively protecting the nest from ectoparasites (Clark and Mason, 1988). Bird ectoparasites are a well-known to be harmful, especially those occupying their nests (Clayton et al., 2010; Hund et al., 2015; also see López-Rull and Macías Garcia, 2015).

In addition to natural materials, two common city birds, *P. domesticus* and *C. mexicanus* weave anthropogenic materials (Van Riper III, 1976), including cotton fibers from discarded cigarette filters into the nest lining (Suárez-Rodríguez et al., 2013). Cigarette butts have a repellent effect similar to that of green materials (Suárez-Rodríguez et al., 2013), yet they also contain many toxic substances in addition to Nicotine, such as ethylphenol, heavy metals (e.g., titanium dioxide), propylene glycol, diverse insecticides, and even cyanide (Witschi et al., 2002; Jensenl et al., 2004; Moriwaki et al., 2009; Slaughter et al., 2011; Register, 2013). Thus chick erythrocytes show signs of genotoxic damage proportional to the amount of cigarette butts used to line their nests (Suárez-Rodríguez and Macías Garcia, 2014), probably as the toxic substances enter their bodies through their skins, which are in contact with the nest materials. Although we cannot exclude the possibility that at least those that are volatile are also inhaled. Genotoxic damage occurs when an exogenous or endogenous agent (including reactive oxygen species) breaks DNA chains during mitosis, a process that can lead to mutagenicity and eventually to carcinogenic processes (DeMarini, 2004; Valko et al., 2004; Fenech, 2007). DNA damage results in nuclear abnormalities, with cells containing a nucleus plus one or more micronuclei, or bi-nucleated cells with bridged nuclei. Damage to the mitotic apparatus (microtubules and microfilaments) results in true bi-nucleated cells (with a duplicated genome; Fenech, 2007). While some damage may go undetected upon visual inspection, standardized counts of cells with nuclear abnormalities can be used as direct measures of genotoxic damage (Fenech, 1997).

Contact with discarded cigarette butts is toxic to chicks (Suárez-Rodríguez and Macías Garcia, 2014), and perhaps also to adults. Thus, by lining their nests with such materials, urban *P. domesticus* and *C. mexicanus* may be exposing themselves to a novel breeding cost, which we expect to differentially affect males and females given their unequal share of the breeding activities. Here we conducted standardized counts of damaged erythrocytes to quantify the cost incurred by male and female *P. domesticus* and *C. mexicanus* at different moments in their breeding season (stages). We hypothesized that the amount of direct contact with cigarette butts through manipulation and incubation should be positively correlated with genotoxic damage, and should thus differ between sexes and species in accordance to their breeding roles. We tested the above on the house finch and the house sparrow. These species coexist in North American cities, where they occupy similar ecological niches (Bent and Austin, 1968; Woods, 1968; Lowther and Cink, 2006), to the point that competition often occurs between them (Kalinoski, 1975; Bennett, 1990; McClure et al., 2011).

METHODS

Study Species

Carpodacus mexicanus (Müller, 1776), a native finch from North America (see **Figure 1**), is a socially monogamous bird with well-defined sexual roles and sexual dimorphism in color -adult males displaying bright carotenoid-dependent red crowns, bibs and rumps- as well as a large song repertory. Females are less conspicuous and choose males for their color and their songs (Nolan and Hill, 2004). Females are more actively involved in parental care than males, at least initially. They choose the nest site, build the nest, incubate the eggs and participate in the feeding of the chicks, whereas males feed the female during the incubation and do most of the feeding of fledglings, often also taking over the feeding of pre-fledging chicks (Hill, 2002).

Passer domesticus (Linnaeus, 1758), the ubiquitous house finch (see **Figure 1**), is a socially monogamous European finch long naturalized in the Americas. It is also sexually dimorphic. Male ornaments (maroon and gray crown, black patches around beak and the eyes, a black bib, and dark back and wings that contrast with their pale underparts) are due to melanin, while females lack contrasting patches and seem drabber. Parental care is shared between sexes; both members of the pair choose the nest site, build the nest, incubate the eggs (female more actively) and feed the chicks (male more actively; Voltura et al., 2002; Hoi et al., 2003; Schwagmeyer et al., 2005; Liker et al., 2008).

Study Site and Blood Samples

We conducted this study in a mixed colony of *C. mexicanus* and *P. domesticus* at the main campus of the Universidad Nacional Autónoma de México (UNAM) in the south of Mexico City. These species were breeding in natural conditions, constructing their nests on building structures or in trees. We worked with a mixed-species colony where nests are placed in close proximity (from 30 cm to 200 cm). Here, pairs of both species interact with each other throughout the breading season, feeding and collecting nest materials from the same places. Discarded cigarette butts are available on the ground, and are locally abundant at particular spots around the gates of the surrounding buildings. In the spring of 2014 we monitored the activity of the colony with binoculars (Minox^{TM} BV 10 x 42) to assess the breeding stage and the number of eggs or chicks. We captured both members of each pair by setting mist nets for 3 days at the end of each of four 15-days intervals starting 15 days after the majority of the pairs had begun nest building. We could not recapture both members of the pair at all breeding stages, and this was declared in our statistical models. Thus we sampled breeding adults at end of (a) nest building, (b) incubation, (c) early (chicks $\leq$ 15 days old) and (d) late breeding (chicks $\geq$ 16 days old). Rodnan et al. (1956) reported that after 30–40 days radioactively marked erythrocytes were no longer present in pigeon (*Columba livia*) chick (*Gallus domesticus*) and duck (*Anas platyrhynchos*) blood samples, and from the reported curves it can be inferred that between 40 and 50% erythrocytes are removed from the bird's circulating blood every 2 weeks (see Reddy et al., 1975 for comparable results). Because of their higher metabolic rate (see Speakman, 2005) blood turnover is likely to be faster in smaller species, thus we decided that sampling every 15 days should provide a fair estimate of the condition of the birds (see below) in the preceding 2–3 weeks. We recorded the species, sex and size (tarsus length [mm] and weight [g]) and took a blood sample ($\sim$5µl) from each bird in a heparinized capillary tube after puncturing the brachial vein. Birds were marked with individual combinations of colored plastic rings and one metal ring with a unique number. We identified the nest of each pair with the aid of binoculars and visited each nest to check attendance by the parents and to record the clutch and brood size. Only one of the 58 nests followed was deserted, and it happened before egg-laying (the nest is not included in the analyses). The remaining 57 nests were followed until their chicks fledged. Within 12 h of collection we took the blood samples to the laboratory where we prepared two smears of each individual, to which we applied Schiff staining and counterstained with Hematoxylin. A coverslip was fitted with Entellan resin to each smear, which was inspected under a phase contrast optic microscope (Carl Zeiss^{TM} Axiostar at a 1000X). We assessed 1000 erythrocytes from each smear (two smears per individual), counting every instance of a cell with micronuclei, nuclear buds, bridges in binucleated cells, and binucleated cells. At the end of the season we collected each focal nest, removed its parasites with a Berlese-Tullgen funnel, and separated and weighed all its component materials (Suárez-Rodríguez et al., 2013). We obtained the proportion of cigarette butts by dividing the butts weight between the total weight of the nest.

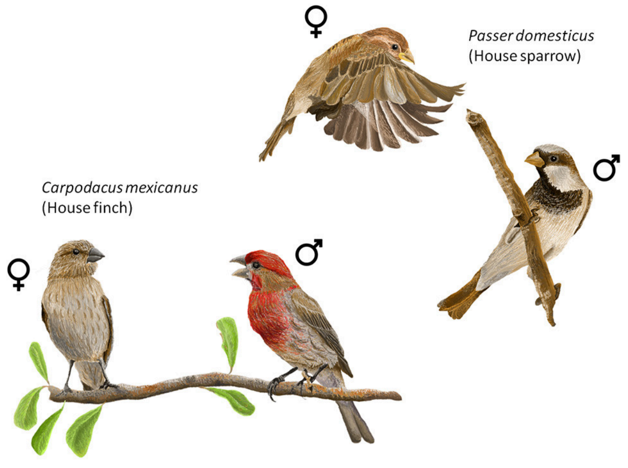

FIGURE 1 | Female (far left) and male *Carpodacus mexicanus* (left), female (right) and male (far right) *Passer domesticus* (drawings by MSR).

Statistical Analyses

We captured and recaptured 41 parents from 26 nests of *Carpodacus mexicanus* and 55 parents from 31 nests of *Passer domesticus*. We analyzed separately the two species, applying one generalized linear model to each, to determine which of our independent variables explained the number of blood cells with genotoxic damage (declaring a Poisson distribution for our dependent variable) using Rx64 s 3.1.2 software. Factors included were breeding stage (four levels), sex (two levels) and weight of cigarette butts in the nest (continuous variable).

After looking at the results we detected that there was an increment in the genotoxic damage experienced by males during the last two breeding stages. At this point, males make the bulk of the contribution to the feeding of the chicks. So, we explored whether the genotoxic damage was actually different from that experienced by females (see results and **Figures 2**, **4**) applying generalized linear models using only the data from the last two stages (early breeding and late breeding) and declaring a Poisson distribution for our dependent variable. We selected the best model using the Akaike Information Criterion (AIC) and ΔAIC, with ΔAIC <2 indicating that two models were equally supported by the data.

RESULTS

Carpodacus Mexicanus

We obtained two models that best explained the number of erythrocytes with evidence of genotoxicity (see Table S1). The best model was a three-way interactive model of the three potentially explanatory factors (proportion of cigarette butts, sex and breeding stage). The amount of cigarette butts in the nest was positively associated with the level of female, but not of male, genotoxic damage (**Table 1**). This effect was evident during nest building, and during incubation; it was much less pronounced during early- (**Figure 2**), but again very substantial in late breeding (**Table 1**; **Figure 2**). Thus the level of genotoxicity experienced by females during breeding is both large and linked to the amount of cigarette butts in the nest (**Table 1**; **Figure 2**).

Males had fewer red-blood cells with signs of genotoxic damage, and their number was consistently independent of the amount of cigarette butts incorporated into the nests. However, the number of damaged cells increased through the breeding stages and appeared to be larger than the damage in females at the last breeding stage (**Table 1**; **Figure 2**). We evaluated whether breeding effort could explain the increase in male genotoxic damage (for instance through endogenous oxidative stress) by running a new model-selection procedure restricted to the early and late breeding stages, and incorporating brood size as a novel predictive factor. The model that best explains the amount of genotoxic damage includes an interaction between sex and brood size. It demonstrates that males attending larger broods had evidence of more genotoxic damage than males attending smaller broods (**Figure 3**). Female genotoxic damage was unrelated to brood size (**Table 2**; **Figure 3**).

Passer Domesticus

The number of cells with evidence of genotoxic damage in *P. domesticus* was best explained by a three-way (sex, cigarette butts and stage of breeding) interactive model (Table S4). In this case, damage in both males and females during nest construction and incubation seemed positively influenced by the amount of cigarette butts in the nest (**Table 3**; **Figure 4**). However, during nest building, incubation and early breeding females had more cells with evidence of genotoxic damage than males. As we

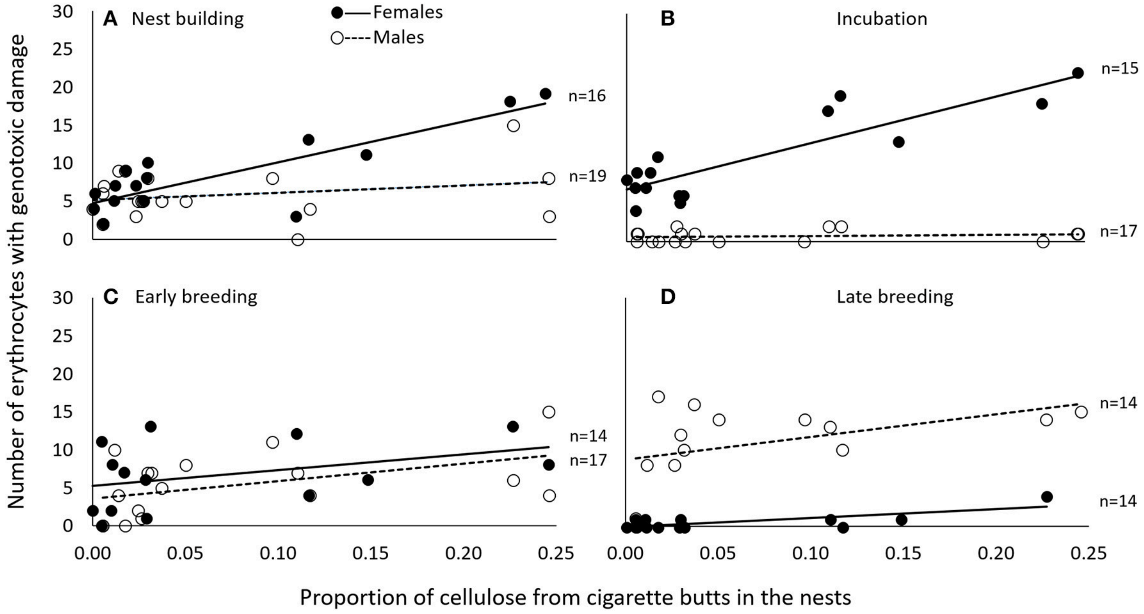

FIGURE 2 | During nest building (A) and incubation **(B)**, genotoxic damage (expressed as the number of cells with nuclear abnormalities per 2000 erythrocytes) was associated in female *C. mexicanus* with the amount of cigarette butts incorporated into the nest. This was not the case during early **(C)** or late breeding **(D)**. Variables are represented in the original scale to facilitate interpretation, but data were analyzed using generalized linear models.

TABLE 1 | Comparison of effects of cigarette butts, sex and breeding stage on the amount of genotoxic damage (number of red-blood cells with nuclear abnormalities per 2000 cells) in *C. mexicanus*.

Breeding moment	Intercept		Slope	
	Females	Males	Females	Males
Nest building	1.656	1.652	5.241*	1.523
Incubation	1.983*	−0.501	4.657*	1.842
Early breeding	1.668	1.347	2.788	3.666*
Late breeding	−1.563	2.189*	12.335*	2.454

*Coefficients[1] come from the best supported model (see Table S1). Model = amount of cigarette butts × sex × breeding stage. Intercept = amount of damage at the onset of the breeding stage; Slope[1] = steepness of the association between amount of cigarette butts in nest and genotoxic damage; * = significant difference between sexes. (An analysis in which each nest is represented only by one if the parents, and only on one breeding stage, yielded essentially the same results; see Tables S8, S9).*

[1] *Both intercepts and slopes in the table are composite (added) values of different coefficients (β) in the full model (see explanation in Table S3).*

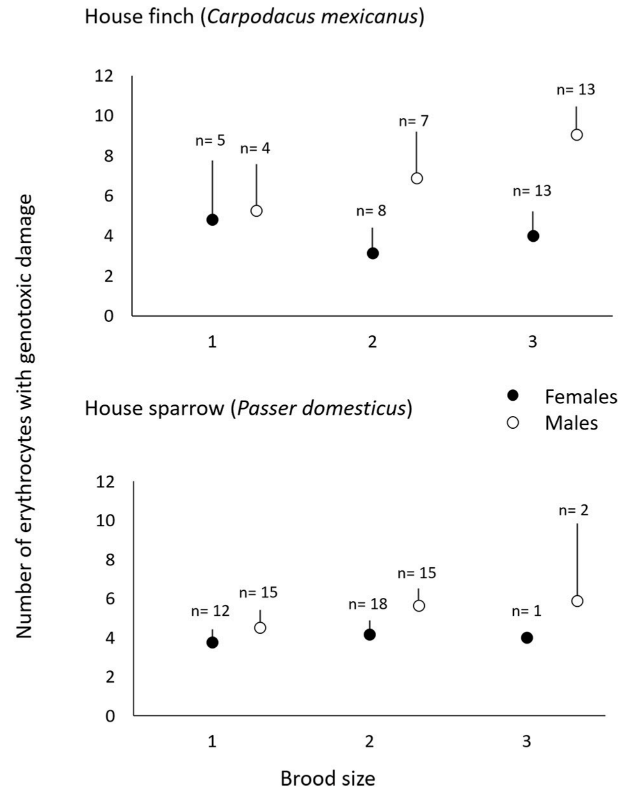

FIGURE 3 | Genotoxic damage in erythrocytes of adult *C. mexicanus* (top) and *P. domesticus* (bottom) of both sexes during early and late breeding, as a function of the brood size (see text).

TABLE 2 | Results of the best model that explains the amount of genotoxic damage in erythrocytes of male *Carpodacus mexicanus* at the last two stages of reproduction (early and late breeding, entered together in the analysis so as not to atomize the sample size).

Variables	Coefficient	SE	Z	*P*
Intercept	1.569	0.204	7.685	< 0.001
Sex: males	0.118	0.281	0.420	0.675
Brood size: 2 chicks	−0.429	0.286	−1.502	0.133
Brood size: 3 chicks	−0.182	0.247	−0.739	0.460
Sex: males x brood size: 2 chicks	0.689	0.373	1.846	0.065
Sex: males x brood size: 3 chicks	0.710	0.326	2.178	0.029

Model = sex × brood size; females and one-chick broods are coded as zero, hence their contribution is included in the intercept.

TABLE 3 | Comparison of effects of cigarette butts, sex and breeding stage on the amount of genotoxic damage (number of red-blood cells with nuclear abnormalities per 2000 cells) in *P. domesticus*.

Breeding moment	Intercept		Slope	
	Females	Males	Females	Males
Nest building	1.997*	0.954	2.696	4.709
Incubation	2.001*	0.271	4.009	5.614
Early breeding	1.688*	0.918	−1.060	−0.261
Late breeding	0.844	2.148*	−0.811	−0.294

*Coefficients[1] come from the best supported model (see Table S4). Model = amount of cigarette butts x sex x breeding stage. Intercept = amount of damage at the onset of the breeding stage; Slope = steepness of the association between amount of cigarette butts in nest and genotoxic damage; * = significant difference between sexes. (An analysis in which each nest is represented only by one if the parents, and only on one breeding stage, yielded essentially the same results; see Tables S10, S11).*

[1] *Both intercepts and slopes in the table are composite (added) values of different coefficients in the full model (see explanation in Table S6).*

expected, the association between amount of cigarette butts in the nest and genotoxic damage was lowest in the last two breeding stages, when parents no longer occupy the nest. However, males had more genotoxic damage than females at the late breeding stage. (**Table 3**; **Figure 4**).

The number of erythrocytes with signs of genotoxic damage in males increased above the number of such cells in females during late breeding, and it was not related to the amount of nest cigarette butts. Consequently, we tested whether, as in *C. mexicanus,* this increase could be explained by endogenous stress related to the effort of feeding chicks. We thus ran the same model-selection procedure looking for a differential effect of brood size on genotoxic damage in male and female parents. Males showed more evidence of genotoxic damage than females during early and late chick breeding, but this damage was not associated with brood size in either sex (**Table 4**; **Figure 3**).

DISCUSSION

We found fluctuating levels of genotoxic damage –measured as the number of red-blood cells with nuclear abnormalities- in breeding *P. domesticus* and *C. mexicanus* of both sexes. In females, at least during the early stages of the breeding attempt (nest building and incubation) such damage can be expressed as a function of the amount cigarette butts that they use to build their nest, and thus reveals a novel breeding cost for birds nesting in cities. This effect is evident in female *C. mexicanus*, which are in contact with the material during nest building and incubation, and in both members of *P. domesticus*, as

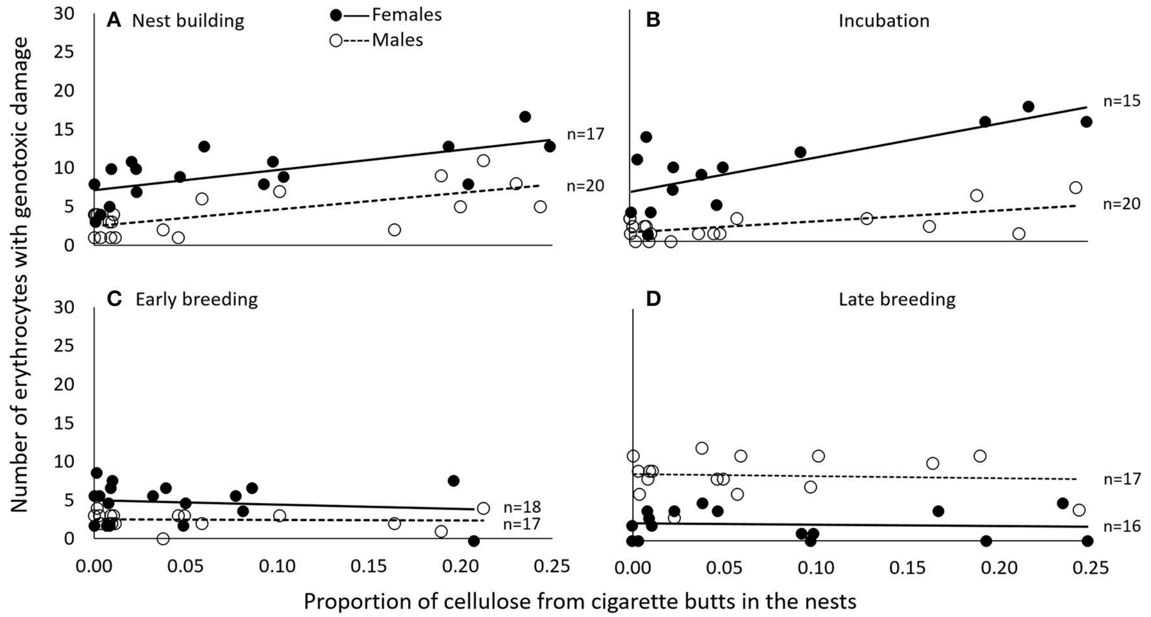

FIGURE 4 | During nest building (A) and incubation **(B)**, genotoxic damage (expressed as the number of cells with nuclear abnormalities per 2000 erythrocytes) was associated in female and in male *P. domesticus* with the amount of cigarette butts incorporated into the nest. This was not the case during early **(C)** or late breeding **(D)**. Variables are represented in the original scale to facilitate biological interpretation, but data were analyzed using generalized linear models.

they share the activities that lead to exposure with cigarette butts. Our results might be explained as a consequence of differences in health or condition between sexes or species. Yet this would not explain the significant link between the amount of butts in the nest and the numbers of erythrocytes with evidence of genotoxic damage; this was found in the sex most exposed to cigarette butts, and was found in the period when it is in a prolonged, direct contact with them. Since foraging is not restricted to areas where cigarette butts are abundant, we have no evidence of sex-specific diets in these species, and given that butts are never so copious that they may substantially leak toxicants into the ecosystem, we think that our results cannot be explained as a result of species/sex differences in diet.

We argue that nuclear abnormalities reveal physiological costs because they are the result of genotoxic damage, and thus can be a proxy to estimate oxidative stress (Bartsch, 2002; Valko et al., 2004) such as that generated by exposure to toxic substances (Fenech, 1993; DeMarini, 2004; Valko et al., 2004; Skarphedinsdottir et al., 2010; López Gordillo, 2012). In addition to revealing damage, abnormalities in the nuclei of erythrocytes can inform approximately when it took place. This is because birds filter their blood through the spleen in cycles of approximately 15–30 days (Rodnan et al., 1956), thus between one half and the whole of the red-blood cells are replaced approximately every 2 weeks, which is similar to the duration of each of the breeding stages that we defined, and hence the span of time between our consecutive samples. Therefore, our measure of genotoxic damage and our sampling program allow us to explore the consequences of exposure to discarded cigarette butts during particular moments of the breeding cycle, when the relative commitment of males and females to the breeding effort may vary.

Our data not only reveal a novel breeding cost for urban birds, but show these costs to be unequally shared by both sexes. Male and female *C. mexicanus* play different roles in parental care (Badyaev and Hill, 2002; Duckworth et al., 2003). Females build the nest, which means they, and not the males, manipulate all the materials that go into its construction. They also perform all the incubation (Badyaev and Hill, 2002; Hill, 2002; Duckworth et al., 2003), thus being virtually continuously in contact with the nest materials. Accordingly, we found that not only females had more evidence of damage than males, but the number of nuclear abnormalities in their blood was tightly correlated with the amount of cellulose fibers from cigarette butts in their nests (= amount of cigarette butts). By contrast, during the same stages the males had very few damaged erythrocytes, and their numbers were independent of the amount of butts that went into the nest construction. This is evidence that a novel, anthropogenic nest material can generate breeding costs that are a function of the sexual differences in parental roles.

Males of *Carpodacus mexicanus* were apparently unaffected by the amount of cigarette butts in their nests, but they also showed evidence of genotoxic damage, particularly later in the breeding attempt. In *C. mexicanus*, males deliver food to the nest (Badyaev and Hill, 2002); this does not bring them into direct contact with nest-lining but there could be a certain amount of exposure to the toxic material, thus inducing variable amounts of genotoxicity. Furthermore, providing food is a demanding activity, particularly after hatching and increasingly so as fledging approaches. Exertions such as this can raise the level of oxidative stress, and we propose that this is the explanation of the increase

TABLE 4 | Results of the best model that explains the amount of genotoxic damage in erythrocytes of male *Passer domesticus* at the last two stages of reproduction (early and late breeding entered together in the analysis so as not to atomize the sample size).

Variables	Coefficient	SE	Z	*P*
Intercept	1.276	0.120	10.668	<0.001
Sex: males	0.279	0.119	2.335	0.020
Brood size: 2 chicks	0.173	0.123	1.403	0.161
Brood size: 3 chicks	0.204	0.267	0.762	0.446

Model = sex + brood size; females and one-chick broods are coded as zero, hence their contribution is included in the intercept.

in the number of nuclear abnormalities in male erythrocytes in the last two breeding stages (**Figure 2**), and of the apparent association of genotoxic damage with brood size (**Figure 3**).

Data from *P. domesticus* were also consistent with our prediction that exposure to cigarette butts promote genotoxic damage in breeding birds. In this case, however, the association between cigarette butts and nuclear abnormalities during nest building was indistinguishable between males and females (**Figure 4**). This may be due to the fact that male and female house sparrow participate in the construction of the nest and in the incubation off eggs (Bartlett et al., 2005). That the association was weaker in males during incubation is consistent with their reduced share in this activity (**Figure 4**). Thus, as in *C. mexicanus*, we uncover a breeding cost resulting from the use of anthropogenic nest materials by urban birds. Furthermore, we confirm that the extent of such cost is linked to the roles played by each pair member during the breeding effort.

In females of both species damage to the red-blood cells was reduced during early and late breeding, and in female house sparrows it was unrelated to the amount of cigarette butts that went into the making of the nest. Nor were nuclear abnormalities in females related to the number of chicks being raised, but we have little statistical power to detect such an effect.

Females of *C. mexicanus* show a late-breeding increase in genotoxic damage, which is linked, once more, to the amount of cigarette butts in the nest. We studied the genotoxic damage of birds raising their first brood, but since our population of *C. mexicanus* make several successive breeding attempts every year, and given their reported practice of clutch overlap (Evenden, 1957), we propose that the cigarette butt related increase in female nuclear abnormalities during late breeding is the consequence of renewed exposure to the nest lining due to incubation of a new clutch (as we systematically monitored the colony, we were able to detect clutch overlap). However, females do not build a new nest, but rather lay new eggs in the old one, thus exposure to butts, and hence genotoxic damage, are only moderately increased at this time. Indeed, we have witnessed clutch overlap within a nest, starting as early as when the chicks were approximately 5 days old.

The nests of *C. mexicanus* and *P. domesticus* contain the same proportion of cigarette butts (Suárez-Rodríguez et al., 2013), yet there appears to be more genotoxic damage in the former (see Supplementary materials). If confirmed, this pattern could be the consequence of male and female sparrows sharing, and thus diluting, the effect of exposure to cigarette butts, yet in both species the extent of damage is similar during early breeding, but lower in house sparrows than in finches (compare **Figures 2C**, **4C**). Another possibility is that this interspecific difference indicates that *P. domesticus*, with a long urban history that should have brought it into contact with anthropogenic pollutants, may have developed adaptations such as enhanced enzymatic activity (e.g., Rainio et al., 2012), that increase its ability to detoxify itself (Schwagmeyer and Mock, 2003). This tantalizing possibility would constitute one of very few cases of phenotypic adjustments to cities that may not be explained just a consequence of plasticity, but as genuine adaptation to urban life (see Suárez-Rodríguez et al. in press).

While we are confident that our measures of nuclear abnormalities are indicative of genotoxic damage, we have no inkling on what the long term consequences of this damage may be. Indeed, there is a paucity of works on the effects of toxicity on the longevity of short-lived Passerine birds. To be of consequence, genotoxicity—the damage of genetic material during cell division- should result in mutagenesis, which involves a modification of the genetic code that is transferred to the next generation of cells (Fenech, 2008). As we worked with cells that are constantly being produced and replaced (Rodnan et al., 1956), it may be that even if exposure to cigarette butts results in mutagenesis, this may not have time to express itself before the cell carrying it being discarded, unless the damage took place in erythropoietic stem cells, which would continue producing damaged erythrocytes. We counted nuclear abnormalities in erythrocytes because 1) their short life span allowed us to track the possible association between breeding activity and damage, and 2) extracting small amounts of blood to make two smears is both easy and relatively little intrusive. Still, damage may be occurring to other cell lineages -notably in the germ line- and in tissues or organs where it may have a more consequential effect on the condition, life expectancy and fertility of birds.

We have uncovered genotoxic consequences of breeding activities of birds using anthropogenic nest materials, and show that the amount of damage is linked to the roles played by both sexes during breeding. Cigarette butts repel nest parasites (Suárez-Rodríguez et al., 2013), presumably because they contain nicotine (Harvey et al., 2007), and arsenic (Slaughter et al., 2011) which have been used as insecticides, as well as polycyclic aromatic hydrocarbons and other metals which could be responsible for the genotoxic damage experienced by both chicks (Suárez-Rodríguez and Macías Garcia, 2014) and adults (this work). Birds in nature also utilize some particular (plant) materials to deter parasites off the nest (Clark and Mason, 1988; Lafuma et al., 2001; Brouwer and Komdeur, 2004; Ontiveros et al., 2007; Gwinner and Berger, 2008; Polo et al., 2010), and it is conceivable that some of the substances involved (Gwinner and Berger, 2006) are also harmful to the birds. Still, both the diversity and the concentration of substances in discarded, smoked-through cigarette butts are likely to be much higher than those found in plants brought to the nests for prophylaxis, and we propose that the costs reported here apply mainly, if not only to birds using cigarette butts to line their nests. On the other hand, the ubiquitous application of insecticides and herbicides to crops, and their subsequent leaking into non-agricultural habitats, should make the exposure of birds to toxicants from nest materials a global reality, with consequences on the costs paid by the parents in accordance to their respective parental roles. This increasing risk of exposure to anthropogenic pollutants could elevate the cost of nest-building and incubation, with potentially negative demographic consequences.

ETHICS STATEMENT

In conducting this research, we adhered to the guidelines for the use of animals in research. We minimized handling stress, kept the sample to the minimum necessary to test the working hypotheses, and verified that no mortality happened as a result of our manipulations. This report has been approved by an ad-hoc ethical committee set up by the Programa Universitario de Bioética (UNAM).

AUTHOR CONTRIBUTIONS

CG and MS designed the study. MS collected the data, performed the analyses, and generated an initial draft of the report. CG provided logistical support and edited the manuscript. RM advised on the measure of toxicological damage, which was conducted in her facilities. All authors reviewed and approved the final version.

FUNDING

MS was awarded a PhD scholarship (480142/280493) by the Consejo Nacional de Ciencia y Tecnología (CONACyT), and CG contributed to the research expenses using the budget allocated to him by the Instituto de Ecología, UNAM.

ACKNOWLEDGMENTS

We thank S. Vargas, A. Vargas, T. Nakamura and V. Argaez for field work help. José J. Zúñiga-Vega, R. Torres, and J. Schondube advised on methodological and statistical aspects and provided commentaries to early drafts. Edgar Ávila provided logistical support. Field work was facilitated by A. Martínez Sigüenza from the Coordinación de Áreas Verdes y Forestación of the Dirección General de Obras y Conservación, UNAM, and by L. Gómez Pérez and J. Andraca Lara of the Dirección General de Presupuesto and the Instituto de Investigaciones Filológicas, UNAM. Luis Serrano-García, Soledad Solís Ángeles, and Omar Arellano-Aguilar advised on and provided logistical support for conducting the genotoxicity analyses. Also, we thank Lynna Kiere for her English revision. This paper is a partial fulfillment for MS's doctoral degree within the Posgrado en Ciencias Biológicas, UNAM under the supervision of CG. MS was the recipient of a scholarship from CONACyT. This research counted with the Ministry for the Environment (SEMARNAT) permit SGPA/DGVS/00610/13. The authors declare that we have no conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00004/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 12 September 2017
doi: 10.3389/fevo.2017.00106

The Influence of Urban Environments on Oxidative Stress Balance: A Case Study on the House Sparrow in the Iberian Peninsula

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Specialty section:
This article was submitted to Urban Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *20 February 2017*
Accepted: *25 August 2017*
Published: *12 September 2017*

Citation:
Herrera-Dueñas A, Pineda-Pampliega J, Antonio-García MT and Aguirre JI (2017) The Influence of Urban Environments on Oxidative Stress Balance: A Case Study on the House Sparrow in the Iberian Peninsula. Front. Ecol. Evol. 5:106. doi: 10.3389/fevo.2017.00106

The House Sparrow is a globally distributed species and is closely associated with anthropised environments. They are well-adapted to urban life; therefore the decline of their populations in Europe represents an unexpected event that demands an investigation into its causes. Causes that have promoted this decline are not well-known, but one of the highlighted hypotheses is an increase of oxidative stress linked to the toxicity of pollution in urban areas. From an ecophysiological perspective, oxidative damage, antioxidant defense, and oxidative balance are considered reliable indicators of environmental stressors such as pollutants. To carry out this study, blood samples were collected from House Sparrows in three different habitats that varied in terms of urbanization degree: urban, suburban, and rural; during the winter and breeding season. According to our results, urban sparrows showed higher levels of oxidative damage and higher activity of antioxidant enzymes, but lower antioxidant capacity in comparison with the rural birds; and these differences especially increase during the breeding season. The maintenance of oxidative balance increases in an urban environment in comparison to a rural one; we suggest that the high level of pollution and the poor quality diet linked to urban environments. The breeding season is expected to be particularly challenging for the oxidative balance of urban birds, when the reallocation of resources between self-maintenance and reproduction may be critical due to the scarcity of antioxidants found in urban areas. This study may contribute to determining the causes of the population decrease of House Sparrows in cities.

Keywords: urbanization, biomarker, pollution, antioxidant defense, oxidative damage, bird

INTRODUCTION

Urban areas have been growing rapidly over the last several decades, and it is expected that they will continue to expand at the expense of natural and rural habitats. From an ecological perspective, urban environments present some novel challenges for birds, such as new predators, new flora, human presence, unique food resources, and high levels of chemical, light, and acoustic pollution (Gaston, 2010; Lepczyck and Warren, 2012; Forman, 2013; Gil and Brumm, 2014). However, these novel and potentially stressful conditions generated by urban environment processes have also provided new opportunities for certain species to gain an ecological advantage by exploiting city resources (Lepczyck and Warren, 2012; Costantini et al., 2014). Such resources are characterized by lower predation rates (Evans et al., 2015), higher environmental temperatures (Tryjanowski et al., 2015), lower competition for resources (Kark et al., 2007), and a constant, abundant, and more predictable food resources supply, in comparison with the surrounding non-anthropogenic areas (Shochat, 2004; Oro et al., 2013; Andersson et al., 2015; Tryjanowski et al., 2015; Marzluff, 2016).

The House Sparrow (*Passer domesticus* L.) is one of these urban exploiter species, meaning that it dominates highly urbanized environments. It is the most ubiquitous and globally distributed bird, probably due to its ecological tolerance that allows them to cope with urban environments: they are generalist, granivorous, gregarious, and sedentary, they nest in cavities, they are unbothered by human presence, and they are able to explore new feeding sources (Anderson, 2006; Kark et al., 2007; Evans et al., 2011). However, in the last few decades, urban populations of House Sparrow have significantly dropped, especially in the highly developed regions of Western Europe (De Laet and Summers-Smith, 2007; Peach et al., 2008; Shaw et al., 2008; De Coster et al., 2015). There is no consensus on the reason for such a decline, but the increasing level of pollution has been highlighted as one of the main driving factors (Shaw et al., 2008; Herrera-Dueñas et al., 2014).

The atmosphere of urban areas tends to accumulate high concentrations of carbon dioxide (CO_2), nitrous oxides (NO_x), sulfur dioxide (SO_2), ozone (O_3), heavy metals and other suspended particulate matters (PM). In cities, these pollutants are mainly derived by burning fossil fuels (Grimm et al., 2008; Gaston, 2010), representing an environmental risk from human to ecosystem levels (Forman, 2013). In relation to human health, a correlation has been described between the level of pollution and higher human mortality rates, mainly associated with cancer, asthma and cardiovascular disorders (Kelly, 2003). To date, deleterious effects on wildlife are less well-known, but pollution has been related to reproductive performance, lower survival rates and shorter lifespans (Isaksson, 2010; Koivula and Eeva, 2010; Salmón et al., 2016). One trait that has been highlighted as responsible for the toxicity of urban pollutants is their high reactivity to biomolecules (Koivula et al., 2011). Therefore, oxidative stress has been described as an underlying mechanism by which urban pollutants cause deleterious effects in living organisms (Isaksson, 2010).

Oxidative stress has been defined as the occurrence of enzymatic and non-enzymatic antioxidants that cannot fully neutralize the free radicals produced in the cells of living organisms. Free radicals are commonly generated as a by-product of normal metabolic processes. Hence all aerobic organisms have evolved multiple defense lines to prevent or counteract the toxicity of reactive by-products, ranging from antioxidant enzymes to low molecular weight endogenous antioxidants (i.e., GSH), complemented by some dietary antioxidant such as carotenoids or vitamins (Halliwell, 2007). Under pro-oxidant conditions, the unquenched free radicals remain in the system long enough to cause further oxidation reactions (Monaghan et al., 2009). Such reactions may damage macromolecules, such as lipids, proteins, and DNA. Severe accumulation of oxidative damage in these molecules leads to cellular dysfunction or apoptosis, which triggers disease and age-related degeneration (Costantini and Verhulst, 2009; Isaksson, 2015). This supports the hypothesis that the resistance to oxidative stress is a key cellular mechanism to cope with urban pollutants, so the capacity to quench the oxidative damage has been highlighted as a critical adaptation to life in the city (Costantini et al., 2014).

The levels of oxidative stress in organisms could also vary with developmental stage, environmental condition, and level of activity (Isaksson, 2015). Reproduction could increase oxidative stress as an inevitable consequence of increased reproductive effort (Romero-Haro et al., 2016) and/or because the developing offspring consume antioxidants otherwise allocated to self-maintenance (Alonso-Alvarez et al., 2004; Moller et al., 2010; Berglund et al., 2014). Therefore, oxidative stress may have a dramatic effect on bird fitness during the breeding season.

Regarding the roles of these antioxidants in maintaining the oxidative balance, four different scenarios could be described: (I) low oxidative damage due free radicals being quenched by the excess supply of antioxidants (endogenous or diet-provided); the maintenance of the oxidative balance in this case is not costly for self-maintenance of individuals, and resources can be reallocated to other functions (i.e., reproduction). (II) Oxidative damage is in balance with antioxidant availability; therefore, there is no surplus of resources, but self-maintenance is not yet costly for individuals. (III) Oxidative damage persists and to counteract it, the individual may increase its antioxidant capacity by up-regulating some antioxidant enzymes; this entails a cost because some resources must be diverted from self-maintenance. (IV) Oxidative damage is uncontrolled; the antioxidant defenses have been overwhelmed and the survival of the individual is compromised (Costantini and Verhulst, 2009; Monaghan et al., 2009) (**Figure 1**).

We predict that the first scenario will correspond to areas with a low degree of urbanization and therefore to lower levels of pollution (such as the rural ones); the second one will correspond to mild stressor environment (such as the suburban areas); whereas the third scenario will correspond to highly urbanized areas, where the high level of pollution may be a challenge for antioxidant defenses. We will explore the oxidative stress balance during winter, but also during the breeding season, when adults are reproducing.

MATERIALS AND METHODS

Area Characterization

House sparrows were sampled at six locations of central Iberian Peninsula: Plasenzuela, a small village 230 km away from big cities such as Madrid (PZ: 39°22′39″ N; 6°02′57″ W and 427 m altitude), Olmeda de las Fuentes, another small village 50 km East of Madrid located in a traditional agricultural area of the region (OF: 40°21′38″ N; 3°12′23″ W and 794 m altitude), El Escorial, a small town 50 km Northwest of Madrid with typical suburban structure (i.e., family houses

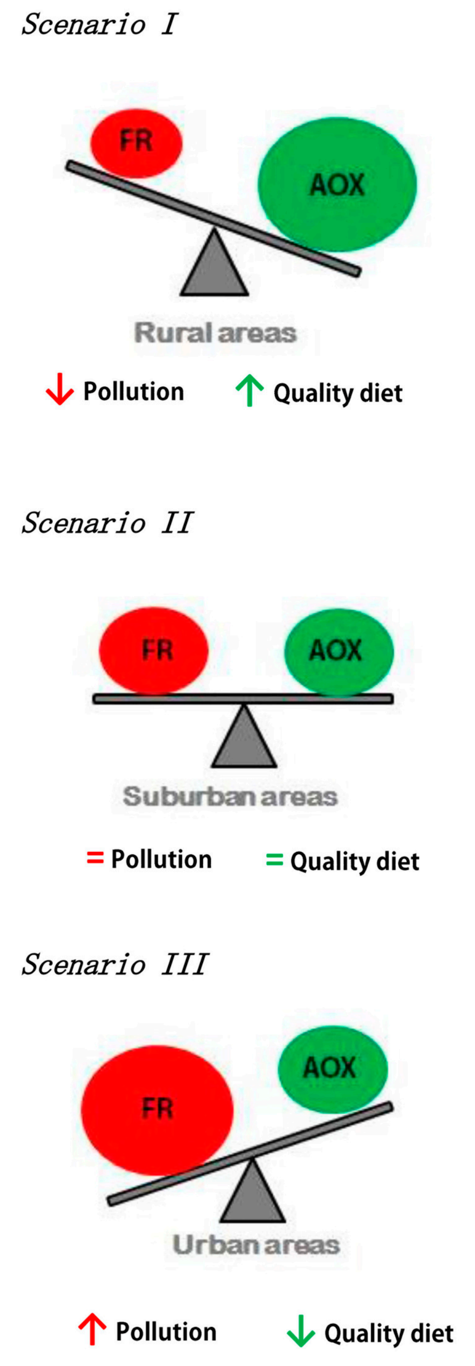

FIGURE 1 | The different scenarios (I, II, and III) depending on the relationship between the environmental stressors (such as pollution) and dietary antioxidants (the scenario IV has been excluded due to it is not expected to be related to urban environments). FR (red color) represents free radical production: intrinsic + induced by environmental stressors; and AOX (green color) represents a number of antioxidants available: endogenous + antioxidant enzymes + dietary antioxidants.

with individual gardens) (EE: 40°34′55″ N; 4°07′41″ W and 1.030 m altitude), Las Matas, a small town 25 km Northwest of Madrid in an area with a similar suburban structure (LM: 40°33′41″ N; 3°53′56″ W and 720 m altitude), Fuenlabrada, a town 25 km South of Madrid in the traditional industrial area of the region (FB: 40°17′07″ N; 3°48′35″ W and 650 m altitude) and Madrid city center, one of the biggest cities of the Mediterranean region (MD: 40°25′03″ N; 3°42′42″ W and 670 m altitude). These sampling areas were chosen because they show strong differences in their landscape and their anthropization degree. In addition they showed high bird densities due to the constant food supply: a horse stable in MD and a farm-school in FB, gardens with hen cages in the suburban areas, and a sheep farm in OF and a poultry farm in PZ. Therefore, it was expected that birds would not show symptoms of starvation and their condition would vary depending on other environmental stressors such as pollutants, and the availability of essential nutritional complements that they should intake (i.e., insects).

One of the most reliable indicators of the urbanization process is land cover (Marzluff, 2008; Gaston, 2010; Niemelä, 2011; Lepczyck and Warren, 2012), which can be complemented by the population density (Marzluff, 2008; Lepczyck and Warren, 2012) and air quality (Gaston, 2010; Forman, 2013). Data referring to air quality (NO_2 and PM_{10}) were collected from the European Environmental Agency (EEA), and data referring to human density and land uses were collected from the Spanish Statistics Institute (INE) and the Information System for Land Uses in Spain (SIOSE), which had a resolution of 0.05 km^2 (**Table 1**). The percentage of each land use (industrial, housing, recreation urban areas, agricultural and natural) was calculated in a 4 km^2 area around the sample point using Photoshop (**Figure 2**), based on the species movements described by Anderson (2006).

Bird Sampling

This study was carried out in accordance with all applicable institutional and national guidelines for the care and use of animals. Bird sampling was performed with permissions from landowners and the regional Consejería de Medioambiente de la Comunidad de Madrid (REFS: JML/ecc-10/038121.9/13; IPZ/ecc-10/068353.9.14; and Delegación de Medioambiente de la Junta de Extremadura REFS: EJD/jmca-CN0004/14/ACA).

A total of 210 adult House Sparrows were captured during the winter period (December 2013 to February 2014) and the breeding season (April 2014 to June 2014) in the different habitats (**Table 2**). The birds were trapped with mist nests or traps and ringed. They were weighed (±0.1 g), their tarsus length was measured (±0.1 mm) and blood samples were collected. Birds were captured in mist nets and retained until manipulation in cloth bags to keep them safe and calm. All sampled birds were released unharmed at the site of capture after manipulation.

Blood samples (∼0.2 ml) were collected (∼15 min after capture) by jugular venepuncture using 30 G needles. The blood was collected in heparinized tubes and kept at 4°C before transporting to the laboratory (maximum 4 h). Blood was centrifuged for 10 min at 1,800 × g (10,000 rpm) to separate the erythrocyte pellet and plasma, and was divided into aliquots to avoid freeze-thaw cycles. All samples were stored at −80°C until use.

TABLE 1 | Characteristic of sampled localities based on the habitat variables: population density (n° inhabitants/km2), land use (%) and air quality (NO_2, SO_2, PM_{10}, and $PM_{2.5}$ in $\mu g/m^3$).

Habitat	Site	Population density	Housing land	Recreation land	Industrial land	Agricultural land	Natural land	NO_2	SO_2	PM_{10}	$PM_{2.5}$
Urban	MD	5,225	58.88	38.34	1.12	0.00	0.00	37.95	5.03	ND	ND
Urban	FB	4,960	33.75	8.27	13.23	34.53	8.72	31.11	ND	28.26	ND
Suburban	LM	1,040	31.35	3.96	0.23	0.87	60.89	27.69	ND	ND	13.4
Suburban	EE	222	17.26	7.91	0.59	9.76	58.83	22.66	ND	23.21	ND
Rural	OF	20	0.97	0.19	0.00	54.19	37.65	7.03	ND	17.30	ND
Rural	PZ	14	0.47	0.03	0.00	41.47	56.18	4.97	2.54	16.05	ND

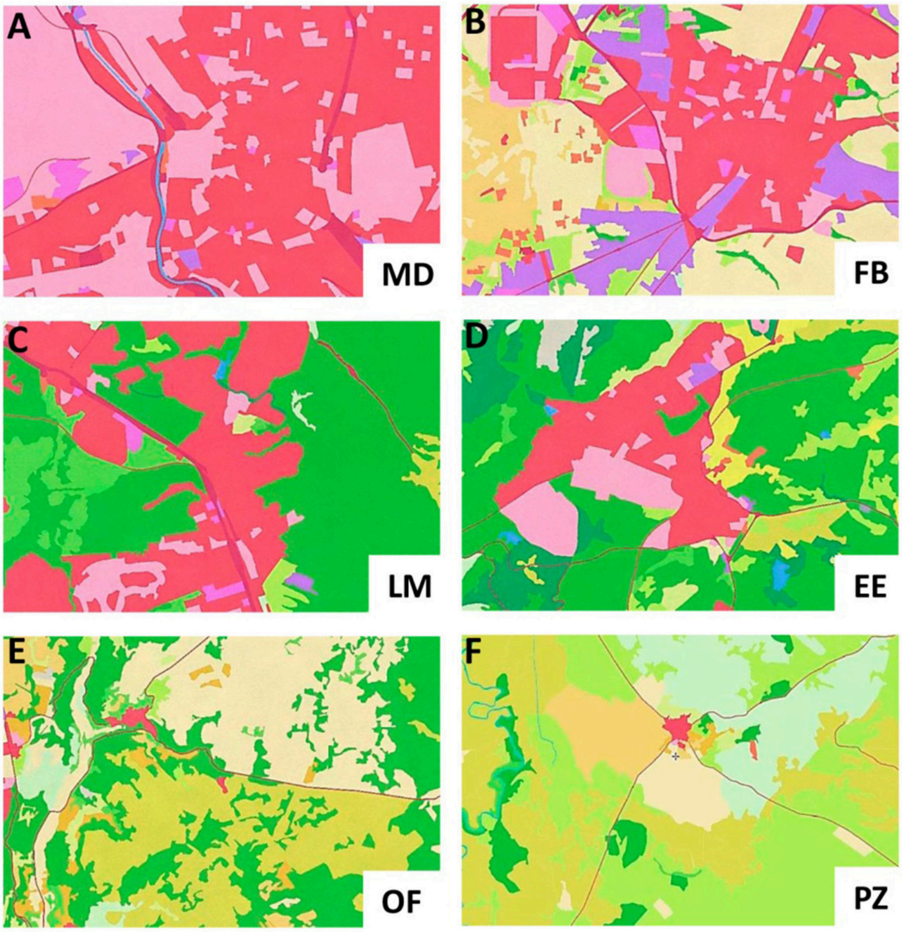

FIGURE 2 | Images used for calculating the land uses at each sample point: Madrid city **(A)**, Fuenlabrada **(B)**, Las Matas **(C)**, El Escorial **(D)**, Olmeda de las Fuentes **(E)**, and Plasenzuela **(F)**. The dark pink areas represent housing and roads, the light pink represent urban parks and recreation facilities, the purple ones represent industrial areas, the yellowish ones represent agricultural lands and the greenish ones represent natural areas like forest or field.

Oxidative Stress Biomarkers

Due to the complex relationship between the different elements constituting the oxidative stress system, at least several oxidative damage and antioxidant capacity biomarkers must be determined to correctly interpret the results (Monaghan et al., 2009). To evaluate the oxidative stress balance of House Sparrows, we determined the antioxidant capacity of plasma, as well as the oxidative damage and activity of some antioxidant enzymes in the erythrocytes.

The total antioxidant capacity (TAC) of plasma is a reliable biomarker to describe the global oxidant/antioxidant balance of individuals. This was determined spectrophotometrically using the ferric reducing ability of plasma (FRAP) method described by Benzie and Strain (1996), with the slight modifications described by Hargitai et al. (2012). This method is based on the ferric

TABLE 2 | Number of individuals captured per locality and season.

		Rural		Suburban		Urban	
		PZ	**OF**	**EE**	**LM**	**FB**	**MD**
Winter	Males	12	12	12	10	7	26
	Females	9	11	11	11	13	14
Breeding season	Males	8	ND	ND	19	ND	8
	Females	11			11		5

reduction: Fe^{3+} (ferric) to Fe^{2+} (ferrous) ion reduction at low pH causes the formation of the colored ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) complex. Briefly, 20 µl of diluted plasma was mixed with 150 µl of ferrous ion and TPTZ at low pH and incubated for 20 min. FRAP values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing Fe^{2+} in known concentration. The parameter was corrected with the uric acid value (Costantini, 2011), which was spectrophotometrically measured at 520 nm by the uricase method (Fossati et al., 1980) using a commercial kit from Spinreact®. The intra-assay and inter-assay coefficient variation (CV), respectively, were 1.72 and 1.97%.

The oxidative damage was determined in the lipid and erythrocyte proteins. The lipid peroxidation was estimated spectrophotometrically by the thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a by-product of the peroxidation of membrane lipids according to the method of Ohkawa et al. (1979). This method is controversial since it has been considered less accurate in comparison with the quantification based on the HPLC method, due to overestimation of the results (Grotto et al., 2009). Although other authors still consider spectrophotometry as a reliable methodology for the determination of lipid peroxidation (Zeb and Ullah, 2016) and we still use it due to technical reasons, this result is less reliable and it should be interpreted carefully and together with other biomarkers of oxidative damage. Briefly, 250 µl of the erythrocyte homogenate was reacted with 500 µl of TBA solution (pH 3.5), the mixture was heated at 80°C for 60 min. Under these conditions, the TBA-reactive substances (mainly MDA) reacted with TBA to yield TBA-MDA adducts, which were detected at 532 nm. The concentration of the chromophore was calculated from a calibration curve prepared with tetramethoxypropane (TMP) solution. The intra-assay and inter-assay CVs, respectively, were 2.89, and 7.24%. The protein oxidation was estimated spectrophotometrically by 2,4-dinitrophenyl hydrazine (DNPH) reaction with the carbonyl groups, by-products of the oxidation of proteins, using the method by Reznick and Packer (1994) with the modifications describe by Arnal et al. (2011). Briefly, 100 µl of the erythrocyte homogenate was subjected to a reaction with 100 µl of DNPH in acid solution of HCl at 37°C in the dark for 30 min; the corresponding hydrazone derivatives were revealed after the addition of 200 µl of NaOH and measured at 505 nm. The concentration of carbonyls was calculated from a calibration curve prepared with a stock solution of sodium pyruvate. The intra-assay and inter-assay CVs, respectively, were 2.56 and 8.67%.

The enzymatic antioxidant system capacity was determined by the evaluation of glutathione peroxidase (GPX) and superoxide dismutase (SOD) activity. The activity of GPX was measured spectrophotometrically by reduction of 5, 5′-dithiobis, 2-nitrobenoic acid (DTNB) for the activity of GPX using the glutathione (GSH) of the cells, as described by Moin (Tkachenko et al., 2014). The reaction was read at 412 nm every 30 s for 3 min. Activity is expressed as µmol GSH/min/ml. The intra-assay and inter-assay CVs, respectively, were 6.92 and 7.69%. The activity of SOD was measured spectrophotometrically by the containment of autoxidation of pyrogallol, as described by Marklund and Marklund (1974). The reaction was read at 420 nm every 60 s for 5 min. Activity is expressed as U/ml. The intra-assay and inter-assay CVs, respectively, were 7.39 and 8.74%.

Samples were randomly distributed among plates. All the assays were running in duplicate. The same assay was running in all the samples during the same lab session.

Data Analysis

The body condition index of individuals was calculated using the scaled mass index (SMI) recommended by Peig and Green (2009) for small animals. The SMI has been calculated according to the equation:

$$\mathrm{SMI} = \mathrm{M_i} \times \left(\frac{\mathrm{L_0}}{\mathrm{L_i}}\right)^{\mathrm{b_{SMA}}}$$

where M_i and L_i are the body mass and tarsus length of individual I, respectively; L_0 is the arithmetic mean value of tarsus length for the study population; and b_{SMA} is the scaling exponent estimated by the standardized major axis (SMA) regression of ln M on ln L.

In order to evaluate the effect of habitat and season, we used a general linear mix model (GLMM). Fix factors included in the whole model were *habitat* (rural, suburban, or urban), *season* (winter or breeding) and their interaction (*habitat × season*) and the capture *locality* as random factor. In all analyses, *sex* (male or female) and *body condition* (as SMI) were also included as covariates. A *post-hoc* assay (Tukey's HSD) was performed only for traits regarding habitat. All the models were tested for residual normality.

All results are expressed as means ± standard error of means ($\overline{X}$ ± S.E.M.). All analyses were performed in *R-Studio* version 3.3.1 using the *lme4*, the *lmerTest,* and the *lsmeans* packages.

RESULTS

Area Characterization

Although the characterization of urban landscapes is complex due to its heterogeneity and huge variation between regions, the sampling sites showed a clear pattern in terms of air quality, land use, and population density. We were able to clearly disentangle three different categories (Marzluff, 2008; Gaston, 2010; Forman, 2013): (I) *urban*, a completely anthropic landscape typical of big cities or town centers, usually characterized by a high percentage of ground occupied by buildings and pavements mixed with facilities (such as shops, museum, schools, hospitals, and sport-centers), with high population densities. These areas are usually associated with high pollutant levels due to traffic and heater emissions. (II) *Suburban*, residential areas located at the edge of big cities and/or smaller towns. It is also characterized by a high building density, but the most common structures are houses with gardens so the percentage of green areas is usually higher and better distributed than in urban areas. In addition, non-anthropized areas, such as forests or fields, contribute to the landscape. The quality of the air usually improves in these areas because of the lower population density; however, it is still high in comparison with rural settlements. Finally, (III) *rural*, villages, or even smaller settlements embedded in natural or agricultural landscapes. In this case, the percentage of buildings and roads is lower when compare to green areas. Its population density is sparse and the quality of air is good according to the standard.

Body Condition and Oxidative Stress Biomarkers

The body condition, calculated as SMI, showed a significant interaction between habitat and season [$F_{(2,\ 113.02)} = 5.92$; $p = 0.003$], driven by the fact that the SMI of urban birds had a trend for being lower during the breeding season compared to winter ($t = -2.86$; $p = 0.052$), whereas rural birds showed the opposite trend (they were bigger during the breeding season). In the suburban population, SMI was similar in both seasons. Overall, the SMI of rural birds was higher in comparison with the other population (**Figure 3**, **Table 3**).

In relation to oxidative stress biomarkers, the season did not show any effects, while the habitat seemed to play a main role. Only in the case of TAC did the model show a significant

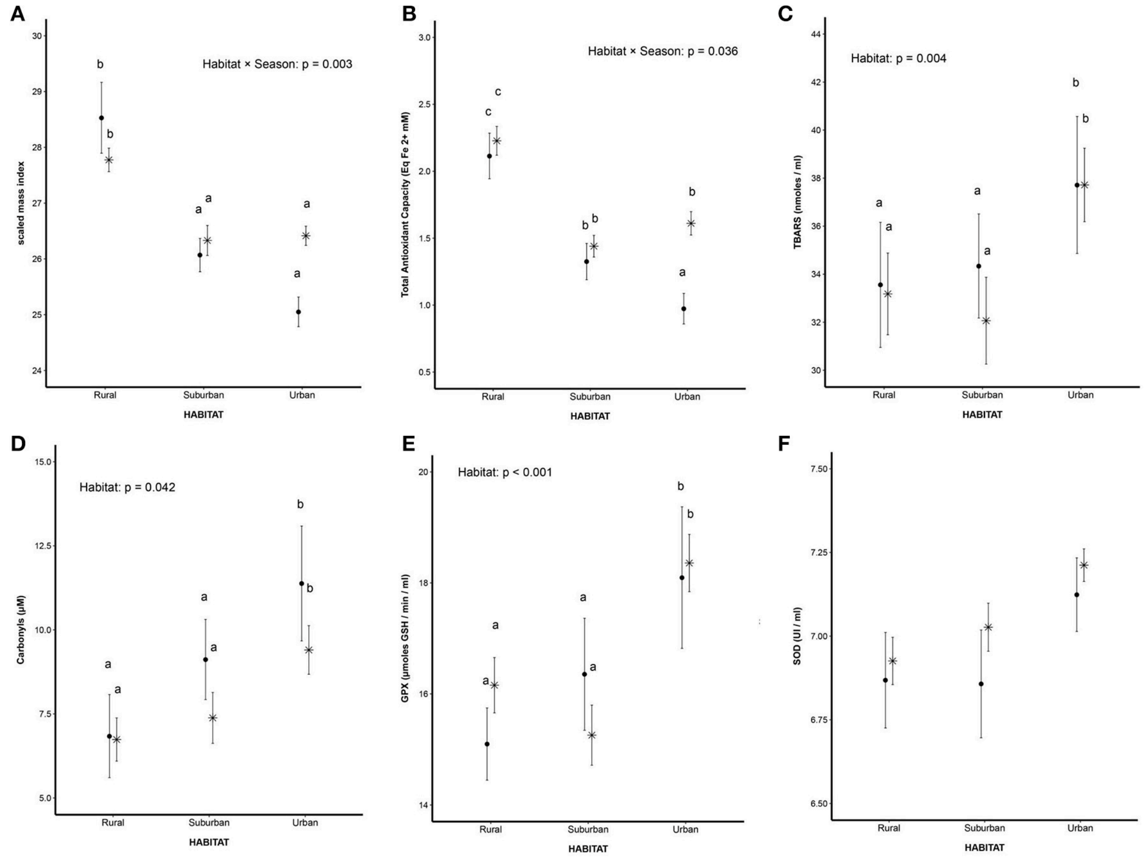

FIGURE 3 | Body condition and oxidative stress biomarkers analyzed. **(A)** Body condition calculated as scaled mass index (SMI). Values are expressed as mean ± S.E.M. Asterisk (*) represent individuals captured at winter and filled circles (•) represent individuals captured during the breeding season. Letters indicate statistical difference: means with the same letter are not statistically different (Tukey's tests, $p \leq 0.05$); **(B)** Total antioxidant capacity calculated as FRAP. Values are expressed as mean ± S.E.M. Asterisk (*) represent individuals captured at winter and filled circles (•) represent individuals captured during the breeding season. Letters indicate statistical difference: means with the same letter are not statistically different (Tukey's tests, $p \leq 0.05$); **(C)** Lipid damage calculated as TBARS. Values are expressed as mean ± S.E.M. Asterisk (*) represent individuals captured at winter and filled circles (•) represent individuals captured during the breeding season. Letters indicate statistical difference: means with the same letter are not statistically different (Tukey's tests, $p \leq 0.05$); **(D)** Protein damage calculated as concentration of carbonyls groups. Values are expressed as mean ± S.E.M. Asterisk (*) represent individuals captured at winter and filled circles (•) represent individuals captured during the breeding season. Letters indicate statistical difference: means with the same letter are not statistically different (Tukey's tests, $p \leq 0.05$); **(E)** Glutathione peroxidase activity (GPX). Values are expressed as mean ± S.E.M. Asterisk (*) represent individuals captured at winter and filled circles (•) represent individuals captured during the breeding season. Letters indicate statistical difference: means with the same letter are not statistically different (Tukey's tests, $p \leq 0.05$); **(F)** Superoxide dismutase activity (SOD). Values are expressed as mean ± S.E.M. Asterisk (*) represent individuals captured at winter and filled circles (•) represent individuals captured during the breeding season.

TABLE 3 | The model for each dependent variable when exploring the effect of the habitat and season on body condition and oxidative stress biomarkers.

Dependent variable	Source of variation	d.f.	*F*	*p*-value
BODY CONDITION				
Scaled mass index (SMI)	Habitat	2, 2.51	14.04	0.043
	Season	1, 125.25	0.80	0.371
	Habitat × Season	2, 113.02	5.92	**0.003**
OXIDATIVE BALANCE				
Total Antioxidant Capacity (FRAP)	Habitat	2, 2.92	17.14	0.024
	Season	1, 51.79	6.04	0.017
	Habitat × Season	2, 37.02	3.63	**0.036**
OXIDATIVE DAMAGE				
Lipid peroxidation (TBARS)	Habitat	2, 177	5.68	**0.004**
	Season	1, 177	0.35	0.549
Protein oxidation (Carbonyls)	Habitat	2, 123	3.23	**0.042**
	Season	1, 123	2.04	0.154
ANTIOXIDANT DEFENSES				
Glutathione peroxidase activity (GPX)	Habitat	2, 141	9.22	**<0.001**
	Season	1, 141	0.05	0.815
Superoxide dismutase activity (SOD)	Habitat	2, 2.78	2.83	0.530
	Season	1, 84.76	0.39	0.213

They were corrected by covariates (sex in all cases, and sex and body condition in oxidative stress biomarkers). Significant factors ($p \leq 0.05$) have been highlighted in bold. Non-significant interactions were excluded from the model.

interaction between habitat and season [$F_{(2,\,37.02)} = 3.63$; $p = 0.036$]: overall, the rural birds showed a higher antioxidant capacity in comparison with the suburban and urban birds. The urban birds showed a significantly lower antioxidant capacity during the breeding season compared to winter ($t = -3.34$; $p = 0.012$) (**Figure 3**, **Table 3**).

Regarding oxidative damage, lipid peroxidation was significantly higher in urban birds in comparison with the suburban ($t = 2.65$; $p = 0.023$) and rural birds ($t = 3.12$; $p = 0.005$) [$F_{(2,\,177)} = 5.68$; $p = 0.004$] (**Figure 3**, **Table 3**). The oxidation of proteins was also higher in the urban population compared to the suburban ($t = 2.49$; $p = 0.036$) and rural population ($t = 1.54$; $p = 0.027$) [$\mathrm{F}_{(2,\,123)} = 3.23$; $p = 0.042$] (**Figure 3**, **Table 3**).

The activity of the antioxidant defense system was also influenced by habitat. The activity of GPX was significantly higher in urban birds compared to suburban ($t = 3.75$; $p < 0.001$) and rural birds ($t = 3.68$; $p = 0.001$) [$F_{(2,\,144)} = 9.22$; $p < 0.001$] (**Figure 3**, **Table 3**). However, the activity of SOD did not show significant differences [$F_{(2,\,151)} = 4.88$; $p = 0.008$] (**Figure 3**, **Table 3**).

DISCUSSION

We found that urban sparrows showed higher levels of oxidative damage and a higher activity of antioxidant enzymes, but a lower antioxidant capacity in comparison with the rural birds. Some of these differences are especially increased during the breeding season.

According to our predictions, the low oxidative damage scenarios (I and II) matches the situation found in the rural and suburban populations, respectively. Oxidative damage seems to be low or the level of dietary antioxidants is high, because the oxidative status (total antioxidant capacity) is balanced. In this case, up-regulation of antioxidant enzymes has not been required, and the depletion of antioxidants is low (Costantini and Verhulst, 2009; Monaghan et al., 2009); therefore, they are available for self-maintenance, which reduces the cost of coping with oxidative damage (Isaksson et al., 2011). The higher antioxidant capacity found in the rural populations (scenario I) could reflect a surplus of dietary antioxidants, probably due to the availability of some dietary antioxidant complements such as vitamins or minerals. Based on the antioxidant capacity found in suburban birds, the pool of antioxidants must be lower or, probably, oxidative damage increases with urbanization process (scenario II) (Costantini et al., 2014; Isaksson, 2015). In both cases (rural and suburban areas), the maintenance of the oxidative balance would not be costly for individuals, and a reproductive investment should not represent an additional challenge for coping with the oxidative balance (Alonso-Alvarez et al., 2004; Wiersma et al., 2004).

On the other hand, the increasing oxidative damage scenario (III) is in accordance with the results found in urban areas. The oxidative damage in lipids, proteins, and GPX activity showed higher values than those in rural and suburban populations; and the antioxidant capacity of urban birds was lower, especially during the breeding season. These results were expected and in line with a previous study which also found a lower TAC linked to urban areas (Herrera-Dueñas et al., 2014). Here, we propose that pollution could be responsible for the variation in oxidative stress balance between urban and rural areas. For instance, major atmospheric pollutants, such as particles (PM) or heavy metals, have been described as pro-oxidant elements (Kelly, 2003; Isaksson, 2010; Koivula and Eeva, 2010) and their deleterious effects on oxidative stress balance have been described in some urban birds such as Great Tits (*Parus major* L.) (Koivula et al., 2011), Feral Pidgeon (*Columba livia* L.) (Kurhalyuk et al., 2009), White Stork (*Ciconia ciconia* L.) (Kaminski et al., 2007). Similar results have also been found in humans (Chuang et al., 2007; Moller et al., 2014). Therefore, the quantification of these pollutants in the blood or feathers of our populations of House Sparrow may be a useful parameter to confirm this hypothesis in the future.

The lower response to TAC in the urban population is also in line with previous studies that reported poor quality of urban food in terms of antioxidant levels such as carotenoids, vitamins, and minerals (Isaksson and Andersson, 2007; Isaksson, 2015; Tryjanowski et al., 2015). In other urban species like Blackbirds (*Turdus merula* L.), it has been reported that rural populations showed higher concentrations of vitamin E and carotenoids than their urban conspecifics, mainly caused by differences in their diet (Moller et al., 2010). Additional studies about the quality of diet in urban birds and its effects on oxidative stress balance could contribute to verifying this theory.

Under pro-oxidant conditions, the up-regulation of antioxidant enzymes may be required and dietary antioxidants may be depleted; both mechanisms entail some costs (Dowling and Simmons, 2009; Isaksson et al., 2011), which may reduce the fitness and reproductive performance of urban birds (Isaksson, 2015). This is a costly status for individuals, as they must allocate all available resources to counteract the oxidative stress imbalance (Costantini and Verhulst, 2009; Monaghan et al., 2009; Isaksson, 2015). The lack of dietary antioxidants during the breeding season in a challenging environment such as an urban area could lead to potentially negative carry-over effects, in nestlings, linked to early-life nutritional constraints (Metcalfe and Monaghan, 2001). It has been widely reported that the poor condition of House Sparrows nestlings in urban areas is linked to a nutritional deficit (Liker et al., 2008; Bókony et al., 2010; Seress et al., 2012; Meillère et al., 2015). Furthermore, an early-life diet that is poor in antioxidants can result in a long-term impairment in the capacity to assimilate dietary antioxidants, like the carotenoids, vitamin A and E in adulthood (Blount et al., 2003; Monaghan et al., 2009; Costantini et al., 2014). Nutritional constraints in early-life further interact to shape the organization of the redox system (Costantini et al., 2014), which could may explain the lack of the antioxidant capacity that we have found in the adults from our urban populations which could be due to the important role that epigenetic mechanism seem to play in the final modulation of oxidative status (Isaksson, 2015).

In our study, we found that rural birds showed better body condition in comparison with their conspecifics in other areas, especially the urban individuals. This result is consistent with the hypothesis of Shochat (2004) in which birds from urban environments show a poorer condition than conspecifics rural birds; and according to previous studies that found these differences between urban and rural populations of House Sparrow (Vincent, 2005; Liker et al., 2008; Bókony et al., 2010; Seress et al., 2012; Herrera-Dueñas et al., 2014). The weak condition of urban birds may be also linked to the nutritional constraints that they suffer during early-life, which could negatively affect body condition in adulthood (Bókony et al., 2010; Seress et al., 2012), even if the restriction diminish at later life stages (Liker et al., 2008). Body condition, especially during development, is often positively correlated with pre- and post-fledgling survival rates (Lamb et al., 2016); consequently, poor condition is often observed in a declining population such as urban House Sparrows (Dulisz et al., 2016).

Apart from the direct effects of poor diet and pollution on oxidative stress, we should also consider that natural selection may have favored different phenotypes in urban and rural populations. For instance, it has been reported that expression of genes that code for metal detoxification enzymes are upregulated in urban populations of Great Tits (*Parus major* L.) in comparison with rural ones. Therefore, epigenetic mechanisms could generate life-long changes in gene expression, and subsequently, phenotypic traits between urban and rural populations (Watson et al., 2017).

Our study has revealed the fragile oxidative stress balance of the urban House Sparrow, promoted either by an excess of harmful free radicals due to pollution, or insufficient antioxidants due to scarce availability of dietary antioxidants, or both in urban environments. Therefore, birds need to invest resources in maintaining the oxidative stress balance, which is costly especially during the breeding season, when the constraints of antioxidants could entail negative consequences not only on the body condition of the individuals, but also on the viability and future fitness of their offspring. The accumulative effects of lower quality individuals and carry over effects at the population level may be one of the causes underlying the population decreases of urban House Sparrow populations across European cities.

AUTHOR CONTRIBUTIONS

JA, MA, JP, and AH conceived and designed the study. JA, JP, and AH performed the fieldwork. JP and AH conducted lab work. AH performed statistical analyses with support from JP. AH drafted the manuscript and all the authors contributing to the final text.

ACKNOWLEDGMENTS

We acknowledge Comunidad de Madrid and Junta de Extremadura for issuing the permissions, and the CMA for the ringing license. We are grateful to Javier de la Puente, Álex García, Yaiza Aragón, Eva Banda, Alicia Jacoste, and Juan from the Royal Palace of Madrid staff, people from Olmeda de la Fuentes and people from Plasenzuela for their assistance during fieldwork; and to Pablo S. and Pablo C. for their assistance during ringing training of AHD. We are also grateful to Javier Pérez-Tris lab for its financial support, and Jasper vH. and Melinda H. for improving this manuscript. Finally, we thank the editors for giving us the opportunity to participate in this issue, and the two reviewers who have contributed to improving this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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in Ecology and Evolution

ORIGINAL RESEARCH
published: 12 May 2017
doi: 10.3389/fevo.2017.00044

Species-Dependent Effects of the Urban Environment on Fatty Acid Composition and Oxidative Stress in Birds

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Ecological impacts of urbanization include the loss of biodiversity and changes in species composition and population densities. However, how the urban environment affects fundamental physiological parameters is largely unknown. Here, we investigated physiological components related to health and nutrition, namely, plasma fatty acids (FA) and lipid peroxidation at inter-habitat and interspecific levels. Specifically, we compared four passerine bird species—the great tit (*Parus major*), the blue tit (*Cyanistes caeruleus*), the house sparrow (*Passer domesticus*), and the tree sparrow (*P. montanus*)—from urban and rural environments. Significant interactions between species and habitat were revealed for the majority of the FAs. Interestingly, the observed inter-habitat variation in FAs was frequently in opposite directions when comparing species from the two families (tits, Paridae; sparrows, Passeridae). These patterns suggest that sparrows and tits feed on different food sources, or modulate their FA metabolism differently, across the urban-rural gradient. By using canonical discriminant analyses (CDA), we further demonstrated species-specific signals in FA composition, with misclassification of species being $<1\%$ within habitats and $<7\%$ between habitats. Finally, the urban-rural FA differences between species and families were manifested in two indices of health. *Firstly*, urban blue tits had a higher total ω-6/ω-3 polyunsaturated FA ratio than rural conspecifics, which is believed to increase inflammatory responses. *Secondly*, urban sparrows of both species showed higher lipid peroxidation indices (indicating a higher susceptibility to lipid peroxidation if exposed to pro-oxidants), and consequently, a higher level of lipid peroxidation compared to their rural conspecifics. Collectively, the species- and habitat-specific differences in plasma FA composition, which are linked to nutrition and metabolism, suggest that the urban environment affect tits and sparrows primarily *via* two different pathways—inflammation and oxidative stress, respectively,—with potential consequences for the health of urban populations.

Keywords: diet, lipid peroxidation, omega-3, omega-6, Paridae, Passeridae, polyunsaturated fatty acid, urbanization

Edited by:
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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *14 February 2017*
Accepted: *25 April 2017*
Published: *12 May 2017*

Citation:
Isaksson C, Andersson MN, Nord A, von Post M and Wang H-L (2017) Species-Dependent Effects of the Urban Environment on Fatty Acid Composition and Oxidative Stress in Birds. Front. Ecol. Evol. 5:44. doi: 10.3389/fevo.2017.00044

INTRODUCTION

Many urban-dwelling birds are declining, despite the suggested benefits of high abundance of anthropogenic food and artificial nesting holes in urban environments (Jokimäki et al., 1996; Jokimäki, 1999; Marzluff et al., 2001; Crick et al., 2002, 2003; McKinney, 2002; Beckerman et al., 2007; Grimm et al., 2008). Species that seemingly do well in urban environments often share a range of traits, such as a high degree of feeding innovation, high storage of dietary antioxidants, high annual fecundity, high adult survival, and large breeding ranges (Møller, 2009; Møller et al., 2010). Despite such similarities, the consequences of inhabiting an urban environment are likely to differ between species, such as between those classified as "urban adapters" vs. "urban exploiters" (McKinney, 2002). The urban adapter species refer to those that take advantage of human-provided resources, but do not depend on them. Urban exploiters, on the other hand, are considered to depend on human-provided resources in order not to decline. Nevertheless, the house sparrow (*Passer domesticus*), an urban exploiter species that is so closely associated with human settlements that its original niche is unknown (Diamond, 1986), has declined with 60% in urban environments over the last 30 years (Crick et al., 2002, 2003). While there is a considerable number of species that exploit human-provided resources in urban habitats, little is known about the associated physiological modulations, and their consequences for urban life (Diamond, 1986; Isaksson, 2015). This is unfortunate, because physiological data, such as biomarkers for health and nutritional status, may provide critical information on the underlying causations for why a species thrives in, or simply just copes with, the urban environment (Isaksson, 2015).

Stress from the urban environment, such as air-, light-, and noise pollution, put city dwellers under considerable stress. This has repeatedly been shown to result in changes in traits with potential fitness links, e.g., behavior, morphology, and reproductive investment (Gorissen et al., 2005; Isaksson et al., 2005; Fuller et al., 2007; Kempenaers et al., 2010). Likewise, several molecular and physiological parameters are also affected by the urban environment, including altered gene expression, endocrine changes, increased oxidative stress, and accelerated telomere attrition (Partecke et al., 2006; Isaksson, 2010; Atwell et al., 2012; Dominoni et al., 2013; Salmón et al., 2016; Watson et al., 2017). Such changes to blood- and cell chemistry may be exacerbated by a suboptimal diet in urban populations (Romieu et al., 2008; Isaksson et al., 2011). For example, while species in their natural environments have evolved unique feeding niches, interspecific dietary differences tend to converge in urban areas due to superabundance of human-provided food items (e.g., sunflower seeds, peanuts, bread, tallow, coconut fat, and leftovers; Jones and Reynolds, 2008). Such food items have a different, potentially poorer, nutrient composition compared to natural food sources (Schoech and Bowman, 2003; Schoech et al., 2004; Jones and Reynolds, 2008; Andersson et al., 2015). Reliance on (exploiters), or high intake of (adapters), human-provided food may therefore further reduce individual health and reproductive success in urban birds. Moreover, natural food sources in urban areas can also be of poorer quality than those in rural areas, which contributes to the lower availability of important macro- and micro-nutrients in cities (Isaksson and Andersson, 2007; Møller et al., 2010; Andersson et al., 2015; Isaksson et al., 2015).

The composition of fatty acids (FAs) has been suggested to differ between anthropogenic and natural food sources (Andersson et al., 2015), but FA variation has rarely been investigated in this context. FAs are obtained *via* dietary sources, or by *de novo* biosynthesis. However, two polyunsaturated fatty acids (PUFAs)—the omega (ω)-3 PUFA α-linolenic acid (α-LNA) and the ω-6 PUFA linoleic acid (LA)—are strictly dietary (i.e., essential) for all birds. These FAs can be elongated to long-chained, highly unsaturated PUFAs. These are especially relevant to study in the urbanization context due to their involvement in the regulation of physiological processes, such as cell membrane fluidity and transmembrane molecular movements, cardiac function, brain development, and immune responses (e.g., Hazel, 1995; Larsson et al., 2004; Pierce et al., 2005; Ben-Hamo et al., 2011; Hulbert and Abbott, 2012). Indeed, a recent study on great tits (*Parus major*) demonstrated that genes encoding enzymes responsible for biosynthesis of long-chain PUFAs were differentially expressed between urban and rural individuals, although the factors underlying this habitat-specific difference, and the resulting physiological consequences, were not determined (Watson et al., 2017). Moreover, in birds, the dietary FA composition can affect several aspects of performance (Twining et al., 2016). From a health perspective, the composition of ω-6 and ω-3 PUFAs is particularly interesting, given their generally opposing effects on inflammatory responses and oxidative stress (Larsson et al., 2004; Romieu et al., 2008; Isaksson, 2015). Accordingly, a high total ω-6/ω-3 PUFA ratio is associated with increased sensitivity to antigens by promoting inflammation (e.g., Calder, 2007, 2009; Romieu et al., 2008). In mammals, a ratio above 3 is considered to induce not only inflammation, but also pro-oxidant production, thereby potentially further increasing oxidative stress in urban-dwelling animals (Simopoulos, 2002; Kiecolt-Glasera et al., 2013). Because the immune system responds to pollution (Romieu et al., 2008; Isaksson, 2015, and references therein), the dietary FA composition, and resultant FA compositions of blood and tissues (Austin, 1993; Pierce et al., 2005; McCue et al., 2009; Ben-Hamo et al., 2011), can play a significant role on birds' health in the urban environment. In addition, long-chain PUFAs are themselves sensitive to oxidative stress by being susceptible to lipid peroxidation.

The present study has two main aims. *Firstly*, we compared the plasma FA profiles of four common species, which falls into the "urban adapter" (great tit, *P. major*; blue tit, *Cyanistes caeruleus*) and "urban exploiter" (house sparrow, *Passer domesticus*; tree sparrow, *P. montanus*) categories, in urban and rural habitats. We predicted (1) that intraspecific FA profiles would differ between urban- and rural-dwelling individuals due to differences in diet composition. We also predicted (2) that the FA composition would show distinct family signals given pronounced diet differences between the exploiters and adapters, but that these signals would converge in the urban habitat due to a more homogenous diet availability. *Secondly*, we investigated whether biomarkers of health, viz. the total ω-6/ω-3 PUFA ratio, lipid peroxidation susceptibility index (peroxidation index, PI; Pamplona et al., 1998; Kang et al., 2004), and lipid peroxidation (malondialdehyde, MDA), differed between the urban and rural individuals of the four species (Gutteridge, 1995). We predicted (3) that urban birds should have a lower overall proportion of both ω-6 and ω-3 PUFAs, but due to the even lower availability of ω-3 PUFAs (compared to ω-6 PUFAs) in the urban habitat (Andersson et al., 2015; Toledo et al., 2016), we expected to find an increase in the ω-6/ω-3 PUFA ratio in urban birds. We also predicted (4) that the adapter species would show a more pronounced difference between the urban and rural environments, given that the exploiter species rely on human-provided foods also in the rural habitat. In addition, we predicted (5) that urban birds should have higher MDA due to higher environmentally induced oxidative stress (Isaksson, 2010). Furthermore, we predicted (6) that if a high ω-6/ω-3 PUFA ratio produces pro-oxidants *via* pro-inflammatory responses, a positive association between the ω-6/ω-3 PUFA ratio and MDA would be revealed in the urban, but not in the rural, environment. Similarly, we predicted (7) a positive association between the PI and the concentration of MDA in the pro-oxidative urban environment, but not in the rural environment.

MATERIALS AND METHODS

Study Species

All four species feed predominantly on nuts and seeds in winter. However, blue- and great tits maintain a proportion of arthropods (e.g., overwintering insects and spiders) in their winter diet, whereas tree- and house sparrows are largely granivorous at this time of the year. To varying degrees, all species exploit human-provided food sources, such as sunflower seeds, peanuts and bread (Jokimäki and Suhonen, 1998). For practical reasons, it was not possible to monitor individual or species-specific diet composition in the present study.

Field Sites and Sampling

The study was approved by the Malmö/Lund Ethical Committee for Animal Research under the Swedish Board of Agriculture (permit no. M454 12:1), and capture was performed under license from the Swedish Museum of Natural History (to CI, license no. 681). Fieldwork was performed in the province of Scania in southern Sweden from 15-Feb-2013 to 6-April-2013. Urban birds were caught at four locations within the city limits of Malmö, the largest city in southernmost Sweden with approximately 300,000 inhabitants (3,600 inhabitants/ km^2; study areas centered at 55°35′ N, 12°59′ E). Samples were collected either in city parks and recreational areas (tree sparrows, blue tits, great tits; two sites), on ruderal land with rich deciduous secondary growth (tree sparrows, blue tits, great tits; one site), or along hedgerows adjacent to granaries (house sparrows; one site). Rural birds were caught at seven locations 15–45 km ENE to NNE of Malmö (with most activity centered on 55°40′ N, 13°31′ E). Sampling was undertaken in deciduous forest stands on grassland (blue tits, great tits, tree sparrows; two sites), along hedgerows on rural farms (all four species; five sites), and in granaries on the same farms (house sparrows, tree sparrows; four of five sites). The rural areas were sparsely inhabited by humans (<5 inhabitants per km^2). Sampling took place throughout the day, from 6 a.m. to 7 p.m., at both urban and rural sites.

All species were caught with mist nets ($n_{\text{tot.}} = 243$ birds), but not all individuals could be measured for all physiological assays due to limited plasma volumes. The assay- and model-specific sample sizes are provided in **Table 1**, and in figure legends when relevant. All birds were individually ringed and (when plumage characteristics permitted) sexed (great tits, blue tits, house sparrows) according to Svensson (1992). Tree sparrows, which are sexually monomorphic, were molecularly sexed using the primers P2 and P8 (Griffiths et al., 1998). A blood sample (100–125 μl) was collected from the jugular vein with a heparinized syringe, and kept on ice until centrifugation (1,800 rpm for 10 min) and separation of blood plasma 0–1 h later. All samples were stored at −80°C until biochemical analyses.

Fatty Acid Extraction and Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

Fatty acids were extracted following the protocol described in Andersson et al. (2015). Briefly, a total lipid extraction of 5 μl plasma was performed for 1 h at room temperature using 50 μl chloroform:methanol (2:1 v/v). The solvent was then evaporated, and the lipid extracts were subjected to base methanolysis for conversion into corresponding fatty acid methyl esters (FAMEs), which were then extracted in *n*-hexane. Extracts were washed with H_2O and then dried over anhydrous sodium sulfate.

The FAME extracts were analyzed using an Agilent 5975 MS coupled to an Agilent 6890 GC equipped with an HP-88 capillary column [(88%-Cyanopropy)aryl-polysiloxane; 30 m, 0.25 mm id, d_f 0.20 μm; Agilent, CA, USA]. The oven temperature was set to 80°C for 1 min, then increased by 10°C/min to 230°C and held for 20 min. Helium was used as carrier gas at a constant flow of 1 ml/min. FAMEs present in plasma were identified by comparing their mass spectra and retention times with those of synthetic standards (Supelco 37-Component FAME Mix, Sigma-Aldrich, Stockholm, Sweden).

Lipid Peroxidation Quantification

We quantified malondialdehyde (MDA), the most frequently used biomarker of overall lipid peroxidation, following the protocol described in Eikenaar et al. (2016). Briefly, 15 μl plasma was mixed with 50 μl O– (2, 3, 4, 5, 6-pentafluorbenzyl) solution (1 mM in sodium acetate buffer, pH 5.0) and the micro-reaction was conducted for 1 h at room temperature. The resulting MDA-bis-(PFB-oxime) derivatives were extracted in 300 μl *n*-hexane containing 1.65 pg/μl of 3-bromofluorobenzene as internal standard, and analyzed using an Agilent 5975 MS coupled to an Agilent 6890 GC. A non-polar capillary column, HP-5MS (30 m, 0.25 mm id, d_f 0.25 μm; J&W Scientific, USA), was used for GC/MS analysis, and the characteristic ion at *m/z* 250 was measured under selected ion monitoring mode to quantify the target derivatives of MDA. The GC oven was programmed to 60°C for 1 min, followed by 15°C/min to 150°C, and then

TABLE 1 | Relative abundance of plasma fatty acids (% of total fatty acid content) in four passerine species from urban and rural habitats (mean values ± SEM).

			Blue tit		Great tit		House sparrow		Tree sparrow	
Common name	**C:D**	**FA group**	**Urban = 29**	**Rural = 29**	**Urban = 35**	**Rural = 32**	**Urban = 24**	**Rural = 25**	**Urban = 10**	**Rural = 42**
Myristic acid	14:0	SFA	0.16 ± 0.03	0.18 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	0.29 ± 0.03	0.43 ± 0.03	0.18 ± 0.03	0.23 ± 0.02
Palmitic acid	16:0	SFA	12.38 ± 0.34	13.65 ± 0.39	10.71 ± 0.22	10.67 ± 0.17	17.27 ± 0.91	22.41 ± 0.78	15.46 ± 1.00	17.19 ± 0.58
Stearic acid	18:0	SFA	15.82 ± 0.37	13.35 ± 0.39	15.53 ± 0.30	14.69 ± 0.31	13.41 ± 0.41	11.86 ± 0.33	13.73 ± 0.58	11.09 ± 0.26
Arachidic acid	20:0	SFA	0.19 ± 0.01	0.19 ± 0.01	0.22 ± 0.02	0.23 ± 0.02	0.14 ± 0.02	0.07 ± 0.01	0.18 ± 0.03	0.16 ± 0.02
Palmitoleic acid	16:1n-7	MUFA	0.12 ± 0.01	0.49 ± 0.11	0.08 ± 0.01	0.15 ± 0.02	1.15 ± 0.19	2.30 ± 0.16	0.28 ± 0.11	0.97 ± 0.14
cis-Vaccenic acid	18:1n-7	MUFA	0.57 ± 0.03	0.65 ± 0.04	0.60 ± 0.02	0.69 ± 0.02	0.62 ± 0.05	0.76 ± 0.06	0.47 ± 0.03	0.71 ± 0.04
Oleic acid	18:1n-9	MUFA	23.91 ± 1.22	20.33 ± 1.28	21.79 ± 1.24	19.04 ± 0.67	20.21 ± 1.44	26.28 ± 0.87	15.28 ± 1.48	19.65 ± 1.14
Eicosenoic acid	20:1n-9	MUFA	0.42 ± 0.04	0.39 ± 0.05	0.42 ± 0.04	0.40 ± 0.05	0.20 ± 0.02	0.20 ± 0.01	0.22 ± 0.03	0.15 ± 0.01
αLNA	18:3n-3	ω-3 PUFA	0.22 ± 0.04	1.39 ± 0.21	0.11 ± 0.01	0.29 ± 0.06	0.67 ± 0.15	0.20 ± 0.02	1.22 ± 0.31	0.63 ± 0.11
EPA	20:5n-3	ω-3 PUFA	0.14 ± 0.01	0.65 ± 0.14	0.10 ± 0.01	0.29 ± 0.05	0.26 ± 0.05	0.13 ± 0.02	0.33 ± 0.05	0.17 ± 0.02
DPA	22:5n-3	ω-3 PUFA	0.16 ± 0.01	0.32 ± 0.04	0.14 ± 0.01	0.20 ± 0.02	0.11 ± 0.01	0.08 ± 0.01	0.27 ± 0.03	0.14 ± 0.02
DHA	22:6n-3	ω-3 PUFA	1.17 ± 0.12	1.35 ± 0.12	1.02 ± 0.08	0.98 ± 0.08	1.69 ± 0.11	1.57 ± 0.10	2.63 ± 0.20	1.87 ± 0.15
LA	18:2n-6	ω-6 PUFA	14.59 ± 0.59	16.74 ± 0.74	16.03 ± 0.55	18.27 ± 0.54	15.34 ± 1.09	10.31 ± 0.65	17.93 ± 1.05	17.44 ± 1.10
γ-Linolenic acid	18:3n-6	ω-6 PUFA	0.08 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.02	0.08 ± 0.11	0.20 ± 0.03	0.22 ± 0.02
Eicosadienoic acid	20:2n-6	ω-6 PUFA	0.06 ± 0.00	0.09 ± 0.01	0.08 ± 0.00	0.09 ± 0.00	0.14 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.00
DGLA	20:3n-6	ω-6 PUFA	0.36 ± 0.04	0.52 ± 0.04	0.53 ± 0.03	0.52 ± 0.05	0.85 ± 0.07	0.94 ± 0.06	0.61 ± 0.06	0.60 ± 0.04
AA	20:4n-6	ω-6 PUFA	14.85 ± 0.60	12.71 ± 0.80	16.14 ± 0.67	14.79 ± 0.68	11.83 ± 0.61	11.40 ± 0.36	12.78 ± 0.82	10.94 ± 0.60
Adrenic acid	22:4n-6	ω-6 PUFA	0.17 ± 0.01	0.14 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.21 ± 0.01	0.25 ± 0.01	0.15 ± 0.02	0.20 ± 0.01
Mead acid	20:3n-9	ω-9 PUFA	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.13 ± 0.02	0.31 ± 0.04	0.03 ± 0.01	0.11 ± 0.02
	totSFA		28.56 ± 0.40	27.38 ± 0.42	26.58 ± 0.30	25.71 ± 0.32	31.12 ± 0.74	34.76 ± 0.67	29.56 ± 1.04	28.67 ± 0.50
	totMUFA		25.01 ± 1.24	21.87 ± 1.32	22.90 ± 1.27	20.27 ± 0.70	22.17 ± 1.64	29.54 ± 1.01	16.25 ± 1.58	21.46 ± 1.31
	totPUFA		31.84 ± 0.72	34.02 ± 0.85	34.49 ± 0.83	35.75 ± 0.56	31.37 ± 1.16	25.37 ± 0.82	36.26 ± 1.33	32.42 ± 0.73
	ω-6 PUFA		30.11 ± 0.72	30.28 ± 0.76	33.10 ± 0.80	33.98 ± 0.54	28.51 ± 1.21	23.10 ± 0.85	31.78 ± 1.07	29.51 ± 0.70
	ω-3 PUFA		1.69 ± 0.16	3.71 ± 0.38	1.37 ± 0.10	1.76 ± 0.16	2.73 ± 0.19	1.98 ± 0.11	4.45 ± 0.50	2.81 ± 0.21
	ω-6/ω-3 PUFA		21.12 ± 1.66	11.90 ± 1.59	28.10 ± 1.96	23.89 ± 1.85	12.26 ± 1.52	12.74 ± 1.05	8.34 ± 1.42	15.33 ± 2.04

C:D, number of carbon atoms:double bonds; AA, arachidonic acid; αLNA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; DGLA, dihomo-γ-linolenic acid. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; ω-3 PUFA, ω-3 polyunsaturated fatty acid; ω-6 PUFA, ω-6 polyunsaturated fatty acid.

10°C/min to 270°C, which was held for 5 min. All chemicals were purchased from Sigma-Aldrich (Stockholm, Sweden).

Data Handling and Statistical Analyses

In total, 19 FAs were identified and quantified in all species (**Table 1**). The proportion of each FA was calculated by dividing each peak area with the sum of all FA peak areas in each individual. All FA proportions were logit-transformed ($\log(y/[1-y])$), prior to statistical analyses (Warton and Hui, 2011). Individual saturated fatty acids, SFAs (i.e., $\sum \text{tot}[\text{SFA}] = 14{:}0 + 16{:}0 + 18{:}0 + 20{:}0$) were pooled, because they are all mainly used as metabolic fuel, in contrast to mono- and polyunsaturated FAs (i.e., MUFAs and PUFAs) that typically have specific biological functions.

Firstly, general linear models (GLMs) were performed on nineteen variables; fifteen individual FAs, $_{\text{tot}}$SFA, total ω-6/ω-3 PUFA ratio, peroxidation index (PI), and MDA. The PI was calculated as PI = [(% monoenoic × 0.025) + (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 4) + (% pentaenoic × 6) + (% hexaenoic × 8)] (Pamplona et al., 1998; Kang et al., 2004). PI takes into account that the sensitivity to peroxidation increases as a power function of the number of double bonds in each FA molecule (Pamplona et al., 2000); in other words, the PI provides an estimate of oxidative stress susceptibility in terms of lipid peroxidation. Apart from the GLMs for MDA (see below), all models included two fixed factors: "species" (four levels) and "habitat" (two levels), along with their interaction (species × habitat). In addition, our previous work on great tits has shown that sampling hour affects the proportion of FAs (Isaksson et al., 2015). Therefore, "sampling time" was always included as a covariate in these GLMs. Furthermore, when the interaction between species and habitat was significant in the above model, we proceeded by fitting species-specific GLMs, including time as a covariate. When the main effect of species or habitat was significant, but the interaction between them non-significant, we proceeded by traditional *post-hoc* analyses (Student's *t*-test), and removed the interaction from the final model (Supplementary Material 1). There were no sex differences among any of the FAs (ANOVAs: $F = 0.0002$–2.237, $p = 0.14$–0.96). Accordingly, sex was excluded from all FA models.

Secondly, multivariate canonical discriminant analysis (CDA) was performed to complement the GLMs for the individual FAs. Here, the proportions of each unsaturated FA along with the proportions of $_{\text{tot}}$SFA were included, and the analysis performed both for each habitat separately, and with habitats combined, with species as a categorical variable. These analyses allowed us to test whether the FA profiles showed distinct species- and family (exploiter vs. adapter) signals, and whether these signals were less pronounced in the urban environment due to expected dietary convergence (i.e., reliance on human-provided food sources). The CDA predicts an individual's classification in a category, i.e., species/family, based on observed values of several continuous variables (here, the FAs). A quadratic discriminant method was used, because the covariances between FAs were not expected to be equal across species.

Lastly, MDA was $\log_{10}$-transformed to obtain a Gaussian distribution. The GLM with MDA included the three fixed factors "species," "habitat," and "sex," and the three covariates "sampling hour," "PI" and the "total ω-6/ω-3 PUFA ratio." In addition, we included all possible two-way interactions. For MDA, we derived final models using backward-elimination of non-significant ($P > 0.05$) interactions and covariates, starting with the least significant interaction followed by the covariates.

All analyses were performed in JMP Pro 11.0 (2013 SAS Institute Inc.). For clarity, we only present significant results in the Results Section [see Supplementary Materials 1–3 for all GLMs and species-specific models for FA, FA-derived indices (PI and total ω-6/ω-3 PUFA ratio), and MDA].

RESULTS

Habitat Differences in FA Profiles

Total SFA ($_{\text{tot}}$SFA) and MUFA

There was a significant interaction between habitat and species for $_{\text{tot}}$SFA [$F_{(3,\,217)} = 8.37$, $P < 0.001$]. Specifically, urban house sparrows had lower proportions of $_{\text{tot}}$SFA than their rural conspecifics [$F_{(1,\,46)} = 12.39$, $P = 0.001$], but $_{\text{tot}}$SFA did not differ between urban and rural populations of the other species (**Figure 1A**). Oleic acid was by far the most abundant MUFA in all species (**Table 1**, **Figure 1B**), although proportions differed for the species in the different habitats [$F_{(3,\,217)} = 6.28$, $P < 0.001$]. Urban blue tits had a higher proportion of oleic acid compared to their rural conspecifics [$F_{(1,\,55)} = 6.20$, $P = 0.016$], whereas the opposite pattern was true for house sparrows [$F_{(1,\,46)} = 13.97$, $P < 0.001$] and tree sparrows [$F_{(1,\,51)} = 5.46$, $P = 0.024$]. Habitat-wise differences in oleic acid for great tits tended to follow those of blue tits ($P = 0.057$; **Figure 1B** and Supplementary Materials 1, 2). Two other MUFAs showed significant habitat effects [palmitoleic acid: $F_{(1,\,220)} = 46.36$, $P < 0.001$; and *cis*-vaccenic acid: $F_{(1,\,220)} = 21.81$, $P < 0.001$; **Figures 1C,D**], whereas habitat was non-significant for eicosenoic acid (**Figure 1E**). Across all species, palmitoleic- and *cis*-vaccenic acid were both higher in the rural habitat than in the urban habitat ($P < 0.05$ in all cases; **Figures 1C,D**).

ω-3 PUFA

All statistical models for ω-3 PUFAs revealed significant interactions between habitat and species [α-LNA: $F_{(3,\,217)} = 18.58$, $P < 0.001$; eicosapentaenoic acid (EPA): $F_{(3,\,217)} = 13.59$, $P < 0.001$; docosapentaenoic acid (DPA): $F_{(3,\,216)} = 13.88$, $P < 0.001$; docosahexaenoic acid [DHA]: $F_{(3,217)} = 4.69$, $P = 0.003$; **Figures 2A–D**]. Species-specific habitat models revealed that all ω-3 PUFAs, except DHA, in blue- and great tits were higher in the rural compared to the urban habitat [blue tit: α-LNA: $F_{(1,\,57)} = 41.56$, $P < 0.001$; EPA: $F_{(1,\,57)} = 24.73$, $P < 0.001$; DPA: $F_{(1,\,57)} = 15.49$, $P < 0.001$; great tit: α-LNA: $F_{(1,\,66)} = 5.68$, $P = 0.020$; EPA: $F_{(1,\,66)} = 19.97$, $P < 0.001$; DPA: $F_{(1,\,66)} = 9.96$, $P = 0.002$]. Both sparrow species showed the opposite pattern, with higher proportions of ω-3 PUFAs in the urban compared to the rural habitat [house sparrow: α-LNA: $F_{(1,48)} = 11.84$, $P = 0.001$; DPA:

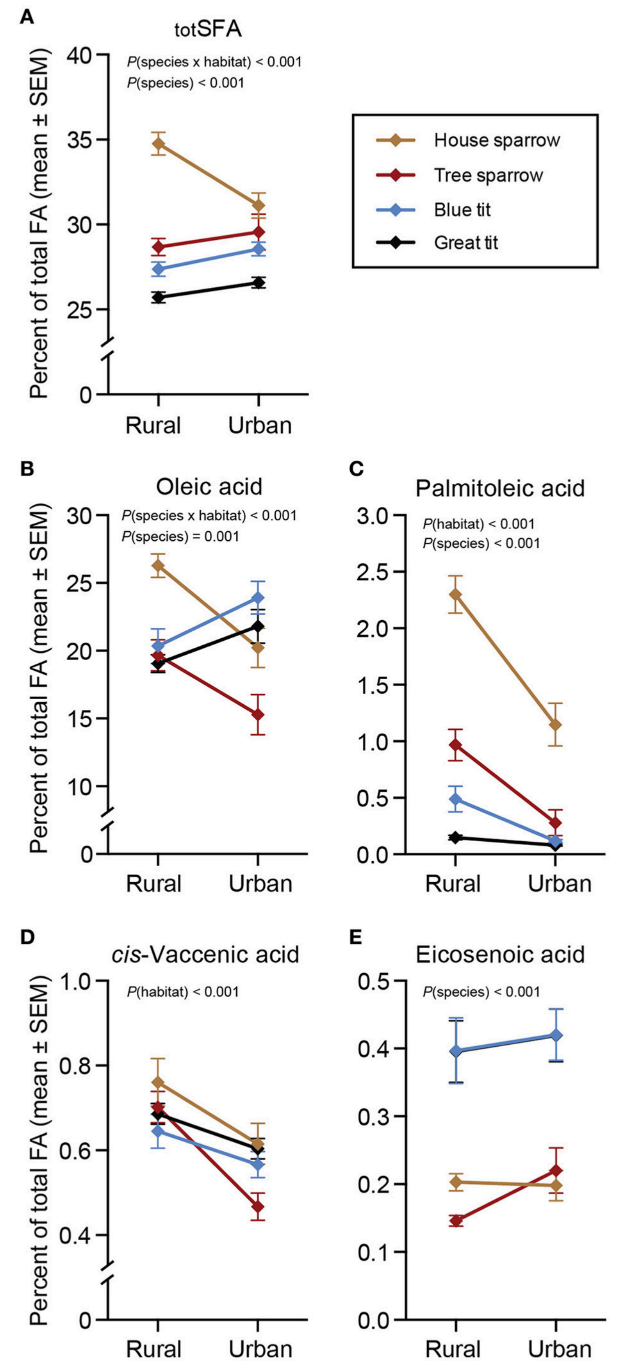

FIGURE 1 | Plasma saturated- and monounsaturated fatty acids (%) in four species of passerines from urban and rural environments. (A) Total saturated fatty acids ($_{tot}$SFA), **(B–E)** four monounsaturated fatty acids (MUFA). Data are presented as means ± standard error of the means (SEM). Note the different scales on the *y*-axes. Significant effects of species, habitat and/or the habitat × species interaction are indicated by the *P*-values. For *n*-values see **Table 1**.

$F_{(1,\,48)} = 4.24$, $P = 0.045$; tree sparrow: α-LNA: $F_{(1,\,51)} = 6.67$, $P = 0.013$; EPA: $F_{(1,\,51)} = 5.22$; $P = 0.027$; DPA: $F_{(1,51)} = 7.88$, $P = 0.007$; DHA: $F_{(1,\,51)} = 5.04$, $P = 0.029$].

ω-6 PUFA

There were significant interactions between habitat and species for all ω-6 PUFAs, except arachidonic acid [LA: $F_{(3,\,217)} = 7.97$, $P < 0.001$; γ-linolenic acid (γ-LNA): $F_{(3,\,217)} = 3.18$, $P = 0.025$; eicosadienoic acid: $F_{(3,\,217)} = 13.71$, $P < 0.001$; dihomo-γ-linolenic acid (DGLA): $F_{(3,\,217)} = 3.00$, $P = 0.032$; adrenic acid: $F_{(3,\,217)} = 4.54$, $P = 0.004$; **Figures 2E–J**]. Urban blue tits had lower eicosadienoic acid [$F_{(1,\,57)} = 20.90$, $P < 0.001$] and DGLA [$F_{(1,\,57)} = 5.73$, $P = 0.020$], and urban great tits had lower LA [$F_{(1,\,66)} = 4.52$, $P = 0.037$] and eicosadienoic acid [$F_{(1,\,66)} = 8.00$, $P = 0.006$] compared to their rural conspecifics. Interestingly, house sparrows showed the opposite result with urban individuals having higher LA [$F_{(1,\,48)} = 20.77$, $P = 0.001$], γ-LNA [$F_{(1,\,48)} = 5.71$, $P = 0.021$], and eicosadienoic acid [$F_{(1,\,48)} = 8.72$, $P = 0.005$]. Similar to urban blue tits, however, urban house sparrows had lower DGLA [$F_{(1,\,48)} = 6.27$, $P = 0.016$] than the rural ones. Finally, urban house- and tree sparrows had lower proportions of adrenic acid [house sparrows: $F_{(1,\,48)} = 11.33$, $P = 0.002$; tree sparrows: $F_{(1,\,51)} = 12.99$, $P < 0.001$] compared to their rural conspecifics.

ω-9 PUFA

There was a significant interaction between species and habitat for mead acid [$F_{(3,\,217)} = 3.45$, $P = 0.017$; **Figure 2K**]. The species-specific models revealed that urban sparrows had lower mead acid proportions than rural sparrows [house sparrow: $F_{(1,\,48)} = 13.99$, $P < 0.001$; tree sparrow: $F_{(1,\,51)} = 4.74$, $P = 0.034$].

Species Differences in FA Profiles

The CDA analyses revealed that the first three discriminant functions significantly discriminated the four species in both urban and rural habitats [urban: $P < 0.001$, eigenvalues: Canonical 1 (C1) = 10.325, C2 = 1.091, C3 = 0.611; **Figure 3A**; rural: $P < 0.001$, eigenvalues: C1 = 11.808, C2 = 1.103, C3 = 0.743; **Figure 3B**]. In the urban habitat, C1 accounted for 85.85%, C2 for 9.07%, and C3 for 5.08% of the variation [Wilk's lambda: 0.026, $F_{(48,\,233)} = 11.65$, $P < 0.001$]. In the rural habitat, C1 accounted for 86.47%, C2 for 8.083% and C3 for 5.45% of the variation [Wilk's lambda: 0.021, $F_{(48,\,325)} = 17.93$, $P < 0.0001$]. 98.97 and 99.22% of the individuals were correctly assigned to the pre-determined groups (species) in the urban and rural habitat, respectively. When birds from the two habitats were analyzed together the correct species assignment was reduced to 93.34%, but the model was still highly significant [Wilk's lambda: 0.044, $F_{(48,\,613)} = 23.80$, $P < 0.001$; **Figure 3C**]. Most misclassified individuals were still assigned to the correct family, with only one blue tit misclassified as a tree sparrow. Similarly, there was a clear distinction between the two families (adapters vs. exploiters), despite the significant species signals [Wilk's lambda: 0.118, $F_{(16,\,208)} = 97.02$, $P < 0.001$].

In the full-species GLMs, there was a clear effect of the main factor "species" for two of the MUFAs: palmitoleic acid [$F_{(3,\,220)} = 54.11$, $P < 0.001$] and eicosenoic acid [$F_{(3,\,220)} = 25.55$, $P < 0.001$]. The *post-hoc* analyses revealed that specifically palmitoleic acid was significantly higher in house sparrows than in any of the other species, and eicosenoic acid was

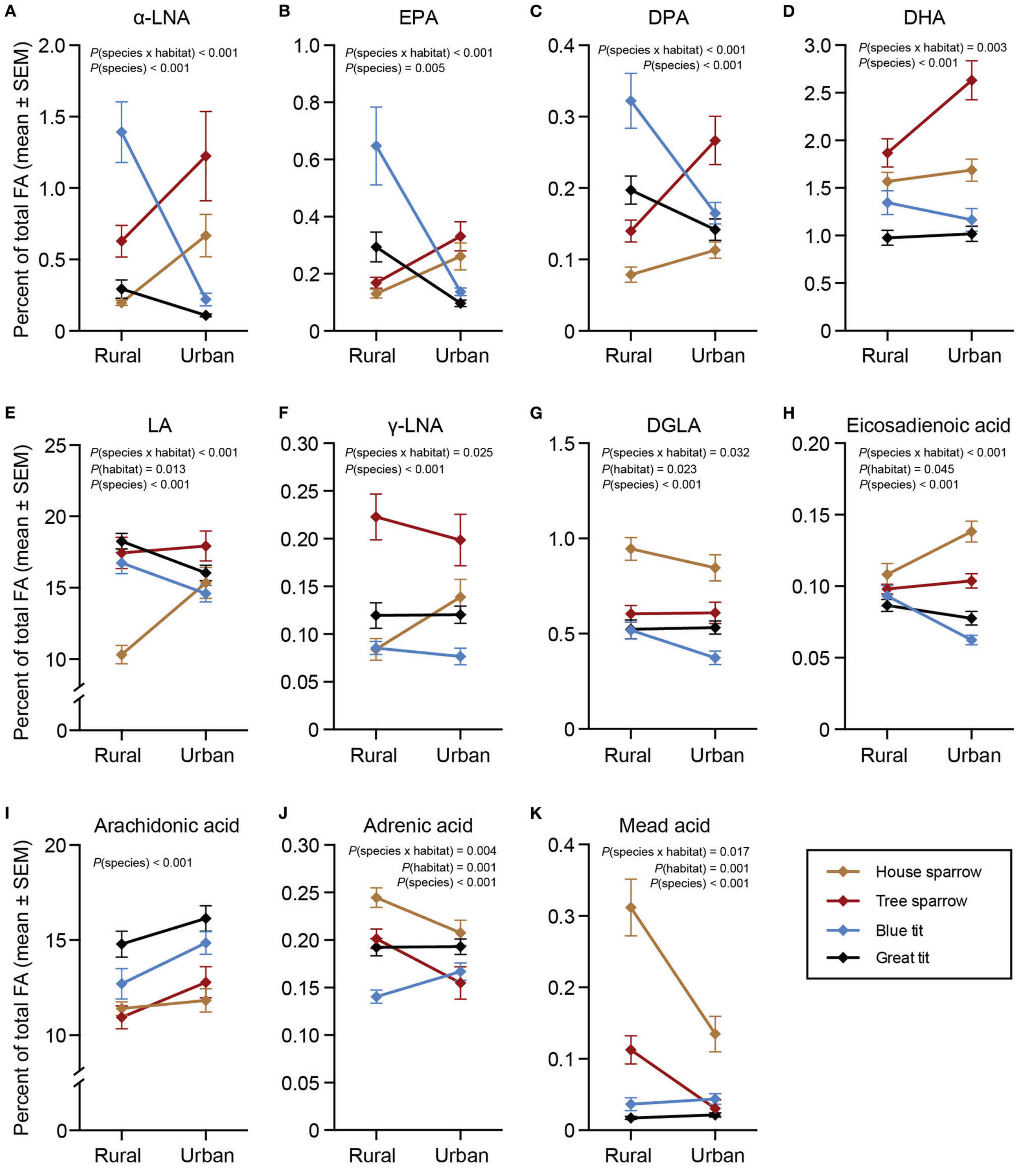

FIGURE 2 | Plasma polyunsaturated fatty acids (%) in four species of passerines from urban and rural environments. (A–D) ω-3 polyunsaturated fatty acids (PUFAs), **(E–J)** ω-6 PUFAs, and **(K)** ω-9 PUFA. Data are plotted as means ± standard error of the means (SEM). Note the different scales on the *y*-axes. Significant effects of species, habitat and/or the habitat × species interaction are indicated by the *P*-values. For *n*-values see **Table 1**. One urban blue tit did not have detectable levels of DGLA, thus the sample size was reduced from 29 to 28. α-LNA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid, DHA, docosahexaenoic acid; LA, linoleic acid; γ-LNA, γ-linolenic acid; DGLA, dihomo-γ- linolenic acid.

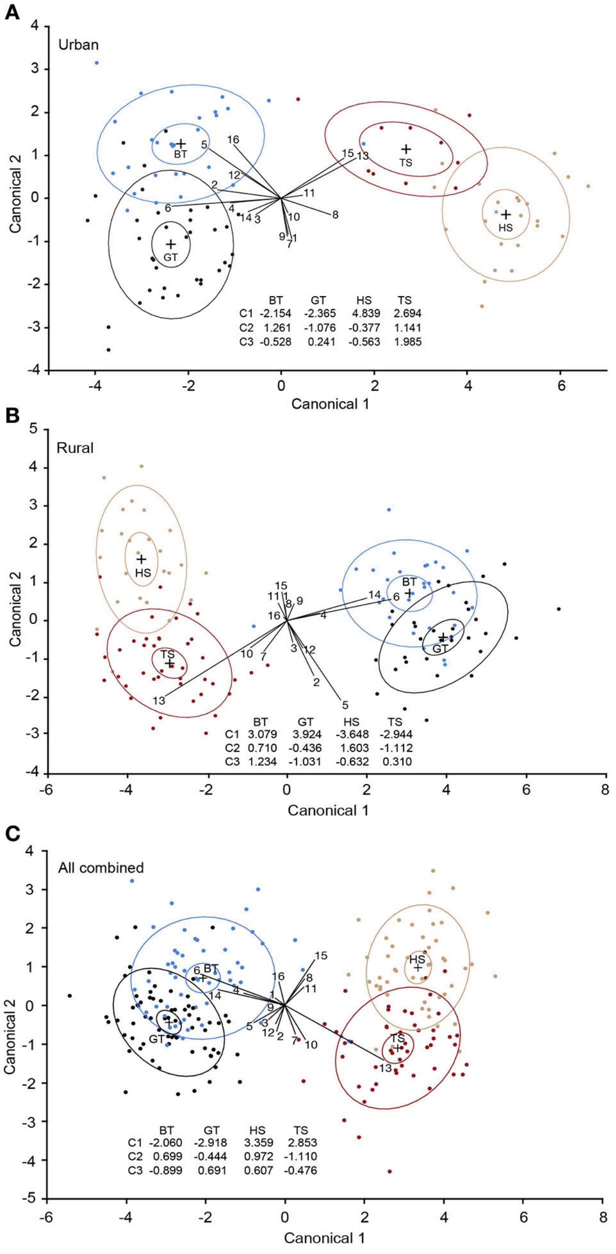

FIGURE 3 | Interspecific comparison of overall plasma fatty acid compositions in four passerine species. (A) Urban birds, **(B)** rural birds, and **(C)** urban and rural birds combined. The plots are based on the first (C1) and the second (C2) discriminant functions estimated from canonical discriminant analyses (CDA) using 15 individual fatty acids (FAs) along with total saturated fatty acids. Although the figure is two-dimensional, the CDA model is based on FA loadings of three functions, hence all three discriminant functions are presented. The ellipses represent 95% of the data clouds of each species. BT, blue tit (blue); GT, great tit (black); HS, house sparrow (tan); TS, tree sparrow (dark red). 1, palmitoleic acid; 2, oleic acid; 3, *cis*-vaccenic acid; 4, eicosenoic acid; 5, linoleic acid; 6, arachidonic acid; 7, γ-linolenic acid; 8, eicosadienoic acid; 9, dihomo-γ- linolenic acid; 10, adrenic acid; 11, α-linolenic acid; 12, eicosapentaenoic acid; 13, docosahexaenoic acid; 14, docosapentaenoic acid; 15, mead acid; 16, total saturated fatty acids.

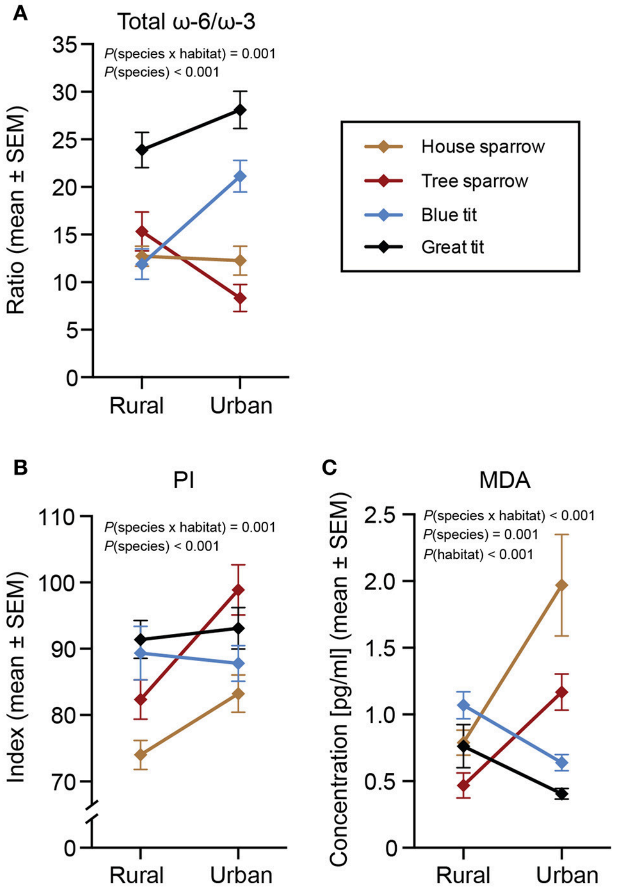

FIGURE 4 | Biomarkers of health in four species of passerines from urban and rural environments. (A) Total ω-6/ω-3 polyunsaturated fatty acids (PUFA) ratio, **(B)** peroxidation index (PI), and **(C)** lipid peroxidation (MDA). Data are plotted as means ± standard error of the means (SEM). Note the different scales on the *y*-axes. Significant effects of species, habitat and/or the habitat × species interaction are indicated by the *P*-values. The *n*-values for **(A,B)** are presented in **Table 1**. The *n*-values for MDA **(C)** were: house $\text{sparrow}_{\text{urban}} = 24$, house $\text{sparrow}_{\text{rural}} = 24$; tree $\text{sparrow}_{\text{urban}} = 12$, tree $\text{sparrow}_{\text{rural}} = 44$; blue $\text{tit}_{\text{urban}} = 32$, blue $\text{tit}_{\text{rural}} = 30$; great $\text{tit}_{\text{urban}} = 34$, great $\text{tit}_{\text{rural}} = 29$.

significantly higher in the two tit species than in the sparrows ($P < 0.05$ in all cases; **Figures 1C,E**). Finally, the ω-6 PUFA, arachidonic acid, differed significantly between species [$F_{(3,\ 220)} = 35.90$, $P < 0.001$], with the two tit species having higher proportions than the two sparrow species ($P < 0.05$ in all cases; **Figure 2I**).

Habitat Differences in Health Markers

ω-6/ω-3 PUFA Ratio

The total ω-6/ω-3 PUFA ratio showed a significant habitat by species interaction [$F_{(3,\ 217)} = 6.04$, $P < 0.001$; **Figure 4A**]. Species-specific analyses revealed that only the blue tit differed between the urban and rural habitats, with rural birds having a lower ratio compared to urban birds [$F_{(1,\ 57)} = 13.84$, $P < 0.001$].

Peroxidation Index (PI)

The interaction between habitat and species was significant also for PI [$F_{(3,\,217)} = 5.54$, $P = 0.001$; **Figure 4B**]. Specifically, the PI was higher for urban house sparrows [$F_{(1,\,48)} = 4.80$, $P = 0.034$] and tree sparrows [$F_{(1,\,51)} = 6.86$, $P = 0.012$] than for their rural conspecifics, whereas there was no significant inter-habitat variation in the two tit species.

Malondialdehyde (MDA)

There was a significant interaction between habitat and species also for MDA [$F_{(3,\,205)} = 26.29$, $P < 0.001$; **Figure 4C**]. Urban exploiters (i.e., sparrows) of both species had higher MDA in the city compared to conspecifics in the rural habitats [house sparrow: $F_{(1,\,47)} = 12.27$, $P = 0.001$; tree sparrow: $F_{(1,\,55)} = 13.42$, $P < 0.001$], whereas urban blue tits had lower MDA than their rural conspecifics [$F_{(1,\,61)} = 11.97$, $P = 0.001$; note that great tits showed a similar tendency: $F_{(1,\,62)} = 3.60$, $P = 0.062$]. In addition, MDA was affected by the interaction between PI and species [$F_{(3,\,205)} = 13.32$, $P < 0.001$], suggesting a negative association between MDA and peroxidizable FAs in tree sparrows, whereas no association was found for the other species. Likewise, we found no support for our prediction about habitat-specific relationships between MDA, PI, and the ω-6/ω-3 PUFA ratio (Supplementary Material 3). Finally, males had higher MDA than females across species [$F_{(1,\,205)} = 6.44$, $P = 0.012$].

DISCUSSION

In line with our predictions, we found large inter-habitat and inter-specific differences in several FAs, often varying in opposing directions for adapters and exploiters. Secondly, the discriminant analyses revealed species- and family-specific FA profiles, suggesting that FA compositions could be informative when investigating inter- and intraspecific variation in nutrition and lipid metabolism. However, in contrast to our prediction, there was no consistent convergence of FA profiles of the four species in the urban habitat (i.e., due to expected dietary similarity). Thirdly, significant interactions between habitat and species were revealed for all three health indices. Urban blue tits had higher total ω-6/ω-3 PUFA ratio than their rural conspecifics, which follows our prediction about the lower availability of ω-3 PUFA in the urban habitat. MDA was higher in the urban than in rural individuals for the two exploiter species, whereas the adapter species showed the opposite pattern. In the exploiters, the peroxidation index (PI) increased from rural to urban, but this was not the case for the adapters. Taken together, the effects of the urban environment on health indices largely followed our general predictions. However, the opposing effects between the adapter and exploiter species were striking and surprising, which we discuss below.

Habitat Differences in Fatty Acid Profiles

The exploiter and adapter species often showed opposite differences in plasma FA composition between the urban and rural environments (**Figures 1**, **2**). For instance, urban blue tits had higher proportions of the main MUFA oleic acid (great tits showed a similar tendency), whereas both urban house- and tree sparrows had lower oleic acid than their rural counterparts. For $_{tot}$SFA, a habitat difference was only found in house sparrows, with urban individuals having lower $_{tot}$SFA than the rural conspecifics. Since both SFAs and MUFAs can be biosynthesized by birds, their relative levels can be controlled to a larger extent than the levels of PUFAs. Thus, the results for $_{tot}$SFA and MUFA are likely due to a combination of metabolic regulation, and habitat- and species-specific diets.

Moreover, urban exploiters had lower proportions of several ω-3 PUFAs, whereas urban adapters had higher proportions than their rural conspecifics. Differences between the habitats in plasma levels of the essential ω-3 PUFA α-LNA should be a direct reflection of dietary differences. This could be a result of either different food items being eaten/or available across the habitats, or habitat-specific differences in fatty acid composition of the same food items. Our previous diet experiments on great tits showed that the proportion of all ω-3 PUFA in plasma is highly affected by availability in diet, with plasma amounts rapidly declining (within a few days) after restricting ω-3 PUFAs in the diet (Andersson et al., unpublished). Thus, the results also for the other ω-3 PUFAs in the present study are likely to be at least partly affected by dietary differences across the habitats. In addition, it is also possible that there are species/family-specific effects on metabolic conversion across habitats (Jing et al., 2013; Watson et al., 2017). Regardless, the lower ω-3 PUFA of rural house- and tree sparrows compared to urban ones indicates a "poorer" nutritional status at the rural farms, rather than in the urban habitat. In support of this argument, mead acid—a suggested biomarker of malnutrition or deficiency of essential FAs (Mead and Slaton, 1956; Smit et al., 2004)—was higher in the rural house- and tree sparrows than in the urban conspecifics. Furthermore, the strictly dietary ω-6 PUFA LA was lower for urban great tits, but higher for urban house sparrows compared to their rural conspecifics. Such a contrasting effect of habitat was also evident for eicosadienoic acid, with both adapters having lower levels in the urban habitat, whereas urban house sparrows had higher levels than rural ones. Whether these interspecific differences have any adaptive values in the urban-rural context requires further investigation.

Species and Family Differences in Fatty Acid Profiles

The present study is the first to investigate interspecific differences in FA profiles among terrestrial bird species. Previously, a similar approach for FA profiles was used on pelagic seabirds in relation to their feeding ecology (Iverson et al., 2007). In that study, a captive feeding experiment was conducted along with sampling of adipose tissue in four species of seabirds and their natural prey. Distinct species-specific discrimination of FA signatures was evident, which could be explained by interspecific differences in diet (Iverson et al., 2007). It was suggested that species FA signatures could be a complement or even an alternative to stable isotope analyses, because FA profiles can provide more information about the diet composition and not simply indicate trophic levels. Although the current study cannot link the plasma FA content with the FA profile of specific food items, it supports the previous conclusions given the clear species and family distinction in FA profiles.

More specifically, our CDA revealed that the two bird families (or the adapter vs. exploiter categorization) were separated by the first canonical function (C1) (**Figure 3**). The separation between adapters and exploiters could be a combined result of shared evolutionary history, as well as similar life-styles and diets among the two species within each group. Unfortunately, the relative contribution of each of these factors could not be determined. However, despite the resemblance between the species of the same taxonomic family, and the variation in FAs in birds across the habitats, the four species were also discriminated by the FA loadings on C2 and C3 both within each habitat and across habitats. Overall, 99% of the birds were successfully assigned into the correct species within each habitat, and although the correctness of species classification was reduced when birds from the two habitats were combined, it was still high (93%). Interestingly, both α-LNA and mead acid signified house sparrows with a decrease in α-LNA and an increase in mead acid likely indicating malnutrition or nutrient deficiency (Mead and Slaton, 1956; Smit et al., 2004). The effect of mead acid was only present in the rural habitat, which is in line with the suggestion that the rural house sparrows are more malnourished than urban conspecifics (see above).

Species and Habitat Differences in Health Markers

Despite the interspecific differences across the urban-rural habitats in several ω-3 and ω-6 PUFAs, only the blue tit differed in the total ω-6/ω-3 ratio between the habitats. As predicted, the urban blue tits had a higher ratio than the rural blue tits, suggesting that urban birds have a diet-induced increased susceptibility to inflammation when exposed to antigens, such as traffic-generated particulate matter and pathogens (Ezenwa, 2004; Romieu et al., 2008; Isaksson, 2015). Since non-pathogenic inflammatory agents are more common in the urban environment, future studies should investigate whether urban blue tits have a higher inflammatory response than their rural conspecifics, and whether this could be explained by their higher ω-6/ω-3 PUFA ratio. However, at the species level, great tits had the highest ratio of all species across habitats (**Figure 4A**). Thus, also great tits are likely to suffer from increased, dietary induced, negative effects, if exposed to pro-inflammatory agents, such as urban air pollution.

MDA is a commonly used biomarker for lipid peroxidation (i.e., oxidative damage) that is generated by pro-oxidant exposure (e.g., Del Rio et al., 2005; Lykkesfeldt, 2007). The two exploiter species followed our prediction, with higher MDA in the urban habitat as compared to the rural habitat. Surprisingly, blue tits had lower MDA in the urban compared to the rural environment. Previous studies on lipid peroxidation in birds have not detected a difference between urban and rural populations (great tits in Gothenburg: Isaksson et al., 2009; house sparrows in Madrid: Herrera-Dueñas et al., 2014). Possibly, our more sensitive quantification of MDA using GC/MS, compared to the absorbance measurements used in the previous studies (thiobarbituric acid [TBARS] assays), is able to more accurately detect smaller differences in lipid peroxidation (Del Rio et al., 2005). Alternatively, lipid peroxidation may be more context-, nutrition-, and species-specific than previously appreciated, particularly since it exhibited the opposite patterns in the two bird families. In support of this argument, both urban exploiter species also showed higher susceptibility to lipid peroxidation, as indicated by the higher PI, than rural birds. This result might be a consequence of population differences in dietary intake or biosynthesis of the long-chain, peroxidation prone, PUFAs, especially the highly unsaturated DHA.

Moreover, due to high levels of traffic-generated air pollution (acting as pro-oxidants) in the urban habitat, we predicted that MDA levels in urban birds would be positively associated with the ω-6/ω-3 PUFA ratio (via increased ROS production during a pro-inflammatory response), and/or the PI (via increased susceptibility to FA peroxidation). However, in contrast to our predictions, there was no significant interaction between habitat and either of the two FA-derived estimates. Instead, there was an interaction between species and PI, revealing a negative association between PI and MDA in tree sparrows, but not in the other species. The direction of this association in tree sparrows is in contrast to our prediction, and also to the results at the population level (where both MDA and PI were higher in the urban compared to the rural environment). Possibly, individual tree sparrows with a high PI have a corresponding increase in dietary antioxidants (Eikenaar et al., 2016, 2017). This could prevent lipid peroxidation to some degree, but not enough to override the population/habitat effect. Alternatively, individuals with a high peroxidation rate, independent of habitat (e.g., due to other unknown factors such as age and life history), might reduce their plasma PUFA levels, thus reducing the PI.

CONCLUSIONS

The present study reveals interspecific and inter-habitat differences in FA profiles and health markers associated with FA intake and physiology (cf. Fokidis et al., 2008). These differences could make the two urban adapters (blue tits and great tits) more prone to be negatively affected by inflammatory responses via their higher ω-6/ω-3 ratio, whereas the urban exploiters (house- and tree sparrows) are likely to be more affected by oxidative stress, at least in terms of lipid peroxidation.

Moreover, multivariate analyses of FA profiles can provide important information about species-, population-, and individual-level variation in foraging behaviors and feeding ecology, which can be of particular value in the urban context where human-provided foods are abundant. These food sources can reduce winter mortality caused by starvation and possibly predation (Jansson et al., 1981; Hole et al., 2002; Perkins et al., 2007). However, artificial food might also impact fitness negatively by causing deficiency of essential micro-and macro-nutrients (Isaksson and Andersson, 2007; Plummer et al., 2013; Isaksson, 2015). This could be exacerbated by the presumably poorer quality of natural food in the urban habitat. Disentangling the mechanistic underpinnings of how, and when, the negative effects of anthropogenic food outweigh the positive effects will be important for understanding its short- and long-term impacts on wild birds.

ETHICS STATEMENT

The present study was carried out in accordance with a license (Dnr M454 12:1) from the Swedish Board of Agriculture, and capture was performed under C.Isaksson's ringing license (ID: 681) from the Natural History Museum, Sweden.

AUTHOR CONTRIBUTIONS

CI, AN, and MP designed the study. CI, AN, and MP performed fieldwork. CI, MA, and HW performed the laboratory analyses. CI, MA, and AN drafted the manuscript. MP and HW edited the manuscript. All authors reviewed and approved the final version.

ACKNOWLEDGMENTS

We are grateful to Christer Löfstedt for hosting MA and HW. We thank Amparo Herrera-Dueñas for molecular sexing of tree sparrows. We also acknowledge Sydvatten AB and the City of Malmö for access to field localities, and Lennart Blomquist for help with field work in Malmö. CI was funded by a Marie Curie re-integration grant (FP7-*CIG*), Carl Trygger's Foundation, the Royal Physiographic Society in Lund, and the Crafoord Foundation. AN was supported by the Swedish Research Council (grant no. 637-2013-7442).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00044/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CH and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 18 July 2017
doi: 10.3389/fevo.2017.00079

Growing in Cities: An Urban Penalty for Wild Birds? A Study of Phenotypic Differences between Urban and Rural Great Tit Chicks (*Parus major*)

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Urban sprawl is associated with deep and intense modifications of the natural habitats of wild vertebrates. Although, many species are unable to cope with such an environment, a few species can be found in cities and can help us assessing the impact of urbanization on wildlife. Urban-related environmental modifications are multiple and some of them seem beneficial while others seem rather detrimental to wild vertebrates. Moreover, the impact of these modifications on wild vertebrates is likely to vary depending on the phase of the annual life-cycle. Therefore, it is challenging to get a comprehensive picture of the impact of urbanization on wild vertebrates. Overall, urbanization is usually associated with reduced breeding performances in wild birds, but the impact of urbanization on the phenotype and quality of developing offspring has been less studied. In this study, we specifically investigated the impact of urbanization on several proxies of individual quality in great tits (*Parus major*). We concomitantly measured body size (tarsus length and body mass), plumage coloration, and telomere length in 14-days old chicks issued from 4 populations (two pairs of urban/rural populations located in two different geographical areas of France). First, rural chicks were significantly taller and heavier than urban birds although this impact of urbanization on body size/body mass appears only true for the most urbanized site. Interestingly, body size was also affected by the geographical area of capture, suggesting that regional environmental conditions may attenuate or exacerbate the influence of urbanization on nestling growth. Second, the carotenoid-based yellow plumage of rural nestlings was more colorful than that of urban birds, independently of the area of capture. This suggests that urban birds probably have a low-carotenoid diet relative to rural birds. Finally, telomere length did not differ between rural and urban chicks. These results suggest that urbanization probably imposes large developmental constraints in wild vertebrates and that this impact may primarily be related to constraining nutritional conditions.

Keywords: urbanization, carotenoid-based signal, developmental conditions, telomeres, body size, plumage color

Edited by:
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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *31 March 2017*
Accepted: *03 July 2017*
Published: *18 July 2017*

Citation:
Biard C, Brischoux F, Meillère A, Michaud B, Nivière M, Ruault S, Vaugoyeau M and Angelier F (2017) Growing in Cities: An Urban Penalty for Wild Birds? A Study of Phenotypic Differences between Urban and Rural Great Tit Chicks (Parus major). Front. Ecol. Evol. 5:79. doi: 10.3389/fevo.2017.00079

INTRODUCTION

Urban sprawl is a worldwide phenomenon that deeply affects environmental conditions, and consequently, avian biodiversity (Grimm et al., 2008; Shanahan et al., 2014). Thus, cities are characterized by multiple abiotic and biotic modifications of the natural environment such as fragmentation (Crooks et al., 2004), human disturbance (Sauvajot et al., 1998; Fernández-Juricic, 2002), and increased pollutions: noise (Slabbekoorn and Peet, 2003; Meillère et al., 2015b), light (Dominoni et al., 2013; Spoelstra and Visser, 2014), electromagnetic (Engels et al., 2014), and chemical pollution (Roux and Marra, 2007; Isaksson, 2010; Bichet et al., 2013). Urbanization is also associated with altered predation risk (Fischer et al., 2012; Møller and Ibáñez-Álamo, 2012), inter and intra-specific competition (Duckworth, 2014), disease prevalence (Bichet et al., 2013; Giraudeau et al., 2014), food type and abundance (Robb et al., 2008; Harrison et al., 2010; Galbraith et al., 2015). All these modifications raise new important selective constraints for wild vertebrates, and as a consequence, biodiversity is overall impoverished in cities (Clergeau et al., 2006; McKinney, 2008). While some species seem unable to cope with this modified environment (urban avoiders) others can still be found in this specific environment (urban-tolerant species, Chace and Walsh, 2006). Comparative studies have allowed ecologists to determine the specific characteristics that may be compatible with an urban way of life in wild vertebrates (Møller, 2008, 2009; Evans et al., 2011; Sol et al., 2013; Jokimäki et al., 2016). In addition, an individual-based approach is also necessary to fully understand the ecological processes that may constrain the ability of wild populations to cope with urbanization (Gil and Brumm, 2014). Several studies have for instance demonstrated that living in an urban environment is associated with phenotypical changes in vertebrates (e.g., body size: Bókony et al., 2012; Meillère et al., 2017, secondary sexual signals: Fuller et al., 2007; Giraudeau et al., 2015, stress physiology: Bonier, 2012, immunity: Audet et al., 2015). Moreover, a large number of studies have also shown that urbanization affects reproductive performances, such as phenology or the number of young produced (Both et al., 2004; Chamberlain et al., 2009; Ausprey and Rodewald, 2011; Stracey and Robinson, 2012; Deviche and Davies, 2014; Møller et al., 2015).

Surprisingly, the influence of urbanization on the phenotype and quality of developing offspring has been much less studied (Heiss et al., 2009; Seress et al., 2012; Salmón et al., 2016). Early developmental conditions are however crucial to study because they are known to have important long-term consequences on individual phenotypes and adult performances (survival and reproduction, Lindström, 1999; Metcalfe and Monaghan, 2001; Monaghan, 2008). In terms of nutritional conditions, urbanization is very likely to be constraining for most bird species. Several studies have shown that the urban environment is associated with modifications of the arthropod community (Helden et al., 2012), and thus, with a lack of protein-rich diet (McIntyre et al., 2001; Shochat et al., 2004), vitamins, carotenoids (Isaksson and Andersson, 2007; Møller et al., 2010; Giraudeau and McGraw, 2014; Giraudeau et al., 2015), and calcium (Heiss et al., 2009). Similarly, urban-related disturbance is likely to affect the quality of parental care (e.g., incubation commitment or brood provisioning, Ibáñez-Álamo and Soler, 2010; Seress et al., 2011; Meillère et al., 2015a), and therefore the nutritional conditions of offspring. In addition, other urban-related environmental conditions could also constrain the development of offspring through direct or parental-mediated effects (e.g., chemical pollution, light pollution, intra- and inter-specific competition, predation risk, Shanahan et al., 2014).

In this study, we aimed to better understand the consequences of living in an urban environment for developing wild vertebrates. To do so, we propose to study concomitantly several complementary proxies of developmental conditions (growth, carotenoid-based coloration, and telomere length). First, growth is classically used to monitor nutritional developmental conditions in wild vertebrates and poor nutritional conditions are associated with delayed growth, an overall poor health status and a lower probability to fledge and to recruit into the population (Gebhardt-Henrich and Richner, 1998; Naef-Daenzer and Keller, 1999; Naef-Daenzer et al., 2001). Second, carotenoids are also relevant to study because plasma carotenoid levels are known to be positively associated with immunity (Saino et al., 2003b; Biard et al., 2005; Cucco et al., 2006), and growth (Tschirren et al., 2003; Biard et al., 2005, 2006; Berthouly et al., 2008). In addition to these physiological functions, carotenoids are also allocated into carotenoid-based signals (e.g., plumage) in developing animals (Fitze et al., 2003; Isaksson et al., 2006). Such signaling is crucial because it is used by parents to assess the viability/quality of the chicks and therefore to determine their parental investment (Saino et al., 2003a; Pike et al., 2007; Thorogood et al., 2011). Therefore, carotenoids mediate a trade-off between protective physiological functions (e.g., immunity, growth, and antioxidants) and signaling in developing animals. Because carotenoids cannot be synthetized by vertebrates and are only acquired through the diet (Isaksson, 2009), carotenoid-based plumage appear relevant to better assess the environmental constraints associated with an urban way of life in developing vertebrates (Giraudeau et al., 2015). Third, offspring survival is also classically used because it is a direct measure of reproductive success. However, it is often very challenging to follow the young after fledging in wild populations, and therefore, long-term survival is difficult to assess. In that respect, telomere length appears as a promising molecular marker to assess the survival probability of offspring after fledging (Monaghan, 2010, 2014). Telomeres are long repetitive sequences of non-coding DNA that are located at the end of chromosomes. They protect chromosomes from degradation and they also play a crucial role during the replication process (Blackburn, 2000). Telomeres usually shorten as individual age, and importantly, environmental stressors and the occurrence of oxidative stress have been shown to accelerate this shortening (von Zglinicki, 2002). Moreover, telomere length has also been linked with survival (Barrett et al., 2013; Fairlie et al., 2016) and it can be used as a reliable proxy of an individual's longevity (Heidinger et al., 2012).

Several studies have examined the influence of urbanization on these variables. For instance, urbanization often delays and impairs growth in bird species (Chamberlain et al., 2009; Heiss et al., 2009; Seress et al., 2012; Bailly et al., 2016). Similarly, urbanization is often associated with lower offspring productivity in wild vertebrates (Chamberlain et al., 2009; Bailly et al., 2016). However, these results appear somewhat inconsistent and sometimes differ between species or populations (reviewed in Chamberlain et al., 2009). Overall, carotenoid-based plumage color is paler in birds living in cities than in those living in rural areas (Eeva et al., 1998; Hõrak et al., 2000, 2001; Isaksson et al., 2005; Giraudeau and McGraw, 2014; Giraudeau et al., 2015). The influence of urbanization on telomere length has only been investigated a few times in wild animals (Salmón et al., 2016; Stauffer et al., 2017), and further studies are necessary to confirm that urbanization could lead to shortened telomeres in developing wild animals.

In this study, we investigated the influence of urbanization on growth, offspring survival, offspring carotenoid-based coloration and offspring telomere length in a wild bird species, the great tit (*Parus major*). To our knowledge, no study has concomitantly investigated the influence of urbanization on all these variables and their functional links remain unclear in an urbanization context. Moreover, most studies have only compared a rural and an urban population, preventing to assess the influence of the degree of urbanization on these variables. Here, we examined this question by comparing four populations of great tits. In addition to two rural sites, we studied two urban sites with contrasted degrees of urbanization: a very large city (Paris), and a medium-sized city (Niort) in France, Western Europe. Great tit is a relevant species for such a study because it is considered as an urban adapter species (Shochat et al., 2006; Kark et al., 2007), and it can be found in rural sites, but also all along the urbanization gradient (Croci et al., 2008). Moreover, as cavity-nesters, the development of the chicks is very easy to follow (Lambrechts et al., 2010). According to previous studies, we predict that urbanization will be detrimental to developing chicks. First, we predict that growth will be impaired in cities relative to rural areas. Second, we predict, that urban nestlings will be less colorful than rural ones because of a lack of carotenoid-rich diet in cities. Third, we predict that urban nestlings will have shorter telomeres due to increased developmental stress than rural ones. Importantly, we predict that these effects should be reinforced as the degree of urbanization increases. If telomere length and carotenoid-based coloration are two proxies of health status and individual quality, we finally predict that carotenoid-based coloration and telomere length will be positively correlated with growth.

MATERIALS AND METHODS

Study Sites and Data Collection

Data were collected in 2014 in two pairs of rural and urban populations of greattits breeding in France: (i) in the Paris area, and more specifically in city parks of Paris (48° 17′ N, 2° 38′ E) and in the forest surrounding the Foljuif field station, 80 km down south of Paris (CEREEP; 48° 16′ N, 2° 41′ E), and (ii) in the Niort area, and more specifically in the city of Niort (46°18′ N, 0°28′ W) and nearby forest of Chizé-CEBC, 30 km down south of Niort (46° 09′ N, 0° 24′ W). The rural study sites of Foljuif and Chizé respectively contained 120 and 100 nestboxes (Schwegler wood-concrete nest boxes 2 M, Valliance, Saint Pierre La Palud, France, floor area: 113 cm^2) evenly distributed throughout homogenous deciduous woodlands composed mainly of oak (*Quercus* spp.), hornbeam (*Carpinus betulus*) and beech (*Fagus sylvatica*), and used by both blue and great tits (entrance hole diameter: 32 mm). The urban study sites of Paris and Niort contained respectively 60 and 64 nestboxes (Paris: 40 Schwegler nest boxes 2 M, and 20 handmade nestboxes in wooden panel, floor area: 105 cm^2, entrance hole diameter: 32 mm; Niort: 64 Schwegler nest boxes 2 M) installed in several urban parks or cemetery, separated from one another by 1–7 km as the crow flies. Paris is the biggest city in France (∼2 million inhabitants), while Niort is a medium-sized city (∼60 000 inhabitants), allowing us to investigate the potential impact of the degree of urbanization on nestlings' great tits. Paris is a very good example of historical European city with an extremely dense land occupation (housing development, industrial/commercial sites), and relatively small and isolated city Parks, while Niort is a classical "province" city less densely built with relatively larger city vegetation cover. Accordingly, these differences translated in highly divergent urbanization scores between our study sites as measured in another study (Meillère et al., 2017).

Nests were regularly inspected from March to June to determine laying date, clutch size, incubation date, hatching date of the first egg(s), number of hatchlings and unhatched eggs, brood size (at day 14 post-hatch), and number of successfully fledged nestlings. Only first clutches were used in this study. Data were collected for a total of 424 nestlings, originating from 28 nests in the rural sites (14 in Foljuif and 14 in Chizé) and from 26 nests in the urban sites (10 in Paris and 16 in Niort). Nestlings were captured at the nest when aged 14-days old, except for 2 broods aged 13 (one nest from Paris and one nest from Foljuif) and 4 broods aged 15 (two nests from Paris and two nests from Foljuif). Variation in nestling age being confounded with a pair of urban/rural sites and very low (mean nestling age ± s.e.: 14.02 ± 0.34), nestling age was not entered as a covariate in statistical models. Nestling capture spanned from May, 22th to June 10th (mean capture date ± s.e.: 123.63 ± 7.51; with 121=May, 1st), there was no significant difference in phenology among sites [$F_{(3,\ 50)} = 1.31$ $p = 0.28$]. Tarsus length and wing length were measured to the nearest 0.1 mm with a caliper and a ruler, respectively, and body mass to the nearest 0.25 g with a Pesola spring balance. A sample of 5–8 yellow feathers was plucked from the center of the yellow breast for each bird, and stored in individual plastic bags in the dark until later color analysis. A blood sample (20–100 µl) was taken from the brachial vein in heparinized micro-haematocrit tubes, and centrifuged when back in the lab. Packed red blood cells were separated from plasma and stored at −20° C until later DNA extraction.

Telomere Measurements

Genomic DNA was extracted from frozen red blood cells using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA yield and purity were checked using a spectrophotometer (Nanodrop ND-1000; Thermo Scientific, USA) according to previous recommendations (Nussey et al., 2014). All sample yields were >20 $\mathrm{ng\,\mu l^{-1}}$. Moreover, ranges for absorption of all samples were within an acceptable range (between 1.8 and 2.0 for 260 nm/280 nm ratio and between 1.9 and 2.2 for 260 nm/230 nm ratio). Telomeres were measured using real-time quantitative PCR, following a protocol previously validated for birds and successfully used in great tits (Salmón et al., 2016). Briefly, a single-copy gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) was amplified using the primers specifically designed for great tits (Atema et al., 2013). Telomeres primers were Tel1b (5′-CGG TTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTAC CCTTACCCT-3′). qPCR for both GAPDH and telomeres was performed using 5 ng of DNA per reaction. After preliminary optimization procedures, the telomere and GAPDH primers were respectively used at a final concentration of 1,000 and 200 nM. To control for the amplifying efficiency of the qPCR, each plate included a standard curve, which consisted of various concentrations of a great tit DNA pool (50–0.08 $\mathrm{ng\,mL^{-1}}$). The efficiencies of the qPCR were within the acceptable range for both GAPDH and Telomere (Mean ± *SD*; GAPDH: 101.9 ± 1.09; Telomere: 96.7 ± 3.13). Telomere length is expressed relative to the single-copy gene (GAPDH) measured on the same sample of DNA (i.e., T/S ratio, Cawthon, 2002; Salmón et al., 2016). All standard DNA samples were run in duplicates. Moreover, a common sample was run on every plate and the inter-plate CV in TS ratio was 2.5%. Telomeres measurements were obtained for a subsample of 111 randomly chosen nestlings [number of nestlings (number of nests): Foljuif: $n = 36(14)$, Paris: $n = 24(10)$, Chizé: $n = 30(12)$, Niort: $n = 24(9)$].

Feather Color Analysis

Yellow breast feather color was analyzed in the laboratory, using a spectrophotometer (Ocean Optics, IDIL Fibers Optiques, Lannion, France) following previously published methods (Andersson and Prager, 2006), samples being randomly chosen with respect to origin (study site and nest), and among nestlings from the same nest. Feathers were illuminated at an angle of 90° with a deuterium-tungsten lamp (DH2000), and reflected light was measured at the same angle, with a 200 μm fiber-optic reflection probe (QR200-7-SR-BX) connected to the spectrometer (USB2000+) operated using the Spectra Suite software. To exclude ambient light and standardize measuring distance (3 mm), an opaque black tube was fitted at the end of the measuring probe. Feather color was measured against a black short velvet background regularly cleaned and changed to keep its reflectance constant and equal to 0. A set of four feathers was randomly chosen among each sample, and piled up to reflect the bird's plumage (Quesada and Senar, 2006), with their rachis superimposed and parallel to each other. Preliminary tests showed that four feathers were sufficient to get reliable and repeatable spectra while keeping the number of feathers collected to a minimum for ethical reasons. Two sets of four feathers were analyzed for each individual with four independent (probe lift up and placed again on the sample) measures per set. Percentage of reflectance at each 1 nm interval was calculated between 300 and 700 nm, with respect to white (Spectralon diffuse reflectance Standard, WS-1-SL) and dark references, as $R_{(\lambda)} = 100 \times [(\text{sample-white})/(\text{white-dark})]$. From these values, we calculated two independent and objective parameters to characterize carotenoid-based plumage color. Brightness is the total intensity of light reflected by feathers (spectral intensity), and was calculated as the integral of reflectance over [300, 700] nm (e.g., Endler, 1990; Andersson et al., 1998). Brightness reflects the structural quality of feathers and captures the UV component of carotenoid-based feather reflectance (Shawkey and Hill, 2005; Andersson and Prager, 2006). Plumage brightness has been shown to reflect body condition in this species (Galván, 2010). Carotenoid chroma reflects the carotenoid content of feathers and was calculated as the relative difference between the maximal (plateau at wavelength above 500 nm) and minimal (between 445 and 455 nm, corresponding to wavelengths of maximum absorbance of carotenoids) reflectance in the visible part of the spectrum: $[R_{700} - R_{450}]/R_{700}$ (Örnborg et al., 2002; Andersson and Prager, 2006; Peters et al., 2007). Feather reflectance spectra were obtained by a single observer (MN) for 403 nestlings. Repeatability of measurements calculated as the intra-class correlation coefficient (Lessells and Boag, 1987) was always highly significant ($p < 0.0001$) with the following values: (a) repeatability within sets of feathers: brightness: 0.92, carotenoid chroma: 0.75, (b) and repeatability within individuals: brightness: 0.85, carotenoid chroma: 0.63. Average values for the 8 measures per nestling were used in subsequent statistical analyses.

Statistical Analyses

Statistical analyses were run using SAS v9.3 (SAS Institute Inc., Cary, NC, USA). Values are reported as mean ± s. e.

Generalized linear models were used to investigate variation in clutch size, number of hatchlings and brood size (number of nestlings surviving to the day of capture), as a function of site, including date as a covariate (GENMOD procedure with a Poisson distribution and a log link function).

Mixed linear models (MIXED procedure) were used to analyze variation in nestling phenotype (tarsus length, body mass, wing length, plumage color, and telomere length) with nest as a random effect to account for non-independence of nestlings raised in the same nest and sharing genetic and common rearing environment effects, using an unstructured covariance and REML (Restricted/Residual Maximum Likelihood) estimation method. The Wald test of the covariance parameter assessed whether the variance was significantly structured by the random nest effect. The fixed part of the models initially included the effects of site, date and the interaction between site and date. Models were compared with Akaike's Information Criterion (AIC), and the most parsimonious was retained (lowest AIC, Burnham and Anderson, 1998). Final models including significant differences between sites were followed by comparisons of means or least square means with adjusted values of *p*, using the Tukey-Kramer method. Tests of the residuals for normality and homoscedasticity were used to check the validity of the models; feather brightness and carotenoid chroma were log-transformed to meet these constraints. Finally, mixed linear models were also used to investigate whether plumage color and telomere length were related to nestling body mass and growth conditions (date and brood size) and whether telomere length was related to nestling plumage color. As there were significant differences between sites in brood size, nestling body condition and feather carotenoid chroma (see Section Results), these models were run separately for each site to avoid multi-collinearity issues (Graham, 2003).

RESULTS

Clutch size differed significantly among sites [$F_{(3, 50)} = 8.76$, $p = 0.03$] and was significantly greater in Chizé as compared to Paris (**Table 1**), but there was no difference among sites in the number of hatchlings [$F_{(1, 50)} = 5.35$, $p = 0.15$]. Date and its interaction with site were not retained in the final models for clutch size and the number of hatchlings. Brood size, i.e., the number of nestlings surviving to 14-days old, decreased with advancing season [$F_{(1, 49)} = 4.38$, $p = 0.04$, slope estimate ± s.e.: -0.008 ± 0.004] and differed among sites [$F_{(1, 49)} = 3.19$, $p = 0.03$], with great tits in Foljuif rearing more nestlings to fledging than in Niort and Paris, and in Chizé as compared to Paris (**Table 1**).

Nestling growth, i.e., tarsus length, body mass, and wing length, significantly differed among sites (**Table 2A**, **Figure 1**): Nestlings were significantly taller (longer tarsi) in the rural site of Foljuif as compared to the other rural site of Chizé, and both urban sites of Niort and Paris. Nestlings were also smaller in Chizé and Niort as compared to Paris (**Figure 1A**). Nestlings were heavier in the rural site of Foljuif as compared to Chizé, Niort, and Paris (**Figure 1B**). Wing length was greater both in Foljuif and Paris as compared to Chizé and Niort (**Figure 1C**). Regarding plumage color, while yellow feather brightness did not differ among sites, there were significant differences in feather carotenoid chroma (**Table 2B**, **Figure 2**): yellow feathers were significantly more chromatic in both woodland sites of Foljuif and Chizé as compared to both urban sites of Paris and Niort. Finally, telomere length did not differ among sites (**Table 2B**, **Figure 3**). Nestling growth, plumage color, and telomere length did not vary with season in any site (date and its interaction with site were not retained in the final models).

TABLE 1 | Mean ± s.e. great tit clutch size (total number of eggs laid), number of hatchlings and brood size (number of nestlings surviving to 14 days old) in the four study sites.

Site	Clutch size	Number of hatchlings	Brood size
Chizé	10.14 ± 0.49	9.00 ± 0.48	8.14 ± 0.48
Foljuif	9.15 ± 0.38	8.79 ± 0.39	8.64 ± 0.39
Niort	9.31 ± 0.32	8.18 ± 0.45	7.62 ± 0.44
Paris	8.30 ± 0.49	7.5 ± 0.58	6.90 ± 0.48

A posteriori comparison of means or lsmeans for differences among sites (see Section Materials and Methods and Results for more details on the models and site main effect) showed that clutch size in Chizé differed significantly from that in Paris (z = 2.90, adj. p = 0.004). Brood size in Foljuif differed from that in Niort and Paris (z = 2.12, adj. p = 0.03 and z = 2.77, adj. p = 0.006, respectively), and brood size in Paris differed from that in Chizé (z = 2.09 adj. p = 0.04). All other comparison were non-significant (all z < 1.35 and adj. p > 0.17).

Yellow feather brightness was not related to nestling growth conditions or body mass in any site (all $F < 1.87$ and $p > 0.17$). Similarly, carotenoid chroma was not related to nestling growth conditions or body mass in Niort and Foljuif (all $F < 2.16$ and $p > 0.14$, **Figures 4B,C**). However, carotenoid chroma was positively related to nestling body mass [$F_{(1, 40)} = 8.02, p = 0.007$, estimate ± s.e.= 0.59 ± 0.02, **Figure 4D**], while a marginal negative relationship was found in Chizé [$F_{(1, 98)} = 3.51, p = 0.06$, estimate ± s.e.= -0.02 ± 0.01, **Figure 4A**]. In Foljuif and Niort, telomere length was not related to nestling body mass or growth conditions (all $F < 0.59$ and $p > 0.45$, **Figures 5B,C**). However, telomere length was positively related to nestling body mass in Chizé [$F_{(1, 17)} = 7.71$, $p = 0.01$, estimate ± s.e.= 0.02 ± 0.01], while the opposite pattern was found in Paris [$F_{(1, 13)} = 5.45$, $p = 0.04$, estimate ± s.e.= -0.02 ± 0.01; **Figures 5A,D**]. Finally, telomere length was not related to yellow feather color in any site (all $F < 4.19$ and $p > 0.07$).

DISCUSSION

In this study, we aimed to assess the impact of urbanization on the phenotype and quality of developing offspring by comparing four populations of great tits. To do so, we focused on complementary morphological and molecular components (growth, plumage coloration, and telomere length). According to our predictions, we found that the body size and the carotenoid-based plumage of great tits chicks were affected by urbanization, although the impact of urbanization on body size was only found in the most urbanized site. However, we did not find any strong evidence for an impact of urbanization on telomere length.

Urbanization and Body Size in Nestling Great Tits

Overall and according to our prediction, we found some support for an effect of urbanization on nestling body size and body mass. In the area of the biggest city (Paris), we specifically found that urban nestlings were smaller and lighter than rural ones (Foljuif). However, we did not find any difference in body size and body mass between urban (Niort) and rural (Chizé) nestlings in the area of the smallest city. Supporting these contradictory patterns, some studies previously found that urban great tit nestlings are smaller than rural ones (Riddington and Gosler, 1995; Eeva et al., 2009; Geens et al., 2009) while others did not report such a difference (Isaksson and Andersson, 2007). All together, these results suggest that the influence of urbanization on growth and body size may only be apparent when the degree of urbanization reaches an upper threshold, or/and when urbanization is associated with specific anthropogenic perturbations that vary from one locality to another (e.g., pollution, Eeva et al., 2009; Geens et al., 2009). Thus, the effect of urbanization on body size may only be minor and non-significant for great tits reared in small to medium-sized city, such as Niort (∼70,000 inhabitants) while it may become

TABLE 2 | Mixed linear models investigating variation in great tit nestlings' phenotype as a function of site and season.

Effect	Tarsus length			Body mass			Wing length		
	Estimate ± s.e.	***Z* or F_{df}**	***p***	**Estimate ± s.e.**	***Z* or F_{df}**	***p***	**Estimate ± s.e.**	***Z* or F_{df}**	***p***
(A)									
Nest	0.42 ± 0.09	4.50	<0.0001	1.76 ± 0.38	4.65	<0.0001	13.45 ± 2.87	4.68	<0.0001
Site	C: − 1.76 ± 0.29	$62.77_{3,370}$	<0.0001	C: − 0.40 ± 0.57	$6.44_{3,370}$	0.0003	C: − 4.98 ± 1.57	$8.65_{3,369}$	<0.0001
	F:1.24 ± 0.28			F:1.44 ± 0.57			F:0.99 ± 1.56		
	N: − 1.65 ± 0.28			N: − 0.58 ± 0.56			N: − 4.30 ± 1.53		

Effect	Brightness			Carotenoid chroma			Telomere length		
	Estimate ± s.e.	***Z* or F_{df}**	***p***	**Estimate ± s.e.**	***Z* or F_{df}**	***p***	**Estimate ± s.e.**	***Z* or F_{df}**	***p***
(B)									
Nest	0.011 ± 0.004	2.63	0.004	0.017 ± 0.004	3.99	<0.0001	0.0012 ± 0.0007	1.73	0.04
Site	C:0.04 ± 0.06	$0.56_{3,349}$	0.64	C:0.29 ± 0.06	$11.62_{3,49}$	<0.0001	C: − 0.02 ± 0.02	$0.37_{3,66}$	0.78
	F:0.05 ± 0.06			F:0.23 ± 0.06			F: − 0.01 ± 0.02		
	N:0.08 ± 0.06			N:0.05 ± 0.06			N: − 0.003 ± 0.02		

Nestling phenotype was described at 14-days old by measures of (a) body size (tarsus and wing lengths) and mass, and (b) plumage color (yellow feather brightness and carotenoid chroma) and telomere length. Nest was entered as a random effect to account for the non-independence of nestlings raised in the same nest. The fixed part of the model initially included site, date and their interaction. Final models are presented (see Section Materials and Methods for more details on the models and variable selection). The effects of site (C, Chizé; F, Foljuif; N, Niort; P, Paris) were estimated taking Paris as the level of reference.

important and significant when they grow in large cities, such as Paris (over 2 million inhabitants). Similarly, some studies found an effect of urbanization on body size or growth in other bird species (Heiss et al., 2009; Bókony et al., 2012; Seress et al., 2012), supporting the idea that urbanization can have detrimental effects on growth and development. Interestingly, the influence of urbanization on body size also seems to depend on the degree of urbanization in some of these studies (Bókony et al., 2012; Meillère et al., 2017). For example, Meillère et al. (2017) found that urban house sparrows were not significantly smaller than rural ones when living in a medium-sized city such as Niort, while their body size was significantly reduced when living in bigger cities. To our knowledge, most studies have so far focused on a single pair of sites when comparing urban and rural populations of wild birds. Our study highlights the importance of studying multiple populations all over the urbanization gradient in order to better assess the influence of the degree of urbanization on the morphology of wild vertebrates (see Evans et al., 2009; Bókony et al., 2012; Meillère et al., 2017 for some examples).

At the proximate levels, our results support the idea that developmental conditions may be impoverished in big cities relative to forest areas. Overall, cities are characterized by multiple severe environmental modifications that certainly raise new important developmental constraints for wild vertebrates (Clergeau et al., 2006; McKinney, 2008; Gil and Brumm, 2014). Although, we did not quantify abiotic and biotic constraints in our populations, our study suggests that food abundance could explain the patterns we report because the growth of great tit nestlings has been directly correlated with food supply (Naef-Daenzer and Keller, 1999; Mägi et al., 2009, but see Tremblay et al., 2005). Supporting this interpretation, we also found that clutch size and brood size (number of nestlings reaching 14-days old) were generally reduced in cities relative to woodlands. Interestingly, we found that brood sizes differ between Paris and Foljuif, but not between Niort and Chizé, supporting further the idea that the degree of urbanization may have an impact on the ability of parent great tits to rear numerous young. During the chick-rearing period, great tits rely mainly on arthropods to feed their young and arthropod biomass and diversity is overall reduced in cities (McIntyre, 2000; Helden et al., 2012). Because nestlings rely on protein-rich diet during their development, this arthropod impoverishment is probably a major nutritional constraint for urban developing great tits (McIntyre et al., 2001; Shochat et al., 2004). Supporting this idea, food supplementation experiments have been shown to result in increased body size and nestling survival in urban birds (Zanette et al., 2003; Schoech et al., 2007; Heiss et al., 2009; Meyrier et al., 2017) and cross-fostering experiments demonstrated that reduced nestling body size in urban areas is indeed related to the rearing urban environment (e.g., Seress et al., 2012).

Urbanization and Plumage Characteristics

According to our prediction, we found a strong difference between the plumages of urban and rural nestlings: nestlings from Paris and Niort had less chromatic plumages, indicating their feathers contained less carotenoids (e.g., Peters et al., 2007) than those from Foljuif and Chizé. However, we did not report any effect of urbanization on plumage brightness, i.e., feather structural quality. These results are supported by previous studies that have reported similar results in great tits (Hõrak et al., 2000; Isaksson et al., 2005; Isaksson and Andersson, 2007) and other wild bird species (e.g., Jones et al., 2010; Giraudeau et al., 2015). In wild passerines, the diet seems to be the main factor affecting plumage coloration (Tschirren et al., 2003; Isaksson et al., 2007; Eeva et al., 2009; Giraudeau et al., 2015), and therefore, nestling plumage coloration certainly depends on the ability of parents

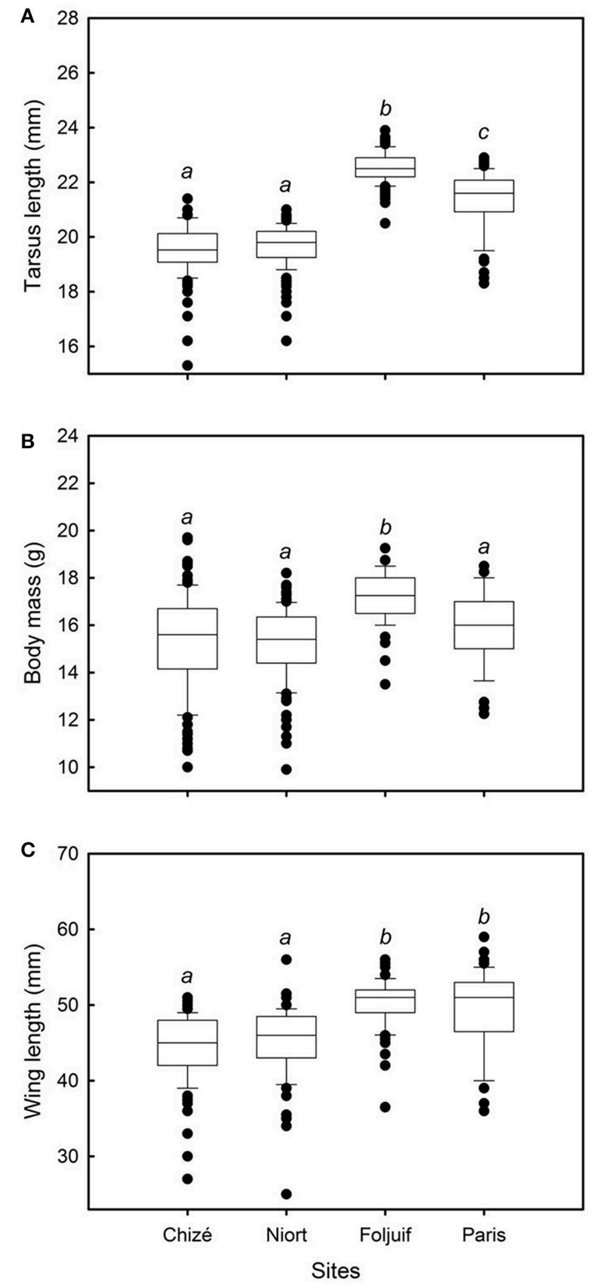

FIGURE 1 | Variation of **(A)** tarsus length, **(B)** body mass, and **(C)** wing length, among breeding sites in great tit nestlings. Nestling tarsus length was significantly greater in the rural site of Foljuif as compared to the other rural site of Chizé, and to both urban sites of Niort and Paris [differences of least squares means: $t_{(df:370)} = 11.67$, adj. $p < 0.0001$; $t_{(df:370)} = 11.58$, adj. $p < 0.0001$; and $t_{(df:370)} = 4.38$, adj. $p < 0.0001$, respectively]. Nestlings were also smaller in Chizé and Niort as compared to Paris [$t_{(df:370)} = -6.20$, adj. $p < 0.0001$; and $t_{(df:370)} = -5.95$, adj. $p < 0.0001$, respectively]. Nestlings were significantly heavier in the rural site of Foljuif as compared to Chizé, Niort, and Paris [differences of least squares means: $t_{(df:370)} = 3.56$, adj. $p = 0.002$; $t_{(df:370)} = 4.03$, adj. $p = 0.0004$; and $t_{(df:370)} = 2.54$, adj. $p = 0.05$, respectively]. Wing length was significantly greater both in Foljuif and Paris as compared to Chizé [$t_{(df:369)} = 4.19$, adj. $p = 0.0002$; and $t_{(df:369)} = 3.18$, adj. $p = 0.01$] and Niort [$t_{(df:369)} = 3.84$, adj. $p = 0.001$; $t_{(df:369)} = 2.82$, adj. $p = 0.03$], respectively. Different letters indicate significant differences.

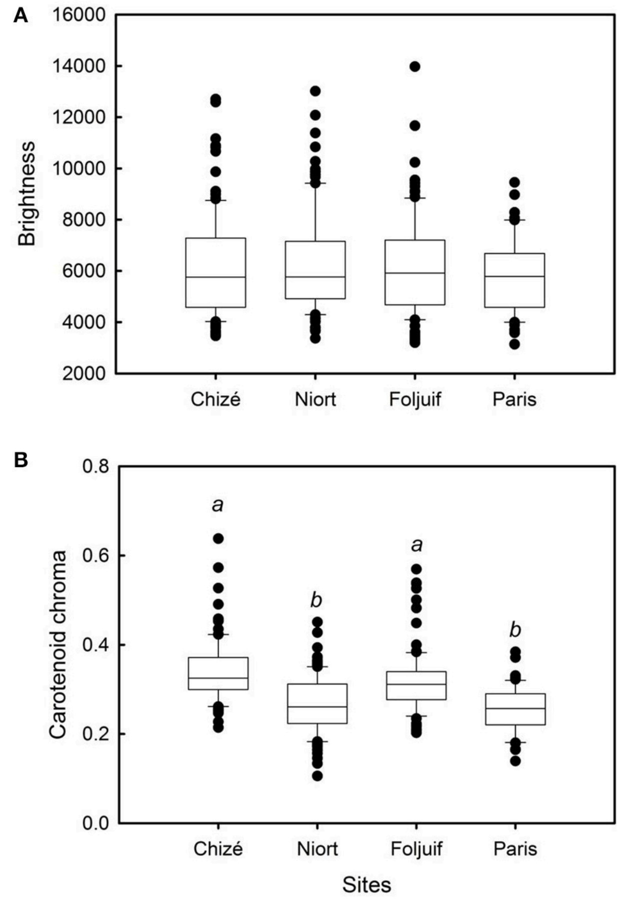

FIGURE 2 | Variation of yellow feather color among breeding sites in great tit nestlings: **(A)** brightness and **(B)** carotenoid chroma. Nestling feathers were significantly more chromatic in both woodland sites of Foljuif and Chizé as compared to both urban sites of Paris [$t_{(df:349)} = 3.68$, adj. $p = 0.001$; $t_{(df:349)} = 4.76$, adj. $p < 0.0001$] and Niort [$t_{(df:349)} = 3.32$, adj. $p = 0.005$; $t_{(df:349)} = 4.57$, adj. $p < 0.0001$], respectively. Different letters indicate significant differences.

to deposit carotenoids into their eggs (Biard et al., 2005, 2007) and to provide their chicks with carotenoid-rich diet (Biard et al., 2006; Isaksson et al., 2006). Our results suggest therefore that carotenoid availability and/or carotenoid uptake is reduced in the urban environment (Isaksson and Andersson, 2007; Isaksson et al., 2007). This interpretation is supported by eco-toxicological studies that were conducted in great tit nestlings: Eeva et al. (1998) and Dauwe and Eens (2008) found that nestlings had a paler plumage when they were raised in more polluted areas, and this effect was mediated by a reduced availability of carotenoid-rich diet in polluted areas (Sillanpää et al., 2008; Eeva et al., 2009). Furthermore, we found that the nestlings from Paris had a similar plumage color as those from Niort despite a large difference in the degree of urbanization between these two cities. Interestingly, this suggests that nestling plumage coloration is not strongly dependent on the degree of urbanization of a city, and more importantly that carotenoid availability or/and uptake by nestlings may not differ between medium-sized and large cities.

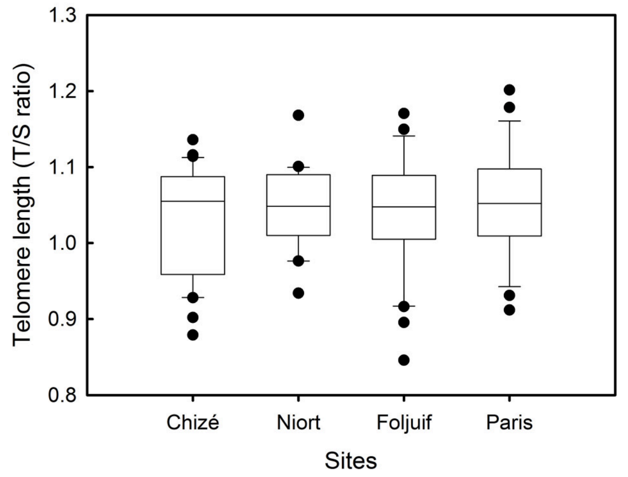

FIGURE 3 | Variation of telomere length among breeding sites in great tit nestlings reared in Chizé (woodland) and Niort (urban), Foljuif (woodland), and Paris (urban).

The diet of nestlings surely differs to a large extent between urban and rural habitats (Isaksson and Andersson, 2007) as shown by contrasted body composition between urban and rural wild birds (e.g., yolk and plasma fatty acids, Andersson et al., 2015; Toledo et al., 2016). However, our results also suggest that the carotenoid availability and/or uptake may only slightly differ within a given habitat type (e.g., between different urban habitats, between different forests). To our knowledge, our study is the first to compare the plumage coloration of two urban populations, but another study did not report any difference in plumage coloration between woodlands differing in size (Ferns and Hinsley, 2008). Similarly, egg yolk fatty acid composition did not significantly differ between different types of woodlands, confirming therefore that the diet may not dramatically differ between different types of woodlands (Toledo et al., 2016).

We did not find any correlation between body mass and plumage coloration in Niort, Foljuif and Chizé (although the relationship was marginally significant in Chizé for feather carotenoid chroma). This suggests that growth and plumage color are relatively independent and may be governed by different pathways, as previously suggested by Hõrak et al. (2000). Actually, carotenoid availability appears as the main driver of plumage coloration whereas structural growth is probably rather related to nutritional conditions that could be independent of carotenoid contents. In contrast to Niort, Chizé, and Foljuif, body mass was positively correlated with plumage carotenoid chroma in Paris (i.e., the most urbanized population in our study). In Paris, the largest chicks were also the most colorful. This suggests that growth and plumage coloration might be simultaneously reduced when nutritional conditions are especially constraining (see also Senar et al., 2003). Supporting the idea that plumage coloration can be related to the nutritional status of individuals under some circumstances, previous studies found that the plumage of infected individuals was paler than that of healthy ones (Møller et al., 2000; Hõrak et al., 2001). Similarly, Tschirren et al. (2003) experimentally demonstrated a positive relationship between growth conditions and plumage coloration in great tit nestlings. Therefore, our study highlights a complex and context-dependent relationship between growth and plumage coloration in developing nestlings (Hõrak et al., 2000; Tschirren et al., 2003; Isaksson and Andersson, 2007). Under constraining environmental conditions, parents may decide to allocate most of the food and most of the carotenoid-rich items to the healthier nestlings, resulting therefore in a positive relationship between growth and plumage coloration.

In the highly-urbanized site (Paris), we clearly found that the development of great tit nestlings was detrimentally impacted (smaller structural size and paler plumage). Although structural growth was not slowed down in the moderately-urbanized site (Niort), we found that plumage color was paler relative to rural nestlings. Because both structural size and plumage color have been related to subsequent performances (e.g., Hõrak et al., 2001; Naef-Daenzer et al., 2001; Blount et al., 2003), our study highlights that urbanization may have multiple effects on developing wild birds. Further studies are now needed to better assess how these urban-related phenotypic modifications translate into fitness consequences.

Urbanization and Telomere Length

Contrary to our prediction, we did not find any effect of urbanization on telomere length in 14-days old great tit nestlings. Thus, we did not detect any difference in telomere length between the four populations (Paris, Niort, Foljuif, Chizé), suggesting no impact of urbanization on telomere length in nestling great tits. Conversely, Salmón et al. (2016) found that telomeres of urban great tit nestlings were shorter than those of rural nestlings and these findings were recently supported by another ecotoxicological study (Stauffer et al., 2017). However, Stauffer et al. (2017) also found that telomere length was not affected by urban-like pollution in nestlings from another passerine species, the pied flycatcher (*Ficedula hypoleuca*). To our knowledge, the impact of urbanization on telomere dynamics has so far rarely been studied in wild vertebrates, and taken all together, these studies suggest that the influence of urbanization on telomere length may depend on the species but also on the environmental characteristics of a given city. It also indicates that urbanization does not necessarily translate into significantly shorter telomeres in wild passerines.

Other hypotheses could also explain why urbanization affects telomere length in some studies but not others. First, these differences could result from regional differences in environmental conditions. In our study, we captured nestlings from four sites (two cities and two forests) that were located in Western Europe. In contrast, Salmón et al. (2016) and Stauffer et al. (2017) studied Northern populations that were located in Scandinavia. Regional differences in life-history strategies or environmental conditions could therefore potentially account for these contrasted results. Second, Niort is a medium-sized city, and thus urbanization may not have been intense enough to induce a strong effect on telomere length in that site. This hypothesis is however quite unlikely because we also did not detect any difference in telomere length in Paris, which is one

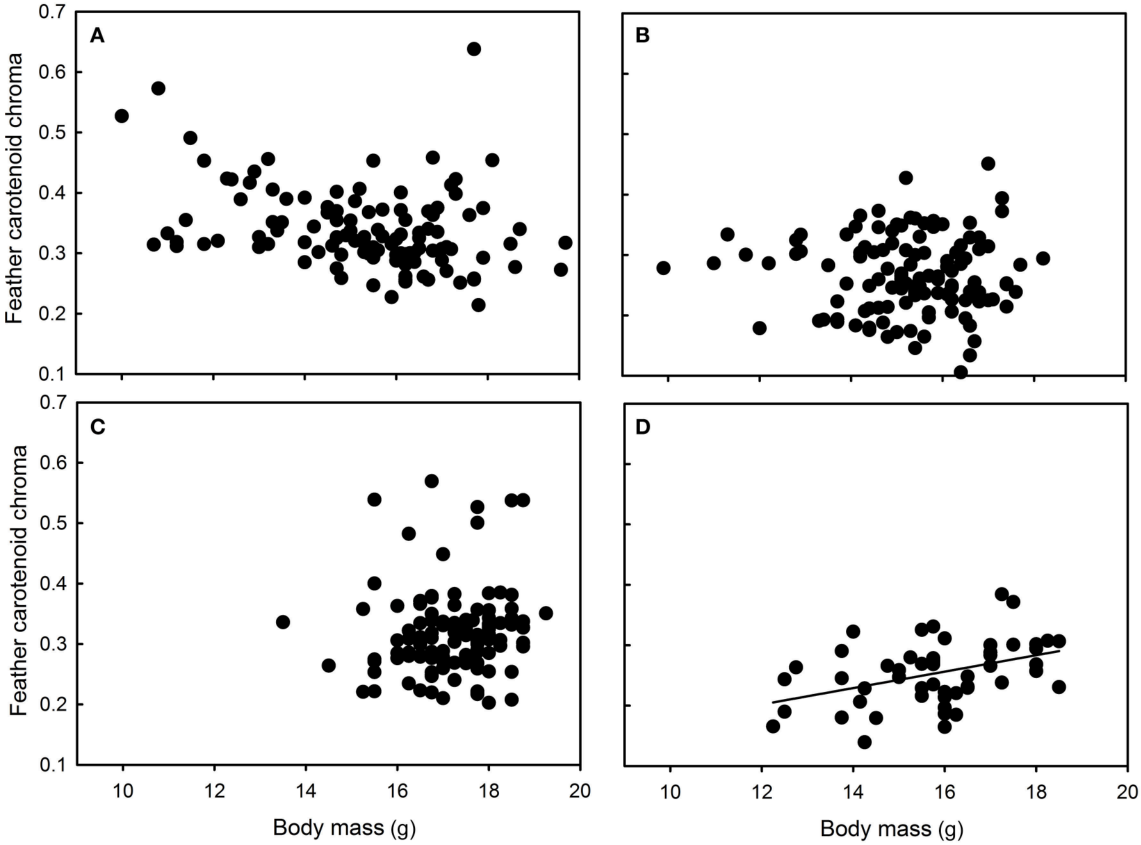

FIGURE 4 | Relationships between feather carotenoid chroma and body mass in great tit nestlings reared in **(A)** Chizé and **(B)** Niort, **(C)** Foljuif, and **(D)** Paris. For information, the marginally negative relationship in Chizé (see text for details), turned significant when the seemingly outlier data point is excluded [$F_{(1,\ 97)} = 8.38$, $p = 0.005$, estimate ± s.e. = −0.03 ± 0.01). There was however no biological or methodological reason to exclude a posteriori this nestling from the data set.

of the most densely inhabited city in Europe, as compared to the other three sites studied. Moreover, Salmón et al., 2016) sampled great tits in Malmö, which is much smaller than Paris. Thirdly, selection processes could have masked a potential effect of urbanization on telomere length (Haussmann and Mauck, 2008). Urbanization is known to be associated with intense brood competition, and thus with brood reduction (reviewed in Chamberlain et al., 2009). Accordingly, we found that brood sizes were overall smaller in cities (Paris and Niort) relative to woodlands (Foljuif and Chizé), although the difference was marginally significant between Niort and Chizé. This brood reduction means that most of the costs are probably borne by a few nestlings while the others are only slightly disadvantaged. Supporting this hypothesis, Stier et al. (2015) found in great tits that the last-hatched nestling has shorter telomeres than its siblings at fledging. Therefore, reduced telomere length could have only occurred in the weakest nestlings that did not reach 14-days old, and were therefore not sampled in our study. This would however mean that urbanization translates into the production of fewer offspring and not necessarily into the production of fledglings with shorter telomeres. Lastly, we may also raise the hypothesis that the telomere length of nestlings may have been preserved at the cost of growth and plumage coloration in the cities we studied (Paris and Niort). Telomere attrition is known to result from oxidative stress (Monaghan, 2014) that is associated with rapid growth (Geiger et al., 2012) and reduced antioxidant protection in wild birds (Monaghan et al., 2009). Because the nestlings from Paris were smaller relative to those from Foljuif, the growth of the nestlings from Paris could have been reduced to limit the oxidative damages. Similarly, most carotenoids could have been allocated to antioxidant functions, and thus telomere protection, at the cost of plumage coloration in urbanized sites (Niort and Paris) although the antioxidant role of carotenoids is debated (Costantini, 2008; Isaksson and Andersson, 2008).

We found that telomere length was not related to growth in Niort and Foljuif, suggesting that developmental nutritional deficit does not necessarily translate into shorter telomeres. However, we found a positive relationship between growth and telomere length in Chizé, suggesting that higher quality nestlings also had longer telomeres. This finding is supported by several studies that have reported a positive relationship between body size and telomere length in chicks or young individuals (Caprioli et al., 2013; Angelier et al., 2015; Parolini

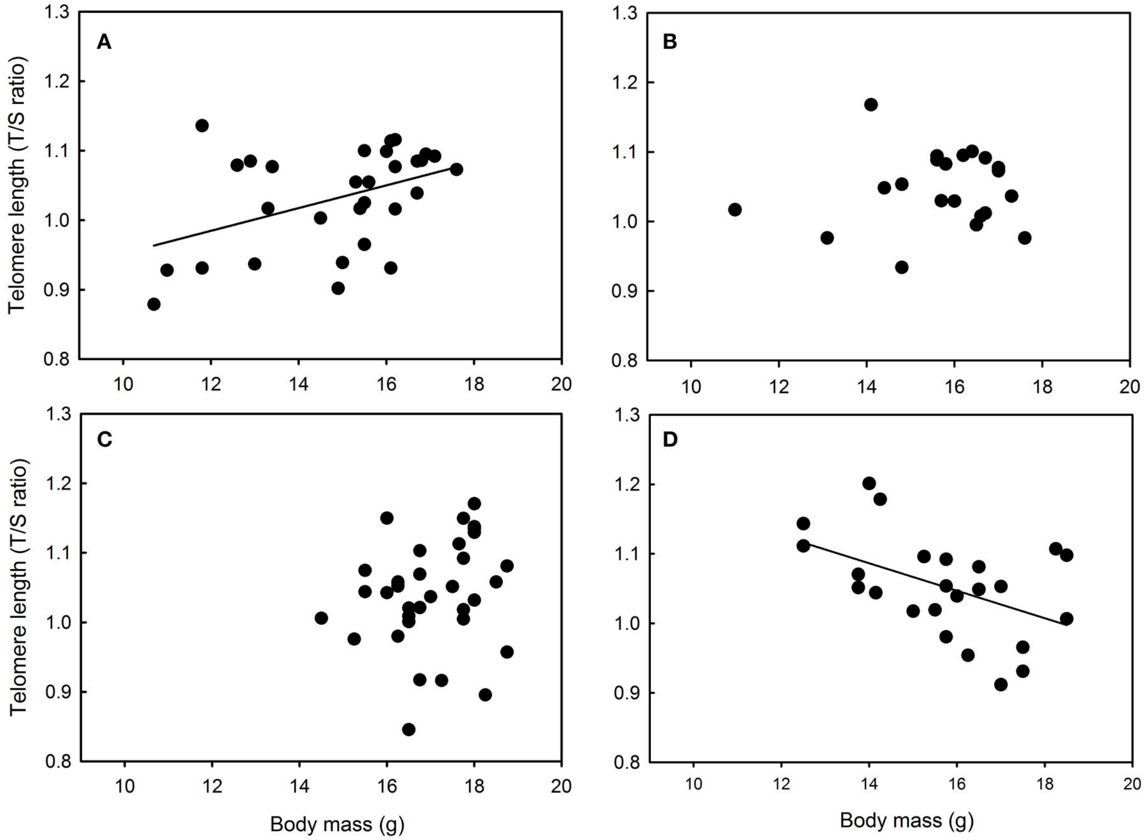

FIGURE 5 | Relationships between telomere length and body mass in great tit nestlings reared in **(A)** Chizé and **(B)** Niort, **(C)** Foljuif, and **(D)** Paris (see text for details of the statistics).

et al., 2015; Mizutani et al., 2016) and it suggests that long telomeres may be a proxy of good nutritional conditions, at least under some circumstances. Surprisingly, we found the opposite pattern in Paris where larger individuals had shorter telomeres. This suggests that structural growth may be made at the expense of telomere protection in highly urbanized populations. Accordingly, previous studies have found that accelerated growth can be associated with oxidative stress and telomere attrition in wild birds (Geiger et al., 2012; Stier et al., 2015). Overall, it remains however unclear why the relationship between telomere length and body size/growth differs to such extent between sites. It emphasizes that the link between urbanization, environmental conditions and telomere dynamics is complex and deserves further studies.

Regional Differences in Nestlings' Morphology and Reproductive Output

Interestingly, we reported a large regional difference in body size between the Paris (Paris and Foljuif) and the Niort geographical areas (Niort and Chizé). Thus, the nestlings from the Paris area (Paris and Foljuif) were overall larger and bigger than those from the Niort area (Niort and Chizé). Although, this difference could result from a different way of measuring the nestlings between sites, this is quite unlikely because all the ringers in France are trained to use similar measurement techniques. Moreover, we found exactly the same pattern for tarsus length and wing length, but also for body mass that is not subject to a handler effect (body mass is measured with an electronic or spring scale). This supports therefore the idea that these differences do not result from different measurements techniques. However, we cannot totally exclude the possibility that some differences in morphological data have to do with variation in measurement techniques Despite this potential bias, our result is supported by the Bergman's rule (i.e., selection of larger body size as temperature decreases, and thus as latitude increases, Ashton, 2002) and by previous studies that have also reported an increase in body size as latitude increases in this species (Snow, 1954; Encabo et al., 2002). Alternatively, other explanations could also be involved in these structural differences between the two areas. For instance, predation risk, food resources and more generally environmental conditions are known to be associated with regional variations in body size (reviewed in Blanckenhorn, 2000). In our study, such hypothesis could explain the differences in body size between populations. For example, environmental conditions (food resources) could have been particularly favorable to nestlings' development (e.g., growth) in Foljuif relative to our other study populations.

More generally, this variation in body size might have important implications when focusing on the impact of urbanization on growth and nestlings' phenotypes because growing a larger structural size requires necessarily more energy and this may be especially challenging in an impoverished habitat, such as the urban one. Consequently, it suggests that the influence of urbanization on reproduction and nestlings' phenotypes might differ between populations of great tits. So far, the influence of urbanization on nestlings' phenotypes of great tits has mainly been studied in Northern environments (e.g., Riddington and Gosler, 1995; Isaksson and Andersson, 2007; Eeva et al., 2009; Geens et al., 2009). Further studies should therefore focus on multiple environmental conditions (populations spread over a large geographic range) in order to better assess the impact of urbanization not only on nestlings and reproductive performances, but also on adult wild birds.

AUTHOR CONTRIBUTIONS

CB: Conceived the research, conducted field work, analyzed data, wrote the manuscript. FB: Conceived the research, conducted field work. AM: Conducted field work, provided comments on the manuscript. BM: Conducted field work. MN: Analyzed feather color. SR: Conducted molecular lab work. MV: Conducted field work, provided comments on the manuscript. FA: Conceived the research, conducted field work, wrote the manuscript.

ACKNOWLEDGMENTS

We are grateful to the ANR (URBASTRESS, ANR-16-CE02-0004-01), the Centre National de la Recherche Scientifique (CNRS), the University of La Rochelle (ULR), the Région Poitou-Charentes, the Conseil Départemental des Deux-Sèvres (DC 79), the Contrat Plan Etat region (CPER ECONAT), and Sorbonne Universités—University Pierre et Marie Curie Paris 06 (Émergence-UPMC EME 1110) for funding this research. This work conforms to French legal requirements, including those relating to conservation and welfare, and was conducted under ringing licenses and authorizations for blood and feather sampling from the CRBPO (National Museum of Natural History) to CB and FA (research programs # 537 and 664, respectively). The protocol was approved by the COMETHEA ethical committee (CE2013-03). We would also like to acknowledge the support of the National Forest Office (ONF) and the cities of Paris and Niort, in allowing us to survey tit populations breeding in state-owned forest and city parks, respectively. We also thank all the students for their help in conducting fieldwork and Janice Duru for her help in the lab with feather color analyses.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 16 June 2017
doi: 10.3389/fevo.2017.00064

Elevated Immune Gene Expression Is Associated with Poor Reproductive Success of Urban Blue Tits

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *25 February 2017*
Accepted: *31 May 2017*
Published: *16 June 2017*

Citation:
Capilla-Lasheras P, Dominoni DM, Babayan SA, O'Shaughnessy PJ, Mladenova M, Woodford L, Pollock CJ, Barr T, Baldini F and Helm B (2017) Elevated Immune Gene Expression Is Associated with Poor Reproductive Success of Urban Blue Tits. Front. Ecol. Evol. 5:64. doi: 10.3389/fevo.2017.00064

Urban and forest habitats differ in many aspects that can lead to modifications of the immune system of wild animals. Altered parasite communities, pollution, and artificial light at night in cities have been associated with exacerbated inflammatory responses, with possibly negative fitness consequences, but few data are available from free-living animals. Here, we investigate how urbanization affects major immune pathways and experimentally test potentially contributing factors in blue tits (*Cyanistes caeruleus*) from an urban and forest site. We first compared breeding adults by quantifying the mRNA transcript levels of proteins associated with anti-bacterial, anti-malarial (TLR4, LY86) and anti-helminthic (Type 2 transcription factor GATA3) immune responses. Adult urban and forest blue tits differed in gene expression, with significantly increased *TLR4* and *GATA3*, but not *LY86*, in the city. We then experimentally tested whether these differences were environmentally induced by cross-fostering eggs between the sites and measuring mRNA transcripts in nestlings. The populations differed in reduced reproductive success, with a lower fledging success and lower fledgling weight recorded at the urban site. This mirrors the findings of our twin study reporting that the urban site was severely resource limited when compared to the forest. Because of low urban survival, robust gene expression data were only obtained from nestlings reared in the forest. Transcript levels in these nestlings showed no (*TLR4*, *LY86*), or weak (*GATA3*), differences according to their origin from forest or city nests, suggesting little genetic or maternal contribution to nestling immune transcript levels. Lastly, to investigate differences in parasite pressure between urban and forest sites, we measured the prevalence of malaria in adult and nestling blood. Prevalence was invariably high across environments and not associated with the transcript levels of the studied immune genes. Our results support the hypothesis that inflammatory pathways are activated in an urban environment and suggest that these differences are most likely induced by environmental factors.

Keywords: urban ecology, inflammation, immunity, gene expression, blue tits, TLR4, LY86, GATA3

INTRODUCTION

Urban areas are the fastest growing land cover globally and are projected to increase a further 30% by 2030 (Seto et al., 2012). The modification of the natural landscape caused by urbanization poses novel challenges to wildlife. For example, urbanization is associated with altered food webs, parasite communities, and chemical, light and noise pollution (Grimm et al., 2008; Alberti, 2015; Isaksson, 2015). To thrive in these novel environments requires changes in behavior and physiology at the phenological and, possibly, genetic level. Such changes have, indeed, been found in urban populations of several species, indicating that they accommodate some of the challenges of city life (Isaksson, 2010; Munshi-South and Kharchenko, 2010; Dominoni et al., 2013a, 2016; Nemeth et al., 2013; Atwell et al., 2014; Gil et al., 2014; Giraudeau et al., 2014; Davies et al., 2016; Watson et al., 2017). Nonetheless, urban environments are linked to reductions in fitness and health in a substantial number of organisms (Chamberlain et al., 2009; Murray et al., 2015). Hence, urban habitats are sometimes considered ecological traps which attract organisms, for example by anthropogenic food availability, but are insufficient for supporting successful rearing of offspring (Plummer et al., 2013; Sumasgutner et al., 2014; Lamb et al., 2017). There is a need, therefore, to understand the mechanisms by which urbanization reduces reproductive success.

Despite their relevance for understanding urban adaptation, the proximate physiological mechanisms that underpin differences in behavior, life histories and fitness between urban and rural populations of wild animals still remain largely elusive (but see Dominoni et al., 2013b; Atwell et al., 2014; Davies et al., 2016; Salmón et al., 2016). Whereas some physiological responses in urban animals have been investigated thoroughly, such as reproductive (Schoech et al., 2004; Partecke et al., 2006; Dominoni et al., 2013b) and stress physiology (Bonier, 2012), others have received little attention. Recently, a study using transcriptome analysis (RNA-seq) has broadly examined the ways physiological systems differ between an urban and rural population of a songbird, the great tit (*Parus major*) (Watson et al., 2017). Among the systems highlighted in this study, immunity showed particularly clear differences, corroborating reports of substantially reduced health of urban populations in several species (Martin et al., 2010; Isaksson, 2015; Murray et al., 2015). Given the central role of the immune system for determining fitness and given its sensitivity to environmental factors, differences in immunity are thus strong candidates for linking urbanization to reduced reproductive success (Martin et al., 2010; Isaksson, 2015; Watson et al., 2017).

Urban environments can affect the immune system by modulating the structure of the parasite community to which animals are exposed and potentially respond to. Cities have been associated with altered parasite communities and altered infection dynamics (Fokidis et al., 2008; Evans et al., 2009; Giraudeau et al., 2014; Neiderud, 2015). For example, foraging behavior in cities is thought to enhance the prevalence of intestinal coccidians (Giraudeau et al., 2014), potentially driven by increasing contact rates at feeders (Becker et al., 2015). Several other factors associated with urban environments can affect the activation of particular immune pathways of wild animals. Prevalent urban conditions, such as chemical pollution and artificial light at night, can act as environmental stressors influencing the immune system and promoting pro-inflammatory immune profiles (Halliwell and Gutteridge, 2002; Bedrosian et al., 2011; Fonken et al., 2013; Isaksson, 2015; Watson et al., 2017). Low-quality and changed diet in urban areas can also enhance pro-inflammatory immune processes, which in turn can impose behavioral and physiological costs on animals (McGraw et al., 2006; Andersson et al., 2015; Becker et al., 2015; Isaksson, 2015). Furthermore, nutritional stress and resource restriction can exacerbate trade-offs between different biological processes, for example through resource allocation to growth vs. immunity (Sheldon and Verhulst, 1996; Norris and Evans, 2000). The findings of the transcriptomics study by Watson et al. (2017) showed that in an urban songbird population, where some of the above factors are effective, inflammatory immune genes were indeed upregulated. Experimental studies that measure candidate genes are now needed to clarify the origin of such differences, and links to fitness need to be established.

Here we examined how urban life affects avian immunity and fitness traits. As a study species, we used another parid songbird that is common in city and forest environments, the blue tit (*Cyanistes caeruleus*). Blue tits reportedly show reduced reproductive investment (e.g., clutch size and egg size) and reduced reproductive success in urban compared to rural populations (Chamberlain et al., 2009; Bailly et al., 2016b). We experimentally studied blue tits at two sites, one in an urban park area in Glasgow (UK), and one in a National Park 40 km away. The same populations were simultaneously investigated in a twin study on food resource availability, food provisioning and stable isotope signatures in eggs and blood samples (Pollock et al., 2017). This parallel study design allowed us to link, for the same sites, comparisons of immunity with resource restriction. We first tested the hypothesis that adult urban blue tits show increased expression of genes associated with inflammation compared to blue tits from a forest habitat. Specifically, using RT-qPCR, we measured the transcript levels of TLR4 and LY86, which are involved in anti-bacterial and anti-malarial responses (Medzhitov, 2001). We also tested the type 2 transcription factor GATA3, which is central to innate and adaptive immunity against parasitic helminths and immune homeostasis (Wang et al., 2011; Tindemans et al., 2014) and showed elevated gene expression in urban great tits (Watson et al., 2017).

We then investigated whether any urban-forest differences in immune gene transcript levels are already present during the nestling stage. Urban-forest immune differences in nestlings could arise either through environmental effects during early life, genetic or maternal effects. Thus, we cross-fostered eggs between and within the urban and forest sites to distinguish whether any difference in immune activation of nestlings was induced by the environment or controlled by genetic or maternal mechanisms (Watson et al., 2017). Finally, from the cross-fostering experiment, we quantified the reproductive success at the two sites and, thus, investigated possible associations between environment, immunity and reproductive success. We also examined prevalence of a common avian disease, avian malaria, as a potential driver of differences in expression of our immune markers (Martin et al., 2014; Videvall et al., 2015).

Increased levels of *TLR4* and *LY86* in adult urban birds are predicted on the basis of the hypothesis that the urban environment enhances inflammation. We also predicted increased levels of *GATA3* in adult urban birds as a consequence of high activation of immune pathways against intestinal parasites, whose transmission is thought to be exacerbated by anthropogenic food provisioning at bird feeders (Becker et al., 2015). Expression of these three genes was also reported to be increased in at least one tissue in the transcriptome study on great tits (Watson et al., 2017). For nestlings blue tits, we predicted that urban-raised offspring might also show heightened expression of immune genes, indicating a direct effect of the environment on their immune profiles within their short postnatal life-span. We further predicted that cross-fostered nestlings should also differ in their immune gene expression profiles by origin, either due to micro-evolutionary change or based on differences in maternal investment. Assuming that higher expression levels in immune genes were selected for in urban birds, we expected higher expression levels in nestlings originating from eggs laid the urban environment regardless of their rearing environment. We also expected lower reproductive investment, fledging success and fledgling body mass in blue tits from the urban compared to the forest environment. The differences in reproductive success of urban and forest nests were indeed striking: 88% fledgling success in the forest compared to only 30% in the city. Consequently, in the cross-fostering experiment we obtained robust sample sizes on gene expression only for nestlings reared in the forest. We acknowledge limitations of our study arising from low sample sizes and from a design that involves only two sites (one urban and one rural) in a single year. Our experimental approach, using cross-fostering, addresses some of these concerns by effectively providing within-site replication. However, urban ecology will require studies in multiple sites and years, and on a broad range of organisms, before major, generalizable advances can be achieved (Watson et al., 2017).

MATERIALS AND METHODS

Experimental Design and Field Protocol

Field work was carried out in one urban and one forest location in Scotland in May and June of 2014. In both sites, existing nest-box study systems were used (woodcrete boxes: 260 × 170 × 180 mm, Ø = 32 mm, Schwegler, Germany). The urban site was located in Kelvingrove Park, Glasgow (55° 52.18N 4° 17.22W), with a total of 60 bird nest-boxes. The forest location was situated in oak woodland near the Scottish Centre for Ecology and the Natural Environment (SCENE; 56° 7.73N 4° 36.79W), with a total of 143 bird nest-boxes. Twenty clutches of blue tits in the city and 22 in the forest were manipulated prior to clutch completion: 10 nests in the city and 12 in the forest were swapped within locations, representing control nests; and 10 nests were swapped across locations, representing experimentally cross-fostered nests (in total, 42 manipulated nests). Clutches within and across locations were matched based on sixth-egg laying date. Before swapping clutches, every egg involved in the experiment was individually marked, weighed (±0.01grams) and kept at 4°C overnight. Clutch size was reduced to six viable eggs at both sites in order to control for possible inter-habitat differences in clutch size. After females laid the sixth egg, clutches were swapped as explained above. When more than six eggs were present in a nest on the day of swapping, the six experimental eggs were randomly chosen. After swapping clutches, nests were checked every other day and newly laid eggs were replaced by dummy eggs. Using dummy eggs, we always kept the original number of eggs laid by females (each nest contained six real eggs plus a variable number of dummy eggs depending on the number of eggs that females actually laid). Dummy eggs were removed from nests after real eggs hatched. The total number of eggs laid by each female was recorded and termed "natural clutch size."

After the 10th day of incubation, nests were monitored daily and hatch date was precisely assigned for every nest. On day 13 after first-egg hatching, blue tit nestlings were weighed (±0.05 grams), ringed with a unique metal ring and between 20 and 75 μL of blood were collected from their brachial veins (stored in 250 μL of RNAlater® for gene expression analysis). Additionally, between 20 and 75 μL of whole blood were stored in >99% ethanol for molecular screening of malaria parasites. Between 10 and 12 days post-hatching, we aimed to capture one or two of the respective parents while provisioning and to sample adults as described for chicks. However, when we realized the low overall breeding success in our urban population we greatly reduced efforts of catching parents to avoid additional risks to the broods. In total, we obtained samples of 24 adult birds (**Table 1**). Nest-boxes were checked 16–20 days after expected fledging dates in search of dead nestlings. Since clutch size was reduced to six eggs, hatching success was defined as number of hatchlings divided by six. Number of fledglings over number of hatchlings represented fledging success. **Table 1** summarizes sample sizes throughout different breeding stages between our urban and forest site, which became progressively disparate because of high chick mortality in the city.

Ethics Statement

All bird sampling was conducted following the directions and legislations of UK Home Office (project license: 70/7899 to BH, and personal licenses to DD and BH), Scottish Natural Heritage (52463 to BH) and British Trust for Ornithology (Scientific C and T licenses to BH and DD, respectively).

Expression of Immune and Reference Genes

Primer Design

Blue tit sequences of *TLR4* (toll-like receptor 4), *LY86* (lymphocyte antigen 86, also known as MD1), *GATA3* (GATA Binding Protein 3) and of the candidate reference genes, *HPRT* (Hypoxanthine-guanine phosphoribosyl transferase), *PMM1* (Phosphomannomutase 1), *SDHA* (succinate dehydrogenase complex, subunit A) and *TFRC* (transferrin receptor protein 1) (Olias et al., 2014) were obtained from the blue tit genome (Mueller et al., 2016). Gene sequences were compared in BLAST against the zebra finch (*Taeniopygia guttata*) genome (Warren et al., 2010) in order to design primers on the

TABLE 1 | Summary of sample sizes that entered the analysis: number of clutches, nestlings and adults of each experimental group at every breeding stage for our two study sites.

		Urban	Forest	Total
Initial number of nests				
Cross-fostered group		10	10	20
Control group		10	12	22
Total		20	22	42
Number of clutches successfully hatched[a]				
Cross-fostered group		7	6	13
Control group		8	9	17
Total		15	15	30
Number of hatchlings[b]				
Cross-fostered group		32	25	57
Control group		30	39	69
Total		62	64	126
Number of fledglings				
Cross-fostered group		9	20	29
Control group		9	36	45
Total		18	56	74
Number of individuals sampled for RT-qPCR analysis[c]				
Nestlings	Cross-fostered group	4	16	20
	Control group	4	24	29
	Total	8	40	48
Adults		13	11	24

Cross-fostered clutches were swapped between sites, control clutches were swapped within sites.

[a] Clutches with at least one egg hatched. [b] Maximum number of hatchlings per clutches was six since every clutch was experimentally reduced to six eggs. [c] With successful gene expression data for both reference genes used in data normalization.

correct gene regions. Primers were designed using Primer Express™ 2.0.0 (Applied Biosystems) as described previously (O'Shaughnessy et al., 2008). In order to avoid genomic DNA (gDNA) amplification, every primer pair was designed to flank an intron of more than 1,000 base pairs whenever possible. All primer sequences (Table S1), as well as the validation of reference genes (Figure S1), are detailed as Supplementary Material.

RNA Extraction, Reverse Transcription and RT-qPCR Protocol

Blood samples stored in RNAlater® were centrifuged for 5 min at 5,000 RPM, and RNA in the cell fraction isolated using TRIzol® reagent (Life Technologies, Thermo Fisher Scientific 2015). Extracted RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript III, Invitrogen Ltd.) (for detailed protocols see O'Shaughnessy and Murphy, 1993; O'Shaughnessy et al., 1994). Levels of mRNA were measured by real-time quantitative PCR using the SYBR Green method (O'Shaughnessy and Murphy, 1993; O'Shaughnessy et al., 1994). The efficiency of the seven employed primer pairs was between 91 and 120% in all the cases. None of the primer pairs amplified gDNA. One unique gene was run in each RT-qPCR 96-well plate along with a non-template control (NTC). Every sample was always run in duplicate and only samples with similar duplicate results were considered for final analysis. The vast majority of samples included in analyses had C_t differences between replicates below one. Five samples showed C_t differences between one and two, and two additional samples in the final analysis had replicate C_t differences larger than two (2.07 and 2.18). The exclusion of the samples with C_t differences above 2 or the samples with C_t differences above 1 did not change the results. For details of RT-qPCR data normalization, see Supplementary Material.

Screening of Malaria Parasites

Avian malaria collectively refers to blood parasites of the three taxa *Leucocytozoon*, *Haemoproteus* and/or *Plasmodium* [PMID:15357072]. We tested for the presence or absence of any of these parasites using a nested PCR approach which identified *Leucocytozoon* in one reaction, and either of *Haemoproteus* and *Plasmodium* in another reaction (Hellgren et al., 2004). In short, DNA from blood samples stored in >99%-ethanol was extracted using a commercial kit (DNeasy whole-blood extraction kit, Qiagen). Eluted DNA was amplified by 20 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 45 s. A 2 μl-aliquot from the result of this reaction was further amplified for 35 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s. Both PCRs were performed in 20-μl reaction mixture using 10 μl of GoTaq® universal PCR master mix (Promega) and 0.6 μM of each primer. Products of the second PCR reaction were examined on a 1% agarose gel. The presence of *Leucocytozoon* and/or *Haemoproteus*/*Plasmodium* parasites was further checked by sequencing 24 PCR positive bands (12 for *Leucocytozoon* and 12 for *Haemoproteus*/*Plasmodium*, corresponding to samples positive for both taxa) (Eurofins Genomics). In all the positive samples that we tested, sequencing confirmed the presence of at least one avian malaria parasite, *Leucocytozoon* (GenBank Accession Numbers KY981756-KY981765) and/or *Haemoproteus* (GenBank Accession Numbers KY981753-KY981755). No *Plasmodium* was detected. In nine out of 12 samples that were positive for both *Leucocytozoon* and *Haemoproteus* by PCR, sequencing revealed that only one of the two parasites was actually present, suggesting that the observed double infections might not always be true but possibly an artifact of the PCR procedure due to cross-reaction of the primers.

Statistical Analysis

Model Selection Approach

For every modeled response variable, data analysis started with a global model including every biologically important predictor (see below). Then, model selection based on Akaike's Information Criterion (AIC, Burnham and Anderson, 2002; Burnham, 2004; Burnham et al., 2011) was applied to investigate alternative hypotheses on the effects of urban–forests conditions and of our experimental manipulation on immune gene expression, breeding investment and breeding success. Models were ranked based on their AICc and considered similarly supported if their ΔAICc value was <2. Poisson and binomial models were checked for over-dispersion by comparing residual deviance and residual degrees of freedom. Linear models and linear mixed model residuals were inspected to check that they met the assumption of normality, and collinearity between pairs of explanatory variables was checked before accepting the results of any statistical model. Data analysis was carried out in R 3.3.2 (R Core Team, 2016) using the *lme4* (Bates et al., 2014) and *MuMin* (Barton, 2016) packages.

Immune Transcript Levels and Malaria Prevalence in Adult Blue Tits

After RT-qPCR data normalization, adult levels of *TLR4*, *LY86*, and *GATA3* were analyzed using linear models (LMs). Habitat (a factor with two levels: urban or forest) and adult body mass 10–12 days after hatching of their clutch were originally included as predictors in every model. Weight information was missed for two adult birds with successful gene expression data. Weight appeared to have little importance in explaining variation in gene expression and we, therefore, present statistical results for models containing weight in Table S2. In the main text, we report results for models without weight but include the two additional birds to increase our sample size and, hence, the confidence in our estimates of the effect of habitat. Malaria prevalence was compared across habitats using Fisher's exact test. Because little variation in infection status was observed in adult birds, we were unable to investigate its association with immune transcript levels.

Immune Transcript Levels and Malaria Prevalence in Cross-Fostered Nestlings

Originally, the main purpose of this analysis was to investigate the effect of the original and rearing environment on immune transcript levels. However, because of the high mortality in our urban environment (see Section Results), sample sizes from urban raised nestlings were very low. Hence, we focus our main analysis only on nestlings raised in the forest and present preliminary results for the full cross-fostering design as Supplementary Material.

Nestling TLR4, LY86, and GATA3 transcript levels were analyzed using linear-mixed models (LMMs). Original environment (a factor with two levels: urban or forest) and weight of chicks on day 13 were included as fixed effects. Malaria infection status (factor with two levels, YES/NO) was also included as a predictor. Nest ID was included as a random factor in every model as several nestlings were measured per nest.

Reproductive Investment and Breeding Success of Urban and Forest Cross-Fostered Nests

We compared several measures of reproductive investment and breeding success of adult females as follows. Female investment was quantified by number (i.e., natural clutch size) and size of eggs laid. Natural clutch size was modeled as a Poisson variable in a generalized linear model (GLM) including habitat, and clutch completion date (as a linear and a quadratic term) to account for temporal trends. We also included experimental group (cross-fostered or control) and its interaction with site to test whether our experimental design differed across habitats. Individual egg weight was modeled using LMMs including as explanatory variables original environment, experimental group, their interaction, natural clutch size and 1st egg laying date (as a linear and a quadratic term). Nest ID was always kept as random factor.

We then examined reproductive success of the urban and forest nests. Hatching and fledging success were analyzed with generalized linear mixed models (GLMMs) using binomial distributions with logit link functions. Due to over-dispersed model residuals, observation-level random factors were employed and yielded good model fit (Harrison, 2015). Original and rearing environment, their interaction, and clutch completion date—for hatching success—or hatch date (linear and quadratic terms)—for fledgling success—were included as explanatory variables. Weight of fledglings on day 13 after hatching was analyzed by a LMM, keeping Nest ID as a random factor and using original and rearing environment, their interaction, hatch date (linear and quadratic terms), brood size on day 13, and the interaction between rearing environment and brood size as explanatory variables.

RESULTS

Immune Transcript Levels and Malaria Prevalence of Urban and Forest Adults

Adult urban blue tits showed higher levels of *TLR4* and *GATA3* than forest blue tits (**Figure 1**). Including habitat as a predictor generated models for these two immune genes that were superior in AICc than intercept-only models (**Table 2**). Removing a high-value outlier in GATA3 transcript levels of urban birds (**Figure 1**) did not qualitatively change the statistical outcome. After removal of this outlier, the model containing habitat as a predictor was still the most supported by the data, with a decrease in AICc of 2.4 compared to the intercept-only model. For *LY86* expression, we did not find statistical evidence for an alteration across habitats (**Table 2**, **Figure 1B**). Body weight of adults 10–12 days after hatching of their clutch was not an important predictor for transcript levels of any immune gene (Table S2).

Every screened adult urban bird scored positive for malaria infection ($n = 6$). Out of 9 forest birds tested, 8 were positive for malaria parasites. These results indicated no association between malaria prevalence and habitat in adult blue tits (Fisher's exact test, $p > 0.90$), suggesting that differential pressure of malaria parasites across environments does not underlay our results of immune gene expression.

Immune Transcript Levels and Malaria Prevalence of Forest-Reared Nestlings

Our data suggested no differences in transcript levels of TLR4 and LY86 in forest-reared nestlings due to habitat of origin, malaria parasite infection or weight 13 days after hatching (**Figure 2**). For the expression of these two genes, intercept-only models were most supported by the data (**Table 3**). The presence of malaria parasites did not predict *TLR4* or *LY86* levels. Malaria infection status was only kept in models with ΔAICc values larger than 2. For *GATA3*, we found some evidence for an effect of the original environment on nestling transcript levels. Urban-originated nestlings reared in the forest had higher levels of *GATA3* than forest-originated forest-reared birds (**Figure 2C**). Four competing models scored very similar AICc values, two of them containing original environment and weight on day 13 as predictors (**Table 3**). However, after removal of an urban-originated outlier with high *GATA3* (**Figure 2C**), the effect of weight and of the original environment lost much importance and the intercept-only model became the most supported one (Table S3). Results for the full cross-fostering experiment, including urban-reared forest birds, are shown in Figure S2.

Prevalence of avian malaria in forest-reared nestlings was 79.16% ($n = 48$, $SE = 5.92\%$), whereas every screened urban-reared nestling was found positive ($n = 4$). Within forest-reared birds, malaria prevalence did not vary based on nestling origin (Fisher's exact test, $p = 0.468$, $n = 38$. Prevalence ± standard error: forest-originated birds = 0.83 ± 0.38, urban-originated birds = 0.72 ± 0.46).

Breeding Success in Urban and Forest Cross-Fostered Nests

Most metrics of breeding investment and success were lower in the urban compared to the forest site (**Figure 3**). Despite observed differences in clutch size between habitats (mean clutch size ± SE: urban clutches = 7.70 ± 0.34, $n = 20$; forest clutches = 8.63 ± 0.48, $n = 22$), they did not received strong statistical support (**Table 4**). Urban-originated eggs were slightly lighter than forest-originated eggs and habitat of origin appeared in three out of four models with ΔAICc < 2 for egg weight (the most supported one amongst them); however, the effect size was small, with urban eggs only 0.045 g lighter than forest ones. Experimental group was also kept in the set of top models showing that cross-fostered eggs were 0.05 grams heavier than control eggs (**Figure 3A**). No predictor was retained

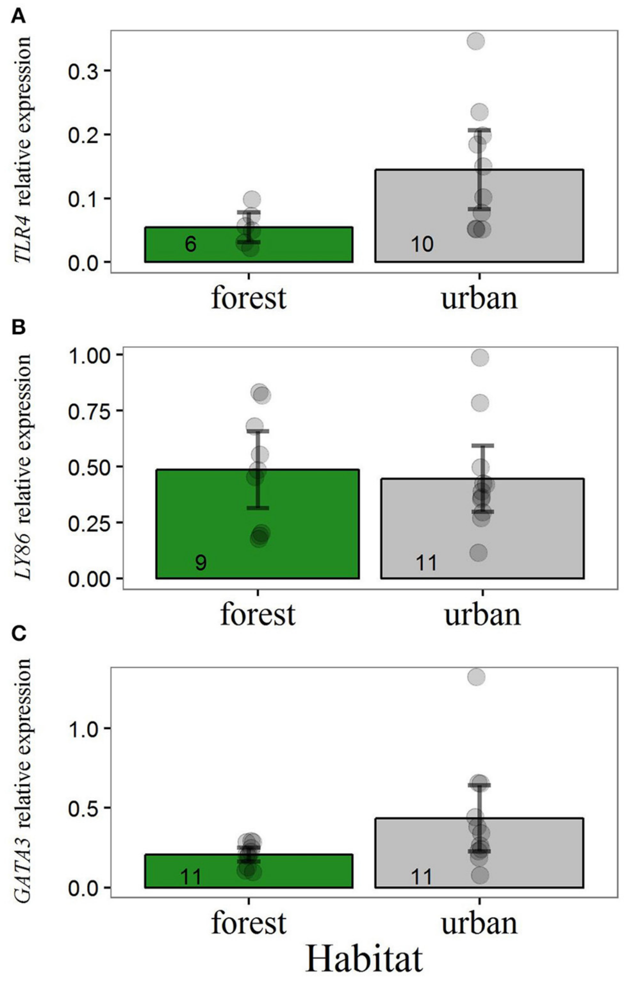

FIGURE 1 | Relative expression of **(A)** *TLR4*, **(B)** *LY86*, and **(C)** *GATA3* for adult, breeding blue tits. Y-axis values refer to levels of gene expression relative to the expression of reference genes. Main bars illustrate raw data mean values and dark error bars illustrate ± 2 standard errors. Dots represent raw data points. Sample sizes are illustrated at the base of each bar.

TABLE 2 | Summarizing table of statistical models employed to explain variation in TLR4, LY86, and GATA3 transcript levels for adult blue tits.

	Estimates of model coefficients (standard error)					
Response term	**Intercept**	**Habitat – urban^a**	***k***	**AICc**	**ΔAICc**	***w***
TLR4 gene expression ($n = 16$; 14 nest-boxes)	0.055 (0.033)	0.090 (0.041) *	3	−29.5	0.0	0.69
	0.111 (0.022)		2	−27.9	1.6	0.31
LY86 gene expression ($n = 20$; 17 nest-boxes)	0.464 (0.055)		2	4.1	0.0	0.79
	0.486 (0.083)	−0.041 (0.112)	3	6.7	2.7	0.21
GATA3 gene expression ($n = 22$, 17 nest-boxes)	0.206 (0.077)	0.229 (0.106) *	3	6.5	0.0	0.72
	0.321 (0.058)		2	8.4	1.9	0.28

*Models are presented along with their coefficient estimates and standard errors [estimate (SE)]. k, number of model parameters; w = Akaike's weight, defined as the probability of a model given the data and the candidate set of alternative models. ^{a}"Habitat – forest" set as reference level and, therefore, equals zero. When top models contained "habitat," this predictor was assessed by a likelihood-ratio test against the intercept-only model and * illustrates a p-value < 0.05. See Table S2 for model estimates including adult weight.*

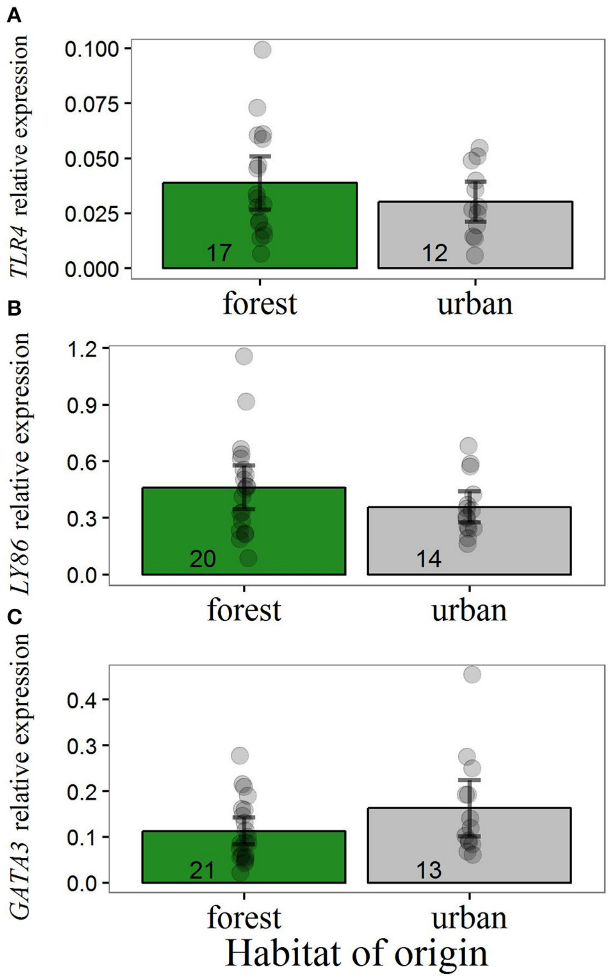

FIGURE 2 | Relative expression of **(A)** *TLR4*, **(B)** *LY86*, and **(C)** *GATA3* for forest-reared nestlings hatched from cross-fostered eggs of the urban or forest site. Y-axis values refer to transcript levels relative to the expression of reference genes. Main bars illustrate raw data mean values and dark error bars illustrate ± 2 standard errors. Dots represent raw data points. Sample sizes are illustrated at the base of each bar. Removal of the urban outlier in *GATA3* analysis **(C)** led to qualitative and quantitative changes in the results regarding this gene (Table S3). Differences in sample size between **Figure 2** and **Table 1** are due to two nestlings with missing information for body weight. The inclusion of these additional data points does not change the results regarding the effect of the rearing habitat.

in the most supported model for hatching success (**Table 5**, **Figure 3B**) and habitat of origin only appeared in a model featuring a ΔAICc value of 1.89 (ranked third in support). These results suggested no differences in hatching success due to habitat of origin or rearing habitat (**Table 5**). The urban rearing environment, however, had a very strong negative effect on fledging success as well as on nestling weight (**Table 5**). Regardless of their origin, forest-reared nestlings were on average 1.33 grams heavier and more than twice as likely to fledge as urban-reared birds (**Figures 3C,D**). Rearing habitat appeared in every model within the Δ2 set for fledging success and nestling weight (**Table 5**).

DISCUSSION

Reproductive success of blue tits was dramatically reduced in the city compared to the forest. Our study adds to existing evidence that urban environments commonly impair fitness and health in wild populations (Chamberlain et al., 2009; Murray et al., 2015; Bailly et al., 2016b). In most avian studies, fledging success and nestling weight were lower in urban than in more rural habitats (Chamberlain et al., 2009; Bailly et al., 2016b; Sprau et al., 2017). These findings are matched by observations in this study and in the parallel study of effects of food availability on other nests at our sites (Pollock et al., 2017). In addition to immediate effects of the environment on developing offspring, such differences could also reflect prenatal maternal investment (e.g., differences in egg composition, Toledo et al., 2016) or genetic differences between urban and rural populations (Mueller et al., 2013). Our cross-fostering experiment does not support these latter hypotheses as we found no strong effects of natal origin on reproductive and fitness traits (**Figure 3**). Slightly larger cross-fostered eggs from both sites were a spurious outcome of our alternating, experimental swaps. Overall, we provide experimental support for the existence of a significant negative postnatal effect of the urban environmental on breeding success in blue tits, although we acknowledge that further replication will be needed to consolidate these results.

These findings converge with a recent study on closely related great tits which also used a cross-fostering experiment and showed that negative implications of urban nesting (in this case, shortened telomeres) arose from the raising environment and not from genetic or maternal factors (Salmón et al., 2016); however, this study did not investigate the possible mechanistic causes of such differences. Our parallel study on food availability and provisioning behavior provided clear evidence of resource limitation and nutritional stress in our urban site compared to our forest location (Pollock et al., 2017). Whereas nestlings in the forest received predominantly caterpillars, the proportion of this preferred, high-quality diet was substantially lower for nestlings in the city. Pollock's data further suggest that parents partly resorted to other food, possibly including anthropogenic sources, to feed their nestlings and presumably themselves (Pollock et al., 2017). Shifts in diet, which were also reported for urban populations in other species (e.g., Murray et al., 2015), can have important effects on the nutritional state of urban animals, and consequently, also on their health (Andersson et al., 2015). Thus, the findings that we present here on immunity have to be interpreted against the backdrop of resource restriction in the urban site.

Our results from adult blue tits on transcript levels of immune genes support some of our initial hypotheses. Urban blue tits showed increased transcript levels of TLR4, a marker of inflammatory processes, although these findings were not paralleled for *LY86*. Several factors have been proposed to explain how the immune system of urban organisms is expected to change in response to the environment (Isaksson, 2015). Malaria parasite infection has been shown to affect the expression of *TLR4* (Martin et al., 2014) and also *LY86* in passerines birds (Videvall et al., 2015). In our study, malaria infection status

TABLE 3 | Summarizing table of statistical models employed to explain variation in TLR4, LY86, and GATA3 transcript levels for forest-reared nestlings.

	Estimates of model coefficients (standard error)								
Response term	**Intercept**	**Original Habitat – urban^{a}**	**Malaria parasites^{b}**	**Weight**	**Original Habitat – urban^{a} X weight**	***k***	**AICc**	**ΔAICc**	***w***
TLR4 gene expression (*n* = 28; 13 nest-boxes)	0.036 (0.004)					3	−128.9	0.00	0.39
	0.104 (0.060)			−0.006 (0.005)		4	−127.4	1.46	0.19
	0.039 (0.005)	−0.007 (0.008)				4	−126.9	1.98	0.14
	0.036 (0.010)		0.001 (0.011)			4	−126.2	2.73	0.10
	0.115 (0.059)	−0.008 (0.008)		−0.007 (0.005)		5	−125.5	3.36	0.07
	0.106 (0.060)		0.004 (0.011)	−0.007 (0.005)		5	−124.6	4.33	0.05
	0.040 (0.011)	−0.001 (0.011)	−0.001 (0.008)			5	−123.9	4.96	0.03
LY86 gene expression (*n* = 32; 13 nest-boxes)	0.418 (0.039)					3	1.7	0.00	0.41
	0.453 (0.050)	−0.086 (0.079)				4	3.1	1.45	0.20
	0.392 (0.091)		0.032 (0.101)			4	4.2	2.52	0.12
	0.357 (0.597)			0.005 (0.053)		4	4.3	2.61	0.11
	0.434 (0.098)	−0.084 (0.079)	0.023 (0.010)			5	5.9	4.23	0.05
	0.504 (0.601)	−0.087 (0.080)		−0.005 (0.053)		5	5.9	4.27	0.05
	0.389 (0.605)		0.032 (0.106)	0.0002 (0.056)		5	7.0	5.35	0.03
	1.183 (0.761)	−1.710 (1.168)		−0.065 (0.067)	0.146 (0.105)	6	7.1	5.44	0.03
GATA3 gene expression (*n* = 32, 14 nest-boxes)	0.548 (0.228)			−0.037 (0.020)		4	−57.6	0.00	0.21
	0.109 (0.020)	0.059 (0.032)				4	−57.6	0.03	0.21
	0.131 (0.017)					3	−57.2	0.37	0.18
	0.458 (0.229)	0.047 (0.031)		−0.030 (0.020)		5	−57.0	0.65	0.15
	0.574 (0.230)		0.021 (0.044)	−0.041 (0.021)		5	−55.0	2.59	0.06
	0.113 (0.040)	0.059 (0.032)	−0.005 (0.041)			5	−54.8	2.85	0.05
	0.137 (0.040)		−0.008 (0.044)			4	−54.6	2.96	0.05
	0.343 (0.292)	0.333 (0.454)		−0.020 (0.025)	−0.025 (0.040)	6	−54.3	3.31	0.04
	0.478 (0.232)	0.046 (0.031)	0.019 (0.042)	−0.034 (0.021)		6	−54.1	3.51	0.04

Models within a ΔICc value of six are presented along with their coefficient estimates and standard errors [estimate (SE)]. k = number of model parameters; w = Akaike's weight, defined as the probability of a model given the data and the candidate set of alternative models. ^{a}"Original habitat – forest" set as reference level and, thus, equals zero. ^{b}Malaria parasite = "NO" fixed as reference level; therefore, the given coefficients represent the change in gene transcript levels associated with the presence of malaria parasites. The statistical importance of weight and habitat in the top two models for GATA3 was further assessed by a likelihood-ratio test comparing such models against the intercept-only one. In both cases, these tests yielded a p-value of 0.08.

probably had little importance for *TLR4* and *LY86* levels across habitats. In contrast to other studies (Evans et al., 2009), malaria prevalence was consistently high at both of our sites; however, our statistical power was small and the lack of differences across habitat needs to be considered cautiously. Other intracellular pathogens found to vary in prevalence between urban and rural sites (Giraudeau et al., 2014) or in association with anthropogenic food provisioning (Becker et al., 2015), may also be important determinants of *TLR4* and *LY86* levels. Because we lack information on the wider pathogen assembly in our study sites, we cannot discard differences in other inflammatory pathogens as a cause of the observed gene expression patterns. Additional environmental factors could also explain differences in immunity between urban and forest populations. Low-quality and restricted diets are known to promote pro-inflammatory immune processes (Blount et al., 2003; McGraw et al., 2006; Isaksson, 2015; Nettle et al., 2017). Given our parallel findings of significant diet differences between our study populations (Pollock et al., 2017), it is likely that resource limitation has contributed to elevated inflammation in the city (Larsson et al., 2004; Isaksson, 2015). Costs of inflammation under resource limitation could have contributed to the birds' low reproductive success via exacerbated physiological trade-offs.

In contrast to *TLR4*, transcript levels of *LY86* in adult blue tits did not match our predictions. Because TLR4 and LY86 interact (Lee et al., 2012), we expected to find a correlated pattern of expression between their gene expression. However, the interaction between these molecules is complex and can vary

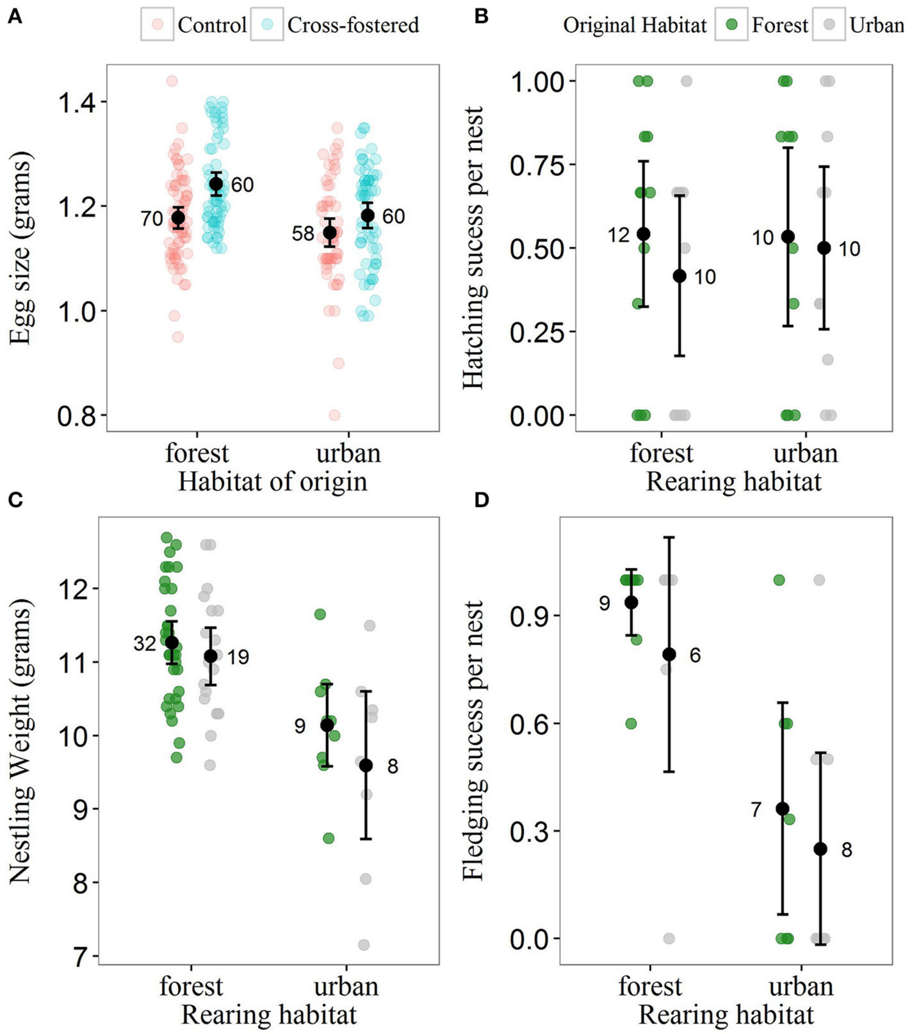

FIGURE 3 | Breeding investment and breeding success for urban and forest blue tits. **(A)** Egg size across original habitats (urban and forest) and experimental groups. Cross-fostered eggs were swapped between sites and were reared in the fostering habitat; control eggs were swapped between nests within site. In the urban rearing habitat, forest- and urban-originated eggs differed in weight, whereas in the forest rearing habitat eggs in both experimental groups had similar weights. **(B)** Hatching success, **(C)** fledging success, and **(D)** weight of 13-day old nestlings across original and rearing habitats. Black dots illustrate raw data mean values and black bars illustrate ± 2 standard errors. Raw data points are represented as partially transparent dots (see legend). Sample sizes are given beside mean values.

between cell types (Divanovic et al., 2005). The lack of differences in *LY86* across habitats, in contrast to our findings regarding *TLR4*, indicates that these two genes might respond differentially to the urban environment. *TLR4* expression may be particularly sensitive to urban-related environmental stressors. As well as acting as a receptor for pathogens (gram negative bacteria molecular patterns), TLR4 is implicated in the recognition of damage-associated molecular patterns that follow tissue damage or cellular apoptosis (Liu et al., 2014). Conceivably, *TLR4* could have been additionally enhanced by urban-associated environmental factors that promote oxidative stress and tissue damage, for example, air pollution and artificial light at night (Isaksson, 2010; Fonken et al., 2013). Our findings on *TLR4* and *LY86* transcript levels can be compared to recent findings of the transcriptomic comparison between urban and rural populations of another parid, the great tit (Watson et al., 2017). In fully grown great tits, expression of *TLR4* (gene ID ENSTGUG00000003342) tended to be elevated in blood and liver also in the city compared to the forest (Watson et al., 2017, Supplementary Datasets 1 and 2). Expression of *LY86* (gene ID ENSTGUG00000002305) also tended to be higher in liver of urban great tits, but not in blood. Largely, therefore, patterns were similar in the two parid species.

The findings of higher *GATA3* transcript levels in adult urban compared to forest blue tits also followed our initial predictions. The type 2 transcription factor GATA3 constitutes a major regulatory component of the immunity against helminth parasites (Tindemans et al., 2014). Although we were unable

TABLE 4 | Summarizing table of statistical models employed to explain variation in natural clutch size and egg weight (in g).

	Estimates of model coefficients (standard error)									
Response term	**Intercept**	**Habitat – urban^{a}**	**Experimental Group^{a} – Cross-fostered**	**Completion Date – linear**	**Habitat × experimental group^{a}**	**Clutch size**	***k***	**AICc**	**ΔAICc**	***w***
Clutch size ($n = 42$)	2.103 (0.054)					Not included	1	186.0	0	0.29
	2.156 (0.073)	−0.115 (0.108)				Not included	2	187.1	1.08	0.17
	1.785 (0.406)			0.008 (0.011)		Not included	2	187.6	1.58	0.13
Egg weight ($n = 248$)	1.185 (0.020)	−0.045 (0.024)	0.050 (0.024)*				5	−632.3	0	0.17
	1.164 (0.017)		0.048 (0.025)				4	−631.0	1.33	0.09
	1.151 (0.054)	−0.043 (0.024)	0.048 (0.024)			0.004 (0.006)	6	−630.7	1.66	0.07
	1.178 (0.022)	−0.031 (0.033)	0.064 (0.033)		−0.029 (0.047)		6	−630.6	1.73	0.07

*Only models within ΔAICc < 2 are presented along with their estimates and standard errors [estimate (SE)]. k = number of model parameters; w = Akaike's weight, defined as the probability of a model given the data and the candidate set of alternative models. ᵃ"Forest" and "Control" categories are set as reference levels for "Habitat" and "Experimental Group," respectively, and, therefore fixed to zero. Correspondingly, estimates for "Habitat × Group" refer to the urban habitat and cross-fostered experimental group. The additional term "Completion Date—quadratic" was included in candidate models but estimates for this variable are not shown as they do not appear in any model within a ΔAICc value of 2. Raw data mean values ± SE for clutch size: urban clutches= 7.70 ± 0.34; forest clutches = 8.63 ± 0.48. Raw data mean values ± SE for egg weight: urban-originated eggs = 1.17 ± 0.01; forest-originated eggs = 1.21 ± 0.01. Model coefficients for clutch size are shown in the scale of the link function (log). The statistical importance of variables in the top model for egg weight was further assessed by a likelihood-ratio test, dropping one predictor at a time, and *illustrates p-values < 0.05.*

TABLE 5 | Summarizing table of statistical models employed to explain variation in hatching success, fledging success and nestling weight (in g).

	Estimates of model coefficients (standard error)								
Response term	**Intercept**	**Original Habitat – urban^{a}**	**Rearing Habitat – urban^{a}**	**Completion – linear**	**Completion – quadratic**	***k***	**AICc**	**ΔAICc**	***w***
Hatching Success ($n = 42$)	−0.126 (0.422)					2	166.4	0.00	0.33
	2.187 (3.142)			−0.060 (0.082)		3	168.2	1.80	0.13
	0.146 (0.578)	−0.565 (0.838)				3	168.3	1.89	0.13
Fledgling Success ($n = 30$)	4.652 (1.876)		−7.046 (3.082)*			3	74.3	0	0.35
	−9.252 (11.493)		−6.503 (2.935)	0.246 (0.213)		4	75.3	1.07	0.20
	5.112 (2.003)	−1.335 (1.615)	−6.780 (2.828)			4	76.2	1.95	0.13
Nestling Weight ($n = 68$)	11.190 (0.180)		−1.328 (0.341) *			4	178.9	0	0.27
	8.822 (2.145)		−1.318 (0.330)	0.042 (0.038)		5	180.0	1.15	0.15
	−32.250 (26.282)		−1.075 (0.350)	1.526 (0.947)	−0.013 (0.009)	6	180.1	1.22	0.15
	11.300 (0.210)	−0.278 (0.310)	−1.322 (0.335)			5	180.4	1.54	0.12

*Only models within ΔAICc < 2 are presented along with their estimates and standard errors (estimate (SE)). k = number of model parameters; w = Akaike's weight, defined as the probability of a model given the data and the candidate set of alternative models. The additional terms "Original habitat × Rearing habitat," "Brood size" and "Rearing habitat × Brood size" were included in global models but estimates for these variables are not shown as they do not appear in any model within a ΔAICc value of 2. ᵃ"Original Habitat – forest" and "Rearing habitat – forest" were set as reference levels for coefficient estimation. Model coefficients for hatching and fledgling success are illustrated in link function scale (logit). The statistical importance of rearing habitat in the top model for fledgling success and nestling weight was further assessed by a likelihood-ratio test against the intercept-only model, and *illustrates p-values < 0.05.*

to monitor intestinal parasite load in our study, our results could be explained by potentially higher helminth pressure in our urban location. Aggregation in feeding stations causes high rates of horizontal transmission of directly-transmitted parasites and, hence, affects parasite distribution (Becker et al., 2015). If increased activation of GATA3-induced immunity in our urban birds was, indeed, caused by increased helminth infection, it might thus reflect a parasitological cost imposed by supplementary feeding and contribute to some of the negative effects of supplementary feeding on fitness (Plummer et al., 2013). Results for *GATA3* expression (gene ID ENSTGUG00000002134) from an urban great tit study in Sweden were comparable for liver, with higher levels in the city, whereas for blood expression did not differ between sites (Watson et al., 2017).

For the nestling stage, our findings from the cross-fostering experiment provide no evidence that urban as compared to forest origin was associated with higher immune transcript levels in nestlings (**Figure 2**). Unfortunately, our sparse data from the urban rearing environment do not allow comprehensive conclusions from the full cross-fostering experiment (Figure S2). However, overall, transcript levels of nestling immune genes appeared to be similar across habitats (Figure S2). Thus, pending confirmation by a larger dataset, our findings suggest a differential impact of urbanization on the immune system, becoming more pronounced in later life-history stages. The contrasting patterns among age groups might be explained by chronic effects of long-term exposure to urban-specific environmental factors in adults (e.g., Bedrosian et al., 2011). Genetic or prenatal maternal causes for increased *TLR4* and *GATA3* transcript levels in adult blue tits are made unlikely by our findings that among nestlings reared in the forest, origin (city or forest) did not strongly affect transcript levels of any gene (**Figure 2**). Hence, the cross-fostering experiment indicates that urban-forest differences in transcript levels of adult blue tits were most likely driven by the environment (Salmón et al., 2016). However, we cannot fully exclude the possibility that genetic or maternal factors are only identifiable in adulthood and not in nestlings. In contrast to our findings, a recent study on great tits did report differences in immune status between urban and rural nestlings (Bailly et al., 2016a). Interestingly, the differences were opposite to those we found for adults blue tits: using physiological assays, rather than gene expression studies, Bailly et al. (2016a) show that urban nestlings produce less haptoglobin (a marker of inflammation) than forest birds, and offer as an explanation that haptoglobin production is compromised by food resource availability in the urban site. Although caution is needed when comparing different species and inflammatory markers, the opposite findings for the age groups might reflect different trade-offs between immunity and development: under resource restriction, growing nestlings might not be able to mount costly responses in the same way as fully grown adults (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Alonso-Alvarez and Tella, 2001).

Our results contribute to a body of evidence suggesting that urban living has reproductive costs and can impact the health of wild animals by altering their immune system (Audet et al., 2016; Bailly et al., 2016a; Watson et al., 2017). We acknowledge that caution is needed when interpreting our result given our sample size and the existence of only one urban-forest study pair and one study year. However, our experimental approach, with within-forest and within-city controls, allows us to draw conclusions on the causal links between immunity, fitness and the urban environment in our study system. Furthermore, the results generally confirm our original predictions and are in line with widespread evidence of reductions in fitness in urban environments (Chamberlain et al., 2009; Bailly et al., 2016b) and with recent discoveries on the effect of the urban environment on gene expression profiles in wild birds (Watson et al., 2017). We find evidence for elevated expression of immune genes in adults of our urban compared to our forest population, and data from our cross-fostering experiment indicate that such changes are best explained by environmental factors. In combination with our twin study on reduced food availability and modified provisioning in the city (Pollock et al., 2017), we tentatively identify links between resource limitation and altered immunity. To better understand the impact of urbanization on the immune system of wild animals, we need fine-scale characterization of the urban environment including parasite assemblies, coupled with a broader immune assessment of wild populations and information on fitness across life stages. Such information would reveal the role of the immune system for adaptation to urban life, as well as the long-term demographic consequences of altered immunity for urban-dwelling species.

AUTHOR CONTRIBUTIONS

PC-L, BH, DD, SB, and TB conceived the original study and designed experimental procedures. PC-L, DD, CP, and BH carried out field work, and PC-L, PO, MM, LW, and FB performed laboratory analysis. Statistical analyses were done by PC-L with advice from DD and BH. PC-L, BH, and DD wrote the manuscript with input from all other co-authors.

FUNDING

PC-L was funded by a postgraduate scholarship from Iberdrola Foundation. Funding of DD and of gene analysis was provided by a Marie-Curie Career Integration Grant to BH [EC CIG (618578) Wildclocks].

ACKNOWLEDGMENTS

We would like to thank Dan Haydon, Barbara Mable, and the IBAHCM for their support of the project. We would also like to thank Jessica Clark, Stephen Larcombe, Steve Duncan, Bernard Lundie, Paul Baker, Stewart White, Kim Mortega, Ruedi Nager, Iain Malzer, Paul Jerem, Ana Monteiro, and Yoana Ivanova for their help in the field and in the laboratory. We acknowledge the Trustees of the RSFS Forest Trust for access to Cashel Forest. Two reviewers provided helpful advice that greatly improved an earlier version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00064/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 30 May 2017
doi: 10.3389/fevo.2017.00055

Artificial Light at Night Reduces Daily Energy Expenditure in Breeding Great Tits (*Parus major*)

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The ecological impact of artificial light at night (ALAN) is an increasingly recognized process that accompanies expanding urbanization. Yet, we have limited knowledge on the impact of ALAN on wild species, and on the potential to mitigate any negative effects by using different light sources and colors. In birds, effects of ALAN on activity levels are reported for several species and, hence, their daily energy expenditure (DEE) may be affected. DEE is a potent mediator of life-history trade-offs and fitness and thus an important aspect to consider when examining the potential long-term ecological effects of ALAN. Previous work has suggested that birds exposed to ALAN show higher levels of provisioning and nocturnal activity, suggesting that white ALAN increases DEE. Other factors regulating DEE, such as provisioning behavior and food availability, might also respond to ALAN and thus indirectly affect DEE. We tested the hypothesis that ALAN increases DEE using an experimental setup where four previously unlit transects were illuminated with either white, green, or red LED light, or left dark as a control treatment. This setup was replicated in eight locations across the Netherlands. We measured DEE of our focal species, the great tit (*Parus major*), using a novel doubly labeled water technique that uses breath rather than blood samples. Contrary to our expectations, birds feeding their offspring under white and green ALAN showed lower DEE compared to birds in the control dark treatment. Differences in chick provisioning activity did not explain this result, as neither visit rates nor daily activity timing was affected by light treatment. However, food availability under white and green light was much higher compared to red light and the dark control. This difference strongly suggests that the lower DEE under white and green ALAN sites is a consequence of higher food availability in these treatments. This result shows that there can be positive, indirect effects of ALAN for breeding song birds which may balance against the negative direct effects shown in previous studies.

Keywords: ALAN, light pollution, doubly labeled water, energy expenditure, *Parus major*

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Specialty section:
This article was submitted to Urban Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *27 February 2017*
Accepted: *11 May 2017*
Published: *30 May 2017*

Citation:
Welbers AAMH, van Dis NE, Kolvoort AM, Ouyang J, Visser ME, Spoelstra K and Dominoni DM (2017) Artificial Light at Night Reduces Daily Energy Expenditure in Breeding Great Tits (Parus major). Front. Ecol. Evol. 5:55. doi: 10.3389/fevo.2017.00055

INTRODUCTION

The alteration of natural light levels in the outdoor environment due to artificial light sources, defined as light pollution (Cinzano et al., 2000), is a globally expanding phenomenon with an estimated increase of 6% per annum (Hölker et al., 2010). It has been recently estimated that at mid-high latitudes more than 23% of land surfaces are exposed to artificial light at night (ALAN)

(Falchi et al., 2016). In very urbanized areas like Europe, this number goes up to 88% (Falchi et al., 2016). ALAN is caused by a wide variety of light sources, such as public lighting of streets and buildings, vehicle lights, and light from advertising. Importantly, ALAN is one of the first signs of expanding urbanization, and rural land is also widely exposed to street lighting, especially in developed countries. Over the last decades, light sources, especially those of street lamps, have been undergoing a general change from narrow to broad spectrum light. A recent example is the widespread shift to LED lights (Gaston et al., 2012; Bennie et al., 2014). However, despite a recent body of studies that examined the ecological consequences of ALAN (Rich and Longcore, 2006; Migaud et al., 2007; Davies et al., 2013; Spoelstra et al., 2015), little is known about how different light spectra might affect the behavioral and physiological responses of wild species. Since LEDs color is easily modifiable, it is important to fill this research gap in order to inform policy-makers and improve future lighting strategies.

ALAN can affect several behaviors of wild animals, from orientation and navigation during dispersal and migration (Rich and Longcore, 2006; Poot et al., 2008), to daily and seasonal rhythms in behavior (Gaston et al., 2013; Robert et al., 2015; Dominoni et al., 2016), to more subtle physiological effects on stress levels, body mass and immune responses (Bedrosian et al., 2011; Ouyang et al., 2015; Raap et al., 2016a). Effects on circadian rhythms of behavior and physiology are particularly evident in a range of species, from insects to mammals (Swaddle et al., 2015). For instance, great tits (*Parus major*) provision their offspring at higher rates when exposed to experimental white ALAN directly at the nest box (Titulaer et al., 2012), and also show more activity at night in the lab (de Jong et al., 2016b), possibly incurring in energetic costs. In nocturnal species, such as mice and bats, ALAN is usually associated with avoidance behavior and lower activity levels at night (Stone et al., 2009; Rotics et al., 2011; Spoelstra et al., 2015). Timing of singing and sleep are also strongly affected by light pollution (Kempenaers et al., 2010; Da Silva et al., 2014; Raap et al., 2015), and such changes are suggested to have physiological consequences (Dominoni et al., 2016). For instance, sleep loss in response to ALAN is associated with reduced body mass and altered concentration of nitric oxide and haptoglobin (Raap et al., 2016b). Such physiological responses to ALAN might differ between urban and rural animals, and suggest the possibility that at least some species might adapt to and thus tolerate the presence of artificial lights. Indeed, bright nights can also offer opportunities, for example extending foraging time found for a number of diurnal species (Santos et al., 2010; Dwyer et al., 2013; Russ et al., 2015; Dominoni et al., 2016), thereby likely providing fitness benefits.

Because of the reported strong changes in activity levels associated with ALAN, we hypothesized that daily energy expenditure (DEE) might also be affected. This measure of energy metabolism is an important aspect to consider when examining the potential long-term effects of light pollution, as changes in energy metabolism are widely recognized as mediators of life-history traits and fitness (Wiersma et al., 2007; Hudson et al., 2013; Careau et al., 2015). Indeed, as the energy available to an individual is usually limited, it needs to be traded off between different various behavioral and physiological processes, such as feeding (Daan et al., 1996), thermoregulation (Kersten and Piersma, 2002), incubation (Cresswell et al., 2004) and territorial defense (Vehrencamp et al., 1989). The factors influencing DEE have been studied extensively in wild birds (Williams, 2012). Body mass explains most of the variation in DEE between and within species (Ricklefs et al., 1996; Nagy, 2005). Yet, environmental variables are also important regulators of energy metabolism, as temperature is usually negatively correlated with DEE (Tinbergen and Dietz, 1994; Te Marvelde et al., 2011; Regular et al., 2014) and environmental stressors are suggested to increase energy expenditure (Weimerskirch et al., 2002; Welcker et al., 2009). Food availability is also a key mediator of energy expenditure. On one hand, energy expenditure might be forced upward by lower food availability, as animals will need to work more and for longer to find food (Tinbergen and Dietz, 1994; Welcker et al., 2009). On the other hand, when resources are plentiful animals might afford to work harder and, for instance, provide more food to their offspring (Tinbergen and Verhulst, 2000; Welcker et al., 2009) or invest more energy and time in territorial defense (Vehrencamp et al., 1989), thereby increasing their DEE. In addition, a potential cost of high DEE at the cellular level is the production of reactive oxygen species, which may lead to oxidative stress (Speakman et al., 2002; Selman et al., 2008; Fletcher et al., 2013; but see also Speakman and Selman, 2011). However, higher food availability can also increase the capacity of organisms to acquire and use anti-oxidants and thus limit oxidative stress (Giordano et al., 2015). Overall, the environmental conditions in which DEE is measured need to be carefully assessed.

We tested our hypothesis using a setup in which previously unlit forest habitats were experimentally illuminated, which we extensively described in previous manuscripts (de Jong et al., 2015; Spoelstra et al., 2015). In this setup, we study the physiological and behavioral responses of rural great tits to three different spectral compositions of LED lighting (green, red and white), while using also a dark control treatment. This setup is repeated across eight different forest locations in the Netherlands. The choice of such light colors was made to cover both short (green) and long wavelengths (red), as well as the broad-spectrum white light. Because sensitivity to light in birds' photoreceptors peaks at mid-short wavelengths (Peirson et al., 2009; Hunt et al., 2014; Dominoni, 2015), we expected white and green light to be associated with stronger responses than red light and the dark treatment. Specifically, we predicted that white ALAN would increase DEE in great tits, because in previous studies we have found that white ALAN was associated with increased provisioning rate during the day (Titulaer et al., 2012), as well as with higher activity levels at night (Ouyang et al., in press), but they never directly measured energy expenditure. In addition, birds breeding under white ALAN showed higher baseline corticosterone levels compared to birds breeding in dark control areas (Ouyang et al., 2015), and this reinforced the prediction that DEE should be higher under such lighting conditions. Importantly, by measuring complementary variables we aimed at providing explanations for potential differences in DEE associated with ALAN. We collected data on the timing and amount of activity using transponders attached to each bird, as well as data on the abundance of caterpillars, which we define as food availability as this is the main food source of great tits nestlings (Perrins, 1991). Food availability has not been measured before in field studies that have tested the effect of artificial lights on songbirds.

METHODS

Experimental Set-Up

We illuminated previously dark natural areas with street lamps from sunset until sunrise, at eight forest sites across The Netherlands (Spoelstra et al., 2015). Lights are turned on at night at all sites since spring 2012, except for one site (Voorstonden) which had lights on since the end of April 2013. Within each site, four different transects were randomly assigned to one of four light treatments. These consists of five lampposts with white, green, or red light (Fortimo white, ClearSky green and ClearField red light, Philips, Amsterdam, The Netherlands), or wooden poles with no lamps—dark control. All three lamp types emit full spectrum light, but green lamps have an increased blue and reduced red, and red lamps have an increased red and reduced blue emission (for details on the spectral power of the light, see (Spoelstra et al., 2015). As these spectra are eventually intended for civil use, we normalized light levels to lux (intensity 7.6 ± 1.2 lux, measured directly under the lamp at ground level). We placed nine nest-boxes in each transect, at different distances from the closest lamppost (median ± SD = 25 ± 21 m, range 1–94 m).

Daily Energy Expenditure

We measured DEE using the doubly labeled water (DLW) technique on breath samples of adult great tits, through a cavity-ringed laser spectrometer (L2120-*i*, Picarro Inc., CA, USA). This technique has been recently validated using blood samples in bird species of similar size of great tits (Mitchell et al., 2015). We caught adult great tits in nest boxes using spring traps during daytime on day 10 of chick rearing. We fitted birds with an individual aluminum ring as well as with an individual radio frequency identification (RFID) transponder, and weighed them to the nearest 0.01 g. Within 15 min of capture, we used a 29-gauge 0.3-cm^3 syringe to inject birds intraperitoneally with 200 μl of doubly labeled water (36.72 g of 98.7% $H_2^{18}O$ and 18.34 g of 99.9% D_2O). The needle was kept parallel to the surface of the bird's belly, and then gently pushed under the skin. After the injection, we placed the birds in a cotton bag for 1 h to allow for equilibration of isotopes in body fluids. After this hour, we removed the bird from the bag and applied a custom-made mask to the bird's head (Figure S1). The mask has two small holes where plastic tubes were inserted. The first of these tubes was used to pump dry air (<500 ppm H_2O) into the mask at a standard rate of 2 l per minute, starting 5 min prior to breath measurements to ensure the spectrometer's cavity was full with dry air. The second tube was used to collect the air flowing out of the mask (consisting of dry air and bird's breath), which was then diverted into the spectrometer. The spectrometer recorded measurements of δ^2H (‰) and $\delta^{18}O$ (‰) approximately every 2 s, as well as 2- and 5-min running averages. All breath samples lasted 5 min, and we used the 2 min running average measured 30 s after the termination of the breath sample (Mitchell et al., 2015) in subsequent analyses of CO_2 production. We recaptured birds after approximately 24 h (mean ± s.d. = 23 h 49 min ± 94 min), and conducted a second breath measurement. In addition to this procedure, we obtained background isotope measurements prior to DLW injection in 25 birds, and used the averaged of such measurements for the remaining individuals. The spectrometer is prone to memory effects, that is, the isotope values obtain in one measurement can be influenced by the previous one. To reduce such effect we followed the procedure used in (Mitchell et al., 2015) and performed measurements at least 20 min apart from one another. In addition, we flushed the spectrometer cavity with ambient air in between measurements, because this has been shown to further limit memory effects (Mitchell et al., 2015).

We calculated CO_2 production according to the single-pool model as in Speakman (1997). We used a respiratory quotient of 0.75 and an energy equivalence of 27.89 kJ/l CO_2following Speakman (1997). The water content of individuals was assumed to be 66% of body mass (Mertens, 1987).

Activity Measurements: Visit Rates and Activity Timing

On day 10, when we captured birds for the first DLW measurement, we equipped the nest box with a transponder reader (Trovan, Dorset Group BV, Aalten, The Netherlands) around the entrance hole. The reader recorded each visit that a bird made to the nest. We used recordings collected on day 11, from the first to the last visit of this day.

Non-independent RFID readings caused by birds residing in the nest box entrance were excluded by removing readings with an interval shorter than 17 s. This "cutoff point" was identified by a peak in the frequency histogram of consecutive recording intervals. We validated this method using nest box visit data from pied flycatchers (*Ficedula hypoleuca*) breeding in the same region. After excluding these data, we calculated the total number of visits per hour per bird and used this variable in all our models. We excluded the first and last hour of recording for each day as these were usually truncated and therefore the calculation of hourly means would have been biased. As the amount of data used per bird per day is clearly affected by lengthening of days during the spring progression, we corrected for this potential bias by including Julian date as explanatory variable in all our models (see below).

We also used the transponder data to calculate the time of onset and offset of activity, by locating the earliest and latest visit time of the adults within a day. We expressed these times relative to the objective (natural) sunrise, sunset and day length time, which we obtained from the US Geological Survey website (http://aa.usno.navy.mil/data/docs/RS_OneYear.php). We calculated the duration of the activity phase as the difference between the offset and onset.

Caterpillar Availability

We assessed caterpillar availability using frass nets, where the biomass of caterpillar above the net can be calculated from the droppings ("frass") of the caterpillars (Tinbergen and Dietz, 1994), in 3 consecutive years, from 2014 to 2016. Frass nets (0.5 × 0.5 m) were placed at 1 m distance from the trunk of the oak closest to the lamppost (Visser et al., 2006). We placed one net in each of the four transects of the site with the highest caterpillar abundance, Voorstonden, as this is the only site consisting of primarily deciduous trees (Spoelstra et al., 2015), while the other sites harbor mostly evergreen species (spruce and pines). In 2016, we also placed nets in all other seven sites under birch trees when these were available, and otherwise under pine trees. Since the total frass collected in these additional sites was extremely low (most samples contained no frass, Figure S2), we decided to use caterpillar biomass data exclusively from Voorstonden for statistical analyses.

Over the breeding period (2014: March 24 to May 22; 2015: April 4 to June 8; 2016: April 18 to June 12), frass nets were emptied on average every 4 days (range 2–14 days) with more regular sampling in the middle of the breeding season (every 2–5 days). Any large debris was removed before collecting. On average, 70 samples were collected over the course of the breeding season each year (range 52–84), with an equal number of measurements for each of the transects (mean = 17 samples per transect per year). In 2015 and 2016, samples were dried at 60°C for 24 h, sorted and weighed following (Visser et al., 2006). In 2014, sorting was performed manually with the use of a microscope. From the dry weights, caterpillar biomass was estimated using the formula by Tinbergen and Dietz (1994), to obtain caterpillar biomass in grams per day per square meter.

Statistical Analyses

We ran all statistical analyses using the software R (R Development Core Team, 2015). Our basic approach was to use linear mixed models (LMMs) with a Gaussian error structure for all our analyses, using the *lme4* package (Bates et al., 2015). We always included site as a random factor, as well as nest box, the latter to correct for non-independent measurements conducted on paired adults that bred in the same box. We also included ID as random factor when repeated measures were conducted on the same animal (for the RFID data). We checked the model fit by inspecting graphs of residuals.

We conducted model selection based on likelihood ratio tests using procedures implemented in the R package *lmerTest* (Kuznetsova et al., 2016). We started with the most complex model with all biologically relevant fixed effects and then sequentially removed non-significant effects until the best model was found. When treatment had a significant effect on the response variable, we tested for differences between light colors with a Tukey *post-hoc* test implemented in the R package *multcomp* (Hothorn et al., 2008). When the interaction treatment*distance was significant, we conducted independent LMMs for each of the four treatments separately.

We ran five main models with the following as response variables: (1) DEE, (2) Visit rates per hour (RFID data), (3) Time of first morning visit activity, (4) Time of last evening visit, (5) Active day length (difference between first and last visits, all from RFID data). In addition, we ran two LMMs to relate individual DEE values (response variable) to visit rates. In all these models we included treatment, distance to closest lamppost, sex, mass, Julian date and the interaction between treatment and distance as fixed effects. In the models for DEE and visit rates, we also included brood size as a fixed effect.

To analyze caterpillar abundance data, we extracted peak height (maximum estimated caterpillar biomass in grams per day) and peak date (date at which maximum was measured) for each tree sampled (Visser et al., 2006). We used these two as response variables in two linear models with treatment and year as fixed effects, as well as their interaction. We log-transformed peak height to meet normality assumptions. We then performed Tukey *post-hoc* tests (see above) to test for significant differences between years and treatments.

RESULTS

Daily Energy Expenditure

We obtained DEE measurements from 55 birds ($N = 34$ females and 21 males; N dark = 18, green = 13, red = 14, white = 10). DEE levels were significantly affected by the light treatment ($\chi^2 = 10.53$, $P = 0.015$). *Post-hoc* tests (treatment-specific linear regressions) showed that the birds in the dark treatment had significantly higher DEE levels compared to birds in both the white and green treatment ($z = -2.73$, $P = 0.032$, and $z = -2.03$, $P = 0.018$, respectively, **Figure 1** and Table S1). All other pairwise comparisons between different light treatments were not significant ($P > 0.4$ in all cases). In addition, brood size had a marginally non-significant and positive effect on DEE ($\chi^2 = 5.34$, $P = 0.060$): that is, parents spent more energy when they had to feed a higher number of nestlings. No other variables were significantly associated with DEE (Table S1).

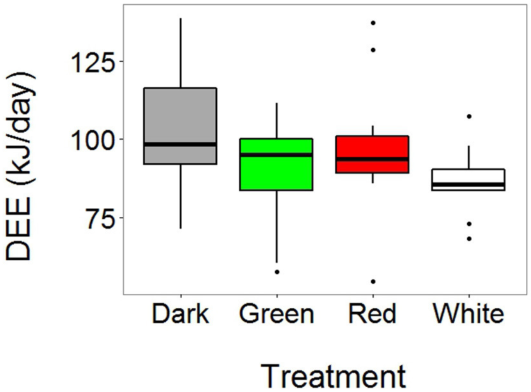

FIGURE 1 | Light at night affects daily energy expenditure (DEE) in free-living adult great tits. Horizontal, black line in boxplots represents median DEE (N dark = 18, green = 13, red = 14, white = 10). Birds were captured at their nest box on day 10 of the chick-rearing period, and injected with doubly labeled water (DLW). We then waited for 1 h to allow equilibration of DLW in body fluids, and subsequently measured isotope concentrations in breath during a 5 min period using a custom-made mask attached to a cavity-ringed laser spectrometer. Birds were released and after approximately 24 h they were recaptured to obtain a new breath measurement. DEE was calculated after (Speakman, 1997). Birds breeding under white and green light at night spent significantly less energy compared to the birds in the dark treatment.

Activity Measurements: Visit Rates and Activity Timing

We obtained RFID data from 70 individuals ($N = 37$ females and 33 males) from 43 nests (dark = 14, green = 10, red = 11, white = 8). Our final model showed a significant effect of the interaction between treatment and distance to the closest lamppost on the number of visits per hour ($\chi^2 = 11.54$, $P = 0.009$, **Figure 2** and Table S2). *Post-hoc* tests showed that in the green treatment there was a negative relationship between the distance to the closest lamppost and the number of visits to the nest ($t = -2.39$, $P = 0.037$). Conversely, such a relationship was absent in all other treatments ($P > 0.28$ in all cases, **Figure 2**). In addition, the full model showed highly significant, negative effects of both Julian date ($\chi^2 = 15.84$, $P < 0.001$) and time of day on the number of visits per hour ($\chi^2 = 101.29$, $P < 0.001$), meaning that birds visited the nest less often both late in the season as well as late in the day. In addition, there was a positive although weak effect of brood size on visit rate ($\chi^2 = 4.34$, P = 0.043). There was no significant difference in visit rates between males and females ($\chi^2 = 0.60$, P = 0.44).

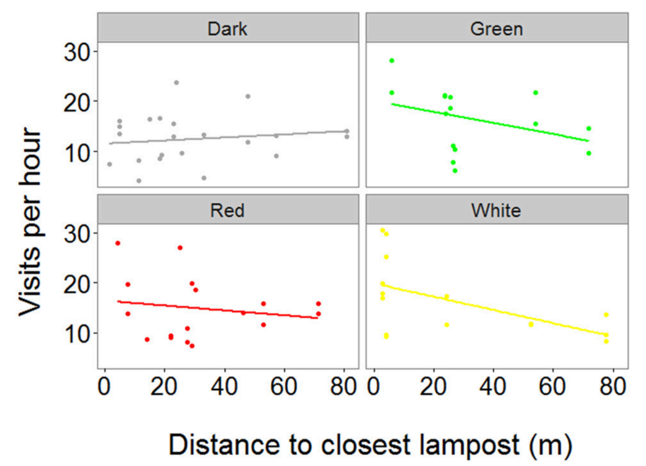

FIGURE 2 | Relationship between the distance to the closest lamppost and visit rates of parent great tits. We found a significant interaction between treatment and distance to the closest lamppost on visit rates, with the birds in the green treatment visiting their nest box more when breeding closer to the light source. X-axis depicts distance of a single nest box (dots) to the closest lamppost. Y-axis depicts the average number of visits per hour that one individual bird made to its nest box. The measurements were conducted during the chick rearing period, when chicks were 10–11 days old, using RFID transponders attached to each individual bird (N dark = 22, green = 15, red = 18, white = 15).

First and last visit to the nest box, as well as the duration of the active day, were not affected by light treatment ($\chi^2 = 2.29$, P = 0.514; $\chi^2 = 4.30$, $P = 0.231$; $\chi^2 = 1.38$, $P = 0.711$ respectively, **Figure 3** and Table S3). The duration of the active day was higher in females than males (females: mean ± sd = 15 h 2 min ± 24 min; males: 14 h 26 min ± 48 min; $\chi^2 = 13.75$, $P < 0.001$). This was due to a sex difference in both the time of the last visit (females: mean ± sd = 25 min ± 30 min before sunset; males: 53 min ± 31 min before sunset; $\chi^2 = 15.19$, $P < 0.001$) and first visit to the nest box (females mean ± s.d = −1.32 min ± 30 min before sunrise vs. males 6.58 ± 35 min after sunrise; $\chi^2 = 7.96$, P = 0.005).

We then correlated the RFID data to the DEE data, and found a significant interaction between treatment and visit rates in predicting DEE ($\chi^2 = 12.49$, P = 0.006, and **Figure 4**). Indeed, independent LMMs run for each treatment showed that visit rates significantly predicted DEE in the dark and red treatment ($t = 2.2$, $P = 0.027$ and $t = 7.9$, $P < 0.001$, respectively, **Figure 4**), but not in the green and white treatment ($t = 1.3$, $P = 0.194$ and $t = 1.4$, $P = 0.195$, respectively, **Figure 4**).

Caterpillar Availability

Peak height in caterpillar abundance, expressed as the maximum estimated caterpillar biomass in grams per day, was significantly affected by treatment [$F_{(3,\,13)} = 11.49$, $P = 0.001$, **Figure 5**, Table S5]. *Post-hoc* tests revealed that abundance was 7 times higher in the green treatment and 6 times higher in white treatment compared to the dark treatment (green-dark: back-transformed estimate = 7.05, $P = 0.002$; white-dark: back-transformed estimate = 6.11, $P = 0.004$). Moreover, both green and white treatments differed from the red treatment, although the differences were smaller, with a 3 times higher caterpillar abundance peak in the green and the white compared to the red (red-green: estimate = 0.30, $P = 0.044$; white-red: estimate = 2.91, $P = 0.079$). There were no differences between years in

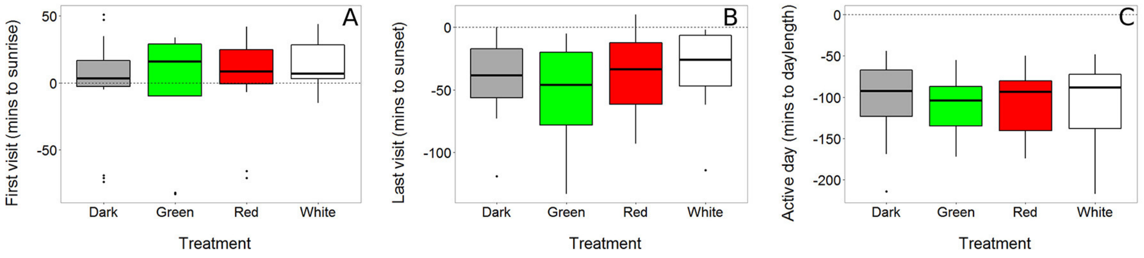

FIGURE 3 | Timing of daily activity was not affected by light at night. First (A) and last visit **(B)** of a bird to its nest box were recorded using RFID transponder data (see **Figure 2**). The time that a bird was active during the day ("active day," **C**), was calculated as the difference between the last and first visit of a day. All data are expressed relative to the natural sunrise, sunset and day length, which are depicted in the graphs as dashed horizontal lines crossing zero (N dark = 22, green = 15, red = 18, white = 15).

peak height [$F_{(2,\ 12)} = 2.6$, $P = 0.123$, **Figure 5**, Table S5]. Light treatment did not have an effect on peak date [$F_{(3,\ 13)} = 1.1$, $P = 0.39$, **Figure 5**, Table S5]. Although in 2015 and 2016 peak date was 7 and 8 days later than in 2014, respectively, we only detected a marginal, non-significant effect of year in our models [$F_{(2,\ 12)} = 3.1$, $P = 0.087$, Table S5].

Given the strong differences in peak height of caterpillar abundance between the green/white sites and the dark sites, we suspected that these could be associated with the lower DEE found in the birds breeding under white and green light compared to the dark control. To test this hypothesis, we created two additional datasets for the DEE measurements with (1) only data from the caterpillar-richest site, Voorstonden (see Spoelstra et al., 2015) for details on this site), and (2) all sites excluding Voorstonden. The effect of treatment on DEE was only present in the dataset with only Voorstonden ($N = 24$, $P = 0.026$, $F = 3.8$). *Post-hoc* tests showed a significantly lower DEE in the green compared to the dark treatment ($t = -3.20$, $P = 0.021$), and a marginally non-significant lower DEE in the white compared to the dark treatment ($t = -2.50$, $P = 0.089$). Conversely, there was no treatment effect on DEE in the dataset with all sites except Voorstonden ($N = 31$, $P = 0.61$, $\chi^2 = 1.82$).

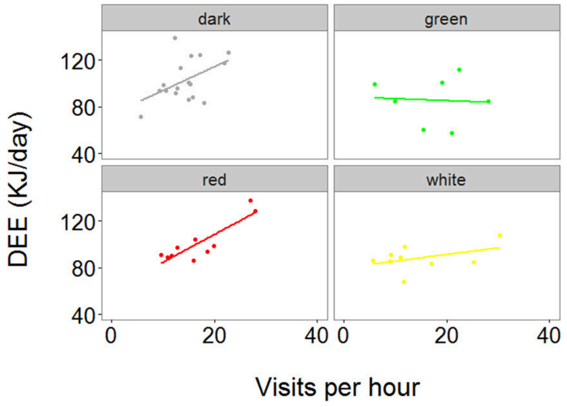

FIGURE 4 | Relationship between DEE and average number of parental visits per hour to the nest during the chick feeding period. Data were collected for 1 day during the chick feeding period (day 10–11 of nestling period). Each point represents one individual. The regression line is the slope estimate of a LMM in which the interaction between treatment and number of visits per hour was found to have a significant effect on DEE.

DISCUSSION

Our study revealed an effect of artificial light at night (ALAN) on the energy expenditure of breeding wild great tits. Birds exposed to either green or white nocturnal light had lower DEE levels compared to the control dark group. Previous studies suggested that adult songbirds exposed to white ALAN showed higher provisioning rates during the day (Titulaer et al., 2012), as well as higher restlessness at night (Dominoni et al., 2013; de Jong et al., 2016a; Ouyang et al., in press), although energy consumption was never directly measured. Thus, we predicted DEE to be higher in the white ALAN group compared to all other treatments. This was clearly not the case. One option why restlessness at night does not lead to a higher DEE is that it is not energetically costly, such as foraging or flying, leaving DEE unaffected. Relative to this, it is important to note that locomotor activity may not be the main

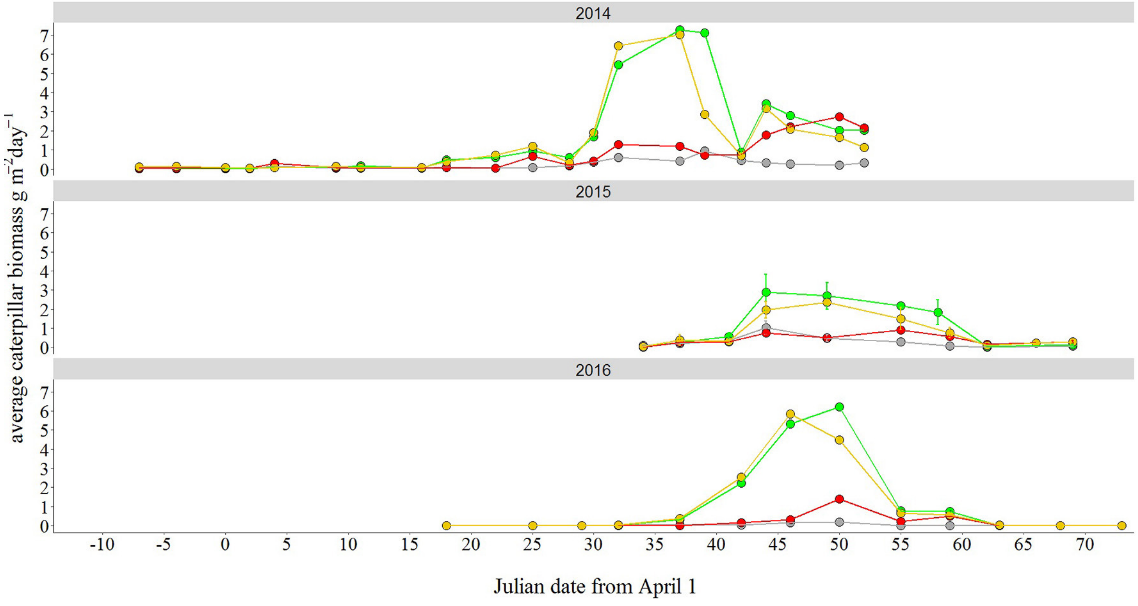

FIGURE 5 | Effect of light at night treatment on caterpillar abundance. Caterpillar biomass was much higher in both the green and white light treatment compared to the dark one. Biomass (in g/m^2 per day) was estimated from caterpillar frass, by placing one frass net in each light transect for only the richest caterpillar biomass site, Voorstonden. Points represent mean values. In 2015 we sampled two trees per treatment, hence we use error bars (SEM). The white light treatment is here depicted in yellow for visualization purposes.

driver of day/night differences in DEE, as birds show a circadian rhythm in energy metabolism that is independent of activity (Pohl, 1977). In addition, higher nocturnal restlessness could simply be attributed to the disruptive effect of white ALAN on sleep (Raap et al., 2015), rather than to an increase in locomotor activity. Some studies in humans and rodents have suggested that sleep disruption may increase DEE (Markwald et al., 2013), but others have found the opposite effect (Benedict et al., 2011). We suggest that other mechanisms may play a far bigger role in explaining our results.

Our data suggest that the increased food availability in the white and green ALAN treatments could have partly mediated the lower DEE levels found in these treatments. Food availability has never been considered in light pollution studies on Parid species. We have measured the abundance of caterpillars during the last three springs, and in all years the green and white ALAN treatments had a considerably higher peak in caterpillar biomass compared to the dark and red treatments, although only in the caterpillar-rich site, Voorstonden (see below for a more thorough discussion on this point). It is therefore conceivable that at this site birds breeding under green and white ALAN had to work less to search and obtain caterpillars for their offspring, thereby spending less energy than birds in dark areas. Indeed, previous research shows a negative relationship between caterpillar abundance and energy expenditure of wild great tits (Tinbergen and Dietz, 1994). In addition, when brood size is manipulated experimentally in great tits, DEE is lower in smaller than larger broods, suggesting that when food availability is relatively large (because parents have to feed less offspring than they planned for), a decrease in DEE is observed. The higher abundance of caterpillars under white and green light is not surprising, as mid and short wavelengths are known to attract more flying insects, and especially Lepidoptera species, than long wavelengths such as red light (van Langevelde et al., 2011). This is also true at our sites (Spoelstra et al in revision). Different light spectra could thus alter the balance between costs and benefits of light pollution for predator birds and their insect preys, an hypothesis that has already been suggested for other types of species interactions (Davies et al., 2013; Sanders et al., 2015).

Obviously, without a direct manipulation of food availability our conclusion that DEE is reduced due to an indirect effect of ALAN on food availability is mostly speculative, but at least two other results support it. First, DEE was lower in the birds in the green and, marginally significant, white ALAN treatments in the site with the highest amount of caterpillar biomass, Voorstonden. This site has the highest concentration of native deciduous trees (oaks and birches) which are known to be the preferred host species of Lepidoptera larvae that Parid species feed on (Visser et al., 2006; Tallamy and Shropshire, 2009; Burghardt et al., 2010). Conversely, the other seven sites are mostly evergreen forests and harbor very little caterpillar biomass, which does not differ between light treatments (Figure S2). When we used DEE data from these sites only, there was no longer a significant difference in DEE between the four treatments. It would be interesting to analyze the diet of great tits in these caterpillar-poor sites, to test whether the lack of caterpillars is compensated with other food sources, as well to sample more deciduous sites in order to avoid pseudo-replication. It could also be possible that the low caterpillar abundance at these evergreen sites precluded accurate measurements of any treatment effect, and/or the flora in some habitats is more responsive to the light stimulation than in others. Second, the relationship between the visit rates and the distance of a nest box to the closest lamppost depended on light treatment. In the green treatment, birds visited the nest box more often when breeding close to the lamppost than further away, while the opposite was found in the dark areas. Sampling caterpillar abundance with increasing distance to the lampposts might help elucidating whether caterpillar biomass varies as a function of the distance to the lamppost.

Interestingly, the relationship between DEE and visit rates depended on light color. While DEE was significantly related to visit rates in the dark and red ALAN treatments, we found no correlation between these two variables in the green and white light. It is well known that visit rate of parent birds during the chick-rearing period does not always relate to DEE, as many other behavioral and physiological factors contribute to energy expenditure aside from provisioning (Tinbergen and Dietz, 1994; Verhulst and Tinbergen, 1997; Williams, 2012). Thus, our results are not contradictory with the existing literature, yet they are very intriguing. It appears that short (green) as well as broad (white) spectrum illumination alters the relationship between work rate and DEE. Thus, there is a striking fit between the results for caterpillar frass, DEE and light treatments. This strongly suggests that the higher food availability found under white and green ALAN might alter foraging behavior and ultimately explain the differences in DEE. Although absolute provisioning rates do not vary between light treatments, birds in the white and green light sites may have to work less to find food for their offspring, disrupting the relationship between DEE and visit rates found under natural dark nights. It has been recently highlighted that environmental as well as endogenous factors can alter the relationship between behavior and physiology (Killen et al., 2013). Our results point in this direction, as we show that DEE and provisioning behavior are not always correlated, but this relationship depends on the environment where birds breed, which in turn is affected by the experimental light source via changes in food availability. Such indirect effects of ALAN have rarely been taken into consideration, but they are likely to be more widespread than currently appreciated.

Activity times of the birds did not differ between treatments. Thus, it is unlikely that differences in active day length could have explained the (observed) lower DEE levels in the green and white treatments. Songbirds are known to extend their activity into night when exposed to light pollution, both in the evening but especially in the morning (Da Silva et al., 2014; Dominoni et al., 2014; Russ et al., 2015; de Jong et al., 2016a), which suggest that ALAN might increase DEE through the lengthening of the active day. However, our results show the opposite trend, with birds in the dark treatments visiting their nest box on average earlier in the morning, and later in the evening, compared to the birds in the light treatments.

Females had a longer active day than males, and this is not surprising as females usually stay in the nest box at night during for most of the breeding period. Therefore, unless males visit the nest box before the female awakes (which is unlikely at least in our own experience studying this species), the female will be the first bird to trigger the transponder reader in the morning (and the last in the evening). In addition, a recent study conducted at our sites showed that onset of dawn song in male birds did not vary between the different light treatments in several different species, including the great tit (Da Silva et al., 2017). This result may be related to our use of relatively low, realistic light intensities for countryside roads in addition to the difference with non-experimental situations where light is correlated with other disturbances (see Spoelstra and Visser, 2013). This might also hint to the fact that any behavioral or physiological changes that we observed between our treatments are unlikely to be a consequence of a direct effect of light exposure, but rather of indirect effects such as changes in food availability.

Although we used experimentally illuminated previously dark habitats, a limitation of our study, and more in general of our experimental set-up, is that we are unable to control for non-random settlement patterns of birds in the different light treatments. Birds with different metabolic rates, personality traits, sensitivity to light, or additional physiological/behavioral characteristics might have settled in the different areas. Thus, our results might be a consequence of such non-random settlements rather than an effect of light on DEE, either direct or indirect. Although this explanation is unlikely (see additional discussion in de Jong et al., 2015), these results have important consequences for our understanding of the ecological impacts of ALAN. They suggest that when artificial illumination is localized in small rural and forest locations, its indirect effect of increased insect availability may offset the negative direct effects on stress and sleep disruption previously reported. However, in more densely urbanized areas with pervasive presence of light pollution and sparse, exotic vegetation, the positive effects of light pollution on food availability that our study suggest might not be present. Indeed, the quantity and quality of caterpillars in urban areas is usually low (Isaksson and Andersson, 2007), and in a previous study DEE was found to be higher in urban compared to rural great tits, although no information was provided on food availability nor on exposure to light (Hinsley et al., 2008). In addition, urban-adapted animals might have very well developed tolerance to the presence of artificial lights (Dominoni et al., 2013). Future studies should focus on experimentally testing the interaction between ALAN, food availability and the behavioral as well as physiological differences between urban and rural animals in response to light pollution.

Taken together with previous research in this field, our results suggest that white and green lights likely have the greatest effect on great tits' behavior and ecology, through both direct and indirect effects. Conversely, longer wavelengths leaning toward the red spectrum seem to induce similar behavioral and physiological responses to living in darkness (but see de Jong, 2016). Such results are comparable to observations on mammals, in particular mice and bats (Spoelstra et al., 2015), and point to red light being a useful opportunity to minimize the impact of light pollution on wildlife. Such finding is not surprising, as most photoreceptors in mammals and birds have peak sensitivity around mid and short wavelengths (Peirson et al., 2009; Hunt et al., 2014). However, more research is needed, especially to understand how generalizable these results are to other avian and mammalian species, but also to other organisms that live in light polluted environments.

ETHICS STATEMENTS

This study was carried out in accordance with the recommendations of the Animal Experimentation Committee (DEC), KNAW, with the protocol number "NIOO 14.05 addendum2."

AUTHOR CONTRIBUTIONS

AW, NV, MV, KS, and DD designed the study. AW, NV, AK, JO, and DD collected the data. AW, NV, and DD analyzed the data. AW and DD wrote the paper. All authors read, commented on and approved the final version of the manuscript.

FUNDING

The setup and maintenance of the experimental facilities of "Licht Op Natuur" is financed by the Dutch Technology Foundation STW, part of the Netherlands Organization for Scientific Research (NWO). The project is supported by Philips and the Nederlandse Aardolie Maatschappij (NAM). We thank Staatsbosbeheer, Natuurmonumenten, the Dutch Ministry of Defence, Het Drentse Landschap and the municipality of Ede for the use of their terrain. This specific study was funded by the NWO grant "Ageing in the light" (260-25310) to DD and MV.

ACKNOWLEDGMENTS

We thank Staatsbosbeheer, Natuurmonumenten, the Dutch Ministry of Defence and Het Drentse Landschap for allowing us to illuminate natural habitat and to work in their terrain. We further thank Lucia Salis and Bart van Lith for their help during field work. Last, we thank Chris Guglielmo and Keith Hobson for their useful suggestions on the laser spectrometer, and Hans Zweers for his help to install the Picarro spectrometer.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00055/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AN and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

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ORIGINAL RESEARCH
published: 17 May 2017
doi: 10.3389/fevo.2017.00046

Two Neural Measures Differ between Urban and Rural Song Sparrows after Conspecific Song Playback

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Urbanization is a critical form of environmental change that can affect the physiology and behavior of wild animals and, notably, birds. One behavioral difference between birds living in urban and rural habitats is that urban males show elevated boldness or territorial aggression in response to simulated social challenge. This pattern has been described in several populations of song sparrow, *Melospiza melodia*. Such behavioral differences must be underpinned by differences in the brain, yet little work has explored how urbanization and neural function may be interrelated. We explored the relationship between urbanization and neural activation within a network of brain regions, collectively called the social behavior network, which contributes to the regulation of territorial aggression. Specifically, we captured free-living, territorial male song sparrows by playing them conspecific songs for 6–11 min, and then collected their brains. We estimated recent neural activation, as indicated by the immediate early gene FOS, and measured levels of a neuropeptide, arginine vasotocin (AVT), which is involved in the regulation of social behavior. Based on previous studies we expected urban males, which are generally more territorially aggressive, to have lower FOS expression in a node of the social behavior network implicated in regulating territoriality, the lateral septum (LS). Additionally, we expected urban males to have lower AVT expression in a brain region involved in the regulation of sociality, the medial bed nucleus of the stria terminalis (BSTm). We found that, compared to rural males, urban male song sparrows did have lower FOS expression in the LS. This pattern suggests that lower neural activation in the LS could contribute to behavioral adjustments to urbanization in male song sparrows. Additionally, counter to our predictions, urban male song sparrows had higher AVT-like immunoreactivity in the BSTm. Future work building upon these findings is needed to determine the causal role of such neural differences across rural and urban habitats. Understanding the mechanisms impacted by urbanization will inform our understanding of the reversibility and consequences of this form of habitat change.

Keywords: urbanization, social behavior network, arginine vasotocin, immediate early gene, territorial aggression, song sparrow

OPEN ACCESS

Edited by:
Diego Gil,
Consejo Superior de Investigaciones Científicas, Spain

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *06 February 2017*
Accepted: *28 April 2017*
Published: *17 May 2017*

Citation:
Sewall KB and Davies S (2017) Two Neural Measures Differ between Urban and Rural Song Sparrows after Conspecific Song Playback. Front. Ecol. Evol. 5:46. doi: 10.3389/fevo.2017.00046

INTRODUCTION

Anthropogenic habitat disturbance is now recognized as impacting the phenotypes of wild animals and is a particular concern for wild birds (Crick, 2004; Both et al., 2006; Caro, 2007; Visser, 2008; Wingfield, 2008; Bonier, 2012; Sol et al., 2013; Wong and Candolin, 2015). Though some species are threatened by anthropogenic habitat disturbance, many animals adjust their behavior and physiology through phenotypic plasticity to cope with such environmental change (Vitousek et al., 1997; Wingfield, 2008; Bonier, 2012; Sol et al., 2013; Wong and Candolin, 2015). Endocrine mechanisms are a major link between organisms' perception of environmental conditions and behavioral and physiological responses, and thus play a central role in mediating phenotypic plasticity or acclimation to urbanization (Lessells, 2008). There is an urgent need to understand the endocrine and neuroendocrine mechanisms that permit animals to cope with changing environments because elucidating how animals adjust their physiology to urbanization will shed light on why some species persist and others decline when faced with a changing environment (Cockrem, 2005; Visser, 2008; Wingfield, 2008; Whitman and Agrawal, 2009; Engel et al., 2011; Hoffmann and Sgrò, 2011; Wong and Candolin, 2015). Reciprocally, determining how novel urban environments alter neuroendocrine and behavioral phenotypes provides insight into the function and evolution of these traits.

Several species of birds and mammals living in urban habitats are more bold or aggressive toward conspecifics than those living in undisturbed habitats (Warren et al., 2006; Parker and Nilon, 2008; Evans et al., 2010; Fokidis et al., 2011; Atwell et al., 2012). Song sparrows are an excellent illustration of this, as multiple research groups have shown that urban male song sparrows are more aggressive toward conspecifics than their rural counterparts (Evans et al., 2010; Foltz et al., 2015b; Davies and Sewall, 2016). Further, male song sparrows living in urban habitats are more bold in response to heterospecific alarm calls and human approach than are rural males, though only response to alarm calls is correlated with conspecific aggression (Evans et al., 2010; Scales et al., 2011; Myers and Hyman, 2016). Understanding the origins of such differences across habitat types requires addressing both ultimate explanations and proximate mechanisms of behavior. Increased boldness or aggression in urban male song sparrows could be a response to resource availability (Foltz et al., 2015b) or differences in conspecific density (though see Davies and Sewall, 2016). However, regardless of the fitness benefit of the behavior, proximate mechanisms must mediate behavioral differences. Despite repeated demonstrations of behavioral differences in song sparrows living along urban-rural gradients, the neural and physiological basis of differences in aggression are not fully understood (Evans et al., 2010; Foltz et al., 2015a; Davies and Sewall, 2016).

Understanding the mechanisms underlying reliable differences in conspecific territorial aggression across habitats is important for predicting the reversibility and fitness consequences of behavioral responses to urbanization. A first step in this process is to compare measures of the physiological processes that underlie behavior in animals living in different habitats to determine which traits vary and therefore could regulate behavioral changes. Prior studies have failed to find reliable differences in levels of the avian stress hormone, corticosterone, between rural and urban songbirds despite predictions that urban habitats could impact stress reactivity (Partecke et al., 2006; Bonier et al., 2007; Schoech et al., 2007; Fokidis et al., 2009, 2011; Atwell et al., 2012; Bonier, 2012; Foltz et al., 2015a). Nor have consistent differences been reported in levels of testosterone, a hormone traditionally thought to promote aggression in vertebrates (Partecke et al., 2005; Fokidis et al., 2011; Deviche and Davies, 2013; Atwell et al., 2014; Davies and Sewall, 2016; Davies et al., 2016). Failure to find differences in circulating hormone levels suggest that reliable behavioral differences may be underpinned by deeper brain mechanisms, yet little work has explored how urbanization and neural function may be interrelated. Therefore, we explored possible relationships between urbanization and neural activation within brain regions involved in the regulation of social behaviors, namely territoriality and aggression, to identify mechanisms that could be impacted by, or reflect behavioral adjustment to, urbanization.

To identify brain mechanisms that could be impacted by urbanization, or underpin adjustments to novel urban environments, we first measured the expression of the immediate early gene (IEG) FOS, a rapidly inducible transcription factor that is a proxy for recent neural activity (Clayton, 2000), within regions of the brain social behavior network. The social behavior network is a taxonomically conserved, reciprocally connected, network of brain regions that play a central role in regulating patterns of social behavior, including regulating male territorial behavior and aggression, across a wide range of taxa (Newman, 1999; Goodson, 2005; O'Connell and Hofmann, 2011). Despite the central role of the social behavior network in regulating conspecific territorial aggression, it remains unclear whether activation of these brain regions in response to social challenge differs across urban and rural habitats. Therefore, we compared FOS expression in the social behavior network of male song sparrows from urban and rural habitats exposed to conspecific song playback. This commonly employed experimental approach (Jarvis et al., 1997; Clayton, 2000) is a first step toward identifying the brain regions underpinning behavioral adjustments to urbanization. Based on previous studies examining territorial aggression and FOS expression in male song sparrows, we expected to find lower FOS-ir in the lateral septum (LS) and paraventricular nucleus (PVN) of urban sparrows (Goodson et al., 2005b).

Additionally, the neuropeptide arginine vasotocin (AVT), the avian homolog of vasopressin (AVP), plays a central role in the modulation of sociosexual behaviors across taxa (Goodson and Bass, 2001; Insel and Young, 2000). Although the AVT and AVP systems have a range of homeostatic functions, including stress responsiveness and osmoregulation (Simon-Oppermann et al., 1980; Madison et al., 2008), this peptide acts within several nodes of the social behavior network to regulate sex and species-typical behaviors (Santangelo and Bass, 2006; Goodson et al., 2009a; Kabelik et al., 2009). In birds, AVT expressing cell bodies in the bed nucleus of the stria terminalis (BSTm) are selectively responsive to social stimuli and are implicated in regulating male territorial aggression, while AVT in the PVN is involved in homeostatic function (Kiss et al., 1987; Panzica et al., 1999; Plumari et al., 2004; Goodson and Wang, 2006; Goodson et al., 2009b; Fokidis and Deviche, 2012). Therefore, we also examined whether AVT expression differed within the BSTm and PVN of male song sparrows living in rural and urban habitats.

Collectively, our approach was designed to determine how neural activation within the brain related to urbanization and thus which regions could be impacted by habitat. Future work can then assess how activity within these brain regions may be involved in regulating persistent differences in territorial aggression between urban and rural male song sparrows (Evans et al., 2010; Foltz et al., 2015b; Davies and Sewall, 2016). Additionally, this work evaluated whether AVT could be a mechanism impacted by urbanization, as it is known to contribute to the regulation of aggression. Based on previous studies, we expected urban birds to have different patterns of neural expression in the social behavior network compared to rural birds, particularly within the BSTm and lateral septum (LS), regions centrally involved in conspecific aggression and territoriality (Kollack-Walker et al., 1997; Goodson, 1998; Goodson et al., 2009b; Motta et al., 2009; Goodson and Kingsbury, 2011). Additionally, based on previous studies, we expected rural birds to have less AVT within the BSTm, but not the PVN (Fokidis and Deviche, 2012). This work moves us toward understanding how "urban adapters" are able to cope with human-impacted habitats and why some species may be limited in such physiological or neural acclimation.

MATERIALS AND METHODS

Subjects

Permission to conduct the procedures described in this study was granted by the US Fish and Wildlife Service (permit MB08005B-0), the US Department of the Interior (permit 23818), the State of Virginia's Department of Game and Inland Fisheries (permit 053668), and Virginia Tech's Institutional Animal Care and Use Committee (protocol 13-074).

Male song sparrows in breeding condition were captured in the wild from one urban ($N = 9$) and one rural ($N = 7$) site within a group of 9 field sites near Blacksburg, VA that are along a rural urban gradient. We calculated urbanization scores using a technique validated by Seress and colleagues, which uses aerial images to quantify land-cover in a 1 km^2 area around each study site (Seress et al., 2014; Davies and Sewall, 2016; **Figure 1**). Larger PC scores from this analysis indicate higher abundance of buildings and paved surfaces, and lower abundance of vegetation. Though the limited number of sites constrains our ability to generalize our findings, it was necessary to collect birds from only two sites and limit impact on the other long-term study populations (each population consists of ∼20–30 breeding pairs). Males from these urban sites are reliably more territorial throughout the breeding season compared to rural male song sparrows (Davies and Sewall, 2016) and this pattern has been found in other rural and urban populations (Evans et al., 2010; Scales et al., 2011). Males were captured between 11 June and 19 June 2014 by placing a speaker (Micro II; JBL, Northbridge, CA, USA) and mist net in the center of a focal male's territory and playing one of 16 conspecific song playback stimuli to simulate a social challenge to the territory holder. Each playback track consisted of two song types recorded from one of 16 males from a population in Durham, NC, USA. Songs were presented at a rate of 1 song per 10 s at an amplitude of 80 dB, 1 m from the speaker. An average of 8.5 (±2) min of playback was required to capture each male; we did not present longer playback to avoid habituation and to maximize our chance of capturing the territory holder. We were unable to collect behavioral data because of insufficient field assistance, but collected blood samples within 3 min. of capture, permitting us to later quantify plasma testosterone. After collecting blood, we held birds in darkness and silence until we collected brains (ca. 40 min) to allow time for FOS protein translation, while minimizing the contribution of additional stimuli to patterns of neural expression (Herdegen and Leah, 1998). We sacrificed males by deeply anesthetizing them with isoflurane before rapidly decapitating them and removing the brain from the skull. We collected brains ca. 50 min from the start of playback, which is shorter than the experimental timeline of previous studies in song sparrows (e.g., 90 min; Goodson et al., 2005b) but longer than the half life of FOS protein (45 min; Herdegen and Leah, 1998). Thus, though the FOS expression we quantified was at least partially induced by hearing and responding to conspecific song, comparison with other FOS studies in song sparrows may be limited. We fixed the brains by immersion in acrolein for 4 h, saturated them in sucrose, flash froze them on dry ice, and stored them at −80°C until sectioning and immunohistochemistry (IHC) was carried out.

Plasma Testosterone

We collected blood samples by veinipuncture of the alar wing vein and stored them on ice until they were centrifuged, the plasma separated, and frozen at –80°C later the same day. We measured plasma testosterone using enzyme-linked immunoassay kits (ADI-900-065, Enzo Life Sciences, Farmingdale, NY, USA), which we have optimized and validated for use with song sparrow plasma (Davies and Sewall, 2016). Briefly, we diluted samples 30 times, added steroid displacement reagent at 0.5% of plasma volume, randomly assigned samples to

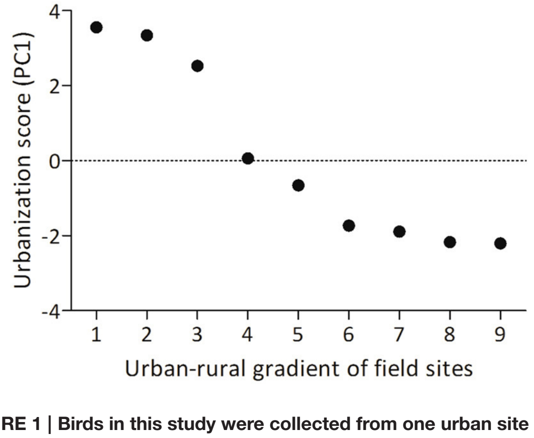

FIGURE 1 | Birds in this study were collected from one urban site (number 1) and one rural site (number 9, above) along an urban rural gradient within 15 km of Blacksburg, VA, USA. The urbanization index for each site was calculated following Seress et al. (2014); larger PC scores on the y-axis indicate higher abundance of buildings and paved surfaces, and lower abundance of vegetation.

assay plates ($N = 3$ plates), and assayed all samples in duplicate. The average assay sensitivity was 1.1 pg/mL. The average intra- and inter-assay coefficients of variation were 8.1 and 5.7%, respectively.

Histology

We coronally sectioned brains at a thickness of 40 μm using a cryostat at −21°C and divided them into three series, which we stored as floating sections in cryoprotectant at −20°C. We immuno-stained two series of brain sections separately, one for FOS and one for AVT, in two runs per antigen, with subjects randomly assigned to runs. For both AVT and FOS, following washes in 1× phosphate buffered saline (PBS), we incubated in 1% sodium borohydride for 30 min to unmask antigens. In the case of AVT, but not FOS, we then incubated three times, each for 5 min, in freshly boiled citrate buffer (10 mM citric acid, 0.05% tween 20, pH 6.0). We then incubated both AVT and FOS sections in 0.3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity, and then 5% normal goat serum for 1 h to block background immunoreactivity. We then incubated sections for ∼24 h at 4°C in either rabbit anti-FOS (Santa Cruz Biotechnology, Dallas, TX, USA, cat. # sc-253 at 1:5,000) or guinea pig anti-AVP (Penisula Laboratories International, San Carlos, CA, USA, cat. # T-5048 at 1:5,000, previously distributed by Bachem, Torrence, CA). Following the primary incubation, we blocked endogenous aviden and biotin by incubating for 15 min in avidin/biotin blocking reagent (Vector Laboratories, Burlingame, CA, USA), then incubated in biotinylated secondary antibody for 1 h (FOS: biotinylated goat anti-rabbit at 1:500; AVT: goat anti-guinea pig at 1:250; Vector Laboratories, Burlingame, CA, USA). We next incubated sections for 1 h in avidin-biotin complex (ABC Vectastain Elite kit at 1:200; Vector Laboratories, Burlingame, CA, USA), then visualized labeling by incubating for 1 min in 3, 3–diaminobenzidine chromagen (Vector Laboratories, Burlingame, CA, USA). Between each incubation described above, we washed in PBS. After mounting on glass microscope slides, we allowed immunolabeled sections to dry at room temperature for 24 h before dehydrating through a graded ethanol series, clearing in xylenes, and affixing coverslips using Permount mounting medium (Fisher Scientific, Pittsburg, PA, USA).

Imaging and Quantification

All quantification of immunoreactivity (ir) for FOS and AVT was carried out by research assistants (T. Breeding and A. Wells) blind to the experimental condition of each subject. We captured gray scale images of each brain region using an AxioCam MR camera attached to a Zeiss Axioimager microscope (Zeiss, USA). The brain regions of interest included the anterior hypothalamus (AH); medial preoptic area (POM); medial extended amygdala, which includes the medial bed nucleus of the stria terminalis (BSTm) and nucleus taeniae (TnA); lateral septum, specifically the caudal ventrolateral portion (LSc.vl; Goodson et al., 2004); ventral tegmental area (VTA); central gray (CG); ventromedial hypothalamus (VMH), which consists of both a lateral and medial portion (Goodson, 2005; Maney et al., 2008); and the paraventricular nucleus of the hypothalamus (PVN). To quantify FOS immunoreactivity (ir) we imaged all brain regions using the 20× objective (200× total magnification).

We located the social behavior network regions following Maney et al. (2008) and references therein. Specifically, we located the AH following Kuenzel and van Tienhoven (1982) and quantified FOS-ir in the region dorsal to the dorsal supraoptic decussation. We located the POM with reference to Alger and Riters (2006) and quantified the region medial to the septomesencephalic tract. To identify the BSTm, we followed Aste et al. (1998) and Maney et al. (2008) and we placed the counting circle dorsal to the anterior commissure. We used Cheng et al. (1999) and Stokes et al. (1974) to locate the TnA. For the caudal ventrolateral portion of the LS, we referenced Goodson et al. (2004) and placed the counting circle medial to the lateral ventricle and beginning rostrally at the level of the anterior commissure. To identify the VTA and CG, we followed Heimovics and Riters (2005) and LeBlanc et al. (2007). For the VTA, we measured the region lateral to the rostral extent of the oculomotor nerve, and for the CG we measured FOS-ir within this region when it resembled the shape of a dolphin's tail. We identified the lateral and medial portions of the VMH at the level of the median eminence following Goodson et al. (2005a) and Maney et al. (2008). We quantified FOS-ir in the PVN starting rostrally from the ventral supraoptic decussation and caudally until the anterior commissure and occipitomesencephalic tract fuse following Davies et al. (2015). We made cell counts within a 0.20 mm^2 circle placed within the AM, BSTm, VTA, and PVN; a 0.15 mm^2 circle in CG and TnA; a 0.13 mm^2 circle inside VMH-m and VMH-l; a 0.05 mm^2 circle placed within the LSc.vl; and a 0.03 mm^2 circle placed in POM. We made counts in the medial most tissue sections of each brain region of interest for a total of 2 sections in AM; 3 sections in CG, POM, VTA, VMH-l, and VMH-m; 4 sections in LSc.vl; 5 sections in BSTm and TnA; and 6 sections in PVN. We used Image J software (ver. 3.1, National Institutes of Health) to view and manually count immunoreactive cells that were visible within the counting frame. All FOS data were calculated as the total number of immunoreactive cells per mm^2.

To quantify AVT-containing cells we counted every immunopositive cell in the BSTm and the PVN, after locating and imaging these brain regions as described above. These data are reported as total cell counts per brain region for each bird. The number of sections in which the brain region of interest was observed is included in analyses (see below) because the value differed across subjects. Though the antibody we used has been validated in avian species (e.g., Kabelik et al., 2010; Kelly and Goodson, 2014) staining in the BSTm was light (**Figure 2**). This could be because of how we prepared the tissue, the ephemeral nature of this cluster of AVT neurons (reviewed in Goodson and Kingsbury, 2011), or cross-reactivity with other non-apeptides. To be conservative, we refer to this hereafter as AVT-like immunoreactivity.

Statistics

We used R statistical software (R Development Core Team, 2008) for all analyses. First, to assess the relationship between habitat type and plasma testosterone we ran a general linear model (GLM) with habitat as a factor and playback duration and the day of year as covariates. We also examined possible relationships between testosterone and AVT cell numbers in the BSTm and PVN using two separate Pearson's correlations.

Because our data are hierarchically structured and to minimize the number of comparisons, we ran one general linear mixed model (GLMM) and one generalized linear mixed model using the lme and lmer package in R (Bates et al., 2015). To examine habitat differences in FOS-ir within the social behavior network we ran a GLMM with habitat type and brain region as fixed factors. We included individual as a random factor to account for the non-independence of samples and nested the number of sections measured per brain region within individual because different numbers of sections were quantified for different brain regions. We used a default Gaussian distribution. The covariates in this model were the day of year, plasma testosterone, and playback duration because reproductive condition, exposure to playback, and hormonal status can influence IEG response (Goodson and Evans, 2004; Goodson et al., 2005b; Maney et al., 2008).

We ran a generalized linear mixed model for AVT in which we tested whether total counts of all AVT cells within a brain region of interest were explained by habitat type, brain region, or the interaction between the two. We specified individual as a random factor to account for non-independence of cell counts. We specified a Poisson distribution for count data (which was supported by reduced residual deviance; Crawley, 2007). We included plasma testosterone and the day of the year as covariates because of the interaction between the testosterone and AVT systems and the effects of breeding state on AVT expression (Panzica et al., 2001). Additionally, we coded the number of tissue sections in which the brain region of interested was found as a covariate because this value differed both between subjects and across brain regions and therefore was not hierarchically structured.

Lastly, we ran separate general or generalized linear models as *post-hoc* tests for each neural marker and brain region of interest, specifying the same covariates and distributions as the main models. We report only main effects from initial models and the main effects of habitat type on measures within each brain region in **Tables 1, 2** but include all model outputs in the Supplementary Materials.

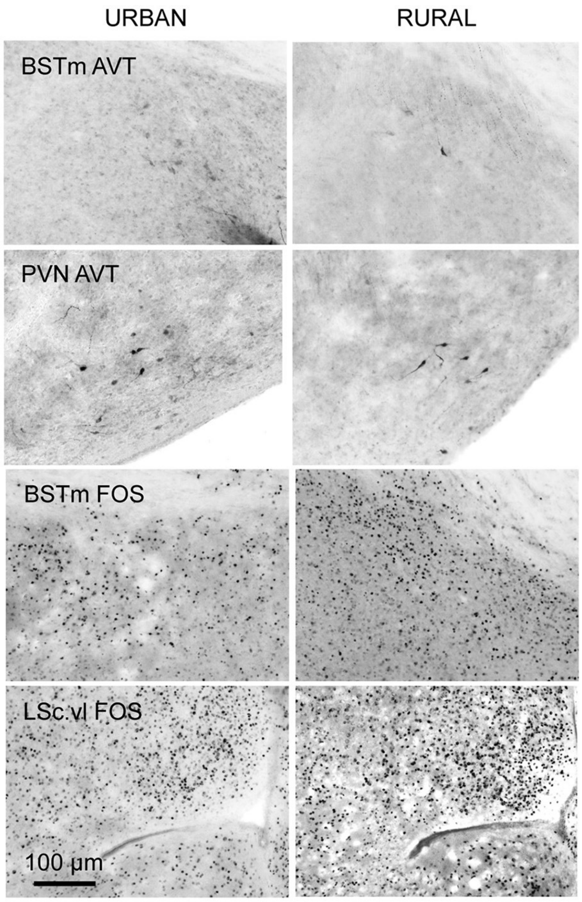

FIGURE 2 | Coronal sections showing examples of AVT-like (top two rows) and FOS induction (bottom two rows) in the BSTm, PVN, and LSc.vl from urban (left column) and rural (right column) subjects. Urban birds had greater AVT-like staining in the BSTm than rural birds but less FOS-ir in the BSTm and LSc.vl than rural birds.

RESULTS

Plasma Testosterone

Though there was a trend for the day of sampling to impact plasma testosterone such that testosterone was counter-intuitively higher later in the season (GLM, effect of day of year, $t = 2.093$, $P = 0.058$), there were no overall differences in testosterone levels as a function of habitat type when day of year was taken into account (mean ± SEM: rural 2.36 ± 0.717 ng/mL;

TABLE 1 | Effects of habitat on FOS-ir quantified as cells per mm^2.

	Estimate	Std. Error	t-value	*P*
Intercept	−2,096.411	928.705	−2.257	**0.028**
Habitat	−2.477	52.105	−0.048	0.963
Brain region	116.402	39.359	2.957	**0.005**
Day of year	14.283	5.684	2.512	**0.029**
Playback duration	2.618	2.797	0.936	0.354
Testosterone	−5.932	12.847	−0.462	0.653
Post-hoc comparisons of effects of habitat on FOS-ir within brain regions (habitat × brain region):				
x AM	26.540	67.801	0.391	0.707
x BSTm	−126.506	58.189	−2.174	0.058
x CG	25.747	90.741	0.284	0.784
x LSc.vl	−126.194	40.638	−3.105	**0.013**
x POM	−64.151	39.246	−1.635	0.137
x PVN	−47.491	96.123	−0.494	0.635
x VMH-l	−102.423	62.235	−1.646	0.151
x VMH-m	−106.808	76.223	−1.401	0.211
x VTA	−41.776	58.080	−0.719	0.495
x TnA	40.998	34.179	1.200	0.258

AM was coded as brain region 1, rural habitat was coded as 0 and urban as 1. Bold text indicates significant ($p < 0.05$) effects and underlined text indicates trends ($p < 0.06$).

TABLE 2 | Effects of habitat on AVT-ir quantified in the PVN and BSTm.

	Estimate	Std. Error	Z-value	P
Intercept	−4.188	5.394	−0.776	0.437
Habitat	1.041	0.284	3.665	**<0.001**
Brain region	3.814	0.190	20.072	**<0.001**
Day of year	0.037	0.033	1.115	0.265
Testosterone	−0.207	0.094	−2.205	**0.027**
Number of tissue sections	−0.028	0.052	−0.545	0.586
Region x Habitat	−1.020	0.216	−4.714	**<0.001**
Post-hoc comparisons of effects of habitat on AVT-ir within brain regions:				
x BSTm	1.355	0.243	5.572	**<0.001**
x PVN	0.2059	0.039	1.478	0.139

The log of plasma testosterone was included as a covariate in the main model and post-hoc GLMs. Rural habitat was coded as 0 and urban as 1; BSTm was coded as brain region 1 and PVN as brain region 2. All cells within each brain region of interest were counted and a Poisson distribution was specified for count data. Bold text indicates significant ($p < 0.05$) effects.

urban 1.35 ± 0.198 ng/mL; GLM effect of habitat, $t = -0.892$, $P = 0.389$). Nor was testosterone correlated with the number of cells showing AVT-like immunoreactivity in either the BSTm ($R = -0.306$, $p = 0.287$) or PVN ($R = -0.325$, $p = 0.279$).

FOS Immunoreactivity

We found a significant effect of habitat type on FOS-ir in the LSc.vl (GLMM, habitat x LSc.vl, $t = -3.105$, $P = 0.013$) and, nearly, in the BSTm (GLMM, habitat x BSTm, $t = -2.174$, $P = 0.058$; **Table 1**; **Figure 3**). Relative to urban birds, rural birds had more FOS-ir neurons in the LSc.vl and a trend of more in the BSTm. Though there was no overall effect of habitat on the number of FOS-ir neurons, the date of the sampling contributed to FOS-ir throughout the brain such that birds collected later in the season had higher FOS-ir (GLMM, effect of day of year, $t = 2.512$, $P = 0.029$; **Table 1**) and there were overall differences in expression across brain regions (GLMM, effect of brain region, $t = 2.957$, $P = 0.005$; **Table 1**). Neither the duration of playback nor plasma testosterone levels significantly explained the number of FOS-ir neurons in the main model or *post-hoc* tests (**Table 1**; Supplemental Materials).

AVT Immunoreactivity

We found a significant effect of habitat on AVT-like immunoreactivity (GLMM, effect of habitat, $z = 3.665$, $p < 0.001$; **Table 2**). Additionally, AVT-like immunoreactivity differed across brain regions (GLMM, effect of brain region, $z = 20.072$, $p < 0.001$), with far higher cell numbers in the PVN than the BSTm (**Figure 4**). Of most importance, there was an interaction between habitat and brain region such that urban male song sparrows had more cells showing AVT-like immunoreactivity in BSTm than rural males (GLMM, habitat x BSTm, $z = 5.572$, $p < 0.001$) though this did not hold in the PVN (**Table 2**; **Figure 4**). Finally, testosterone levels contributed to differences in AVT-like immunoreactivity (GLMM, effect of testosterone, $z = -2.205$, $p = 0.027$).

DISCUSSION

We found that male song sparrows living in urban habitats of Blacksburg, VA have different patterns of immediate early gene and neuropeptide levels in their brains in response to hearing conspecific song, compared to birds living in rural habitats. This work demonstrates that urbanization has impacts on neural processes, and is an important first step toward understanding how habitat change and neural function are interrelated; it is among only a handful of studies exploring how urbanization impacts the brains of wild birds (Maklakov et al., 2011; Fokidis and Deviche, 2012; Davies et al., 2016). The present findings raise two specific hypotheses about how urbanization and neural processes may be interrelated. First, associations between FOS expression and urbanization implicate the social behavior network in the regulation of persistent differences in territorial behavior between urban and rural male song sparrows (Evans et al., 2010; Foltz et al., 2015b; Davies and Sewall, 2016). Second, the finding of elevated AVT-like immunoreactivity in the BSTm of urban male song sparrows supports the hypothesis that these socially sensitive AVT neurons (Goodson et al., 2009b) could also contribute to maintaining behavioral differences between urban and rural song sparrow populations.

Habitat Differences in Neural Response to Conspecific Song

Patterns of IEG expression in the social behavioral network are associated with both perception of social stimuli and behavioral response to those stimuli, with the result that measures differ across social contexts and also as a function of a focal animals' phenotype (Goodson and Wang, 2006; Maney et al., 2008; Kabelik et al., 2009; Goodson and Kingsbury, 2011). Previous studies have found that simulated territory intrusions increase IEG expression in the BSTm, LS, PVN, AH, VMH, CG, and VTA of territorial birds (Maney and Ball, 2003; Goodson and Evans, 2004; Goodson et al., 2005b). However, aggressive response to social challenge is negatively correlated with IEG responses in the AH, PVN, and multiples zones of the LS (Goodson et al., 2005b; Goodson and Kingsbury, 2011) in territorial male song sparrows both during and outside of the breeding season, implicating these regions in the maintenance of persistent differences in territorial behavior (Goodson and Evans, 2004; Goodson et al., 2005b; Goodson and Kingsbury, 2011).

The primary finding in the present study was that urban male song sparrows, who are reliably more territorially aggressive (Evans et al., 2010; Foltz et al., 2015b; Davies and Sewall, 2016), had fewer FOS-ir neurons in the LSc.vl than their rural counterparts (**Table 1**; **Figure 4**). Thus, our finding is consistent with patterns of lower FOS expression in the LSc.vl of animals that show elevated territorial aggression and suggest this region is involved in behavioral adjustments to living in urban areas. Though we also expected to find this pattern of FOS expression across other nodes of the social behavior network (e.g., the AH and PVN; Goodson et al., 2005b) we may not have seen this pattern because our playback was shorter than prior studies in song sparrows (10 min instead of 30 min; Goodson and Evans, 2004; Goodson et al., 2005b; though see Maney and Ball, 2003

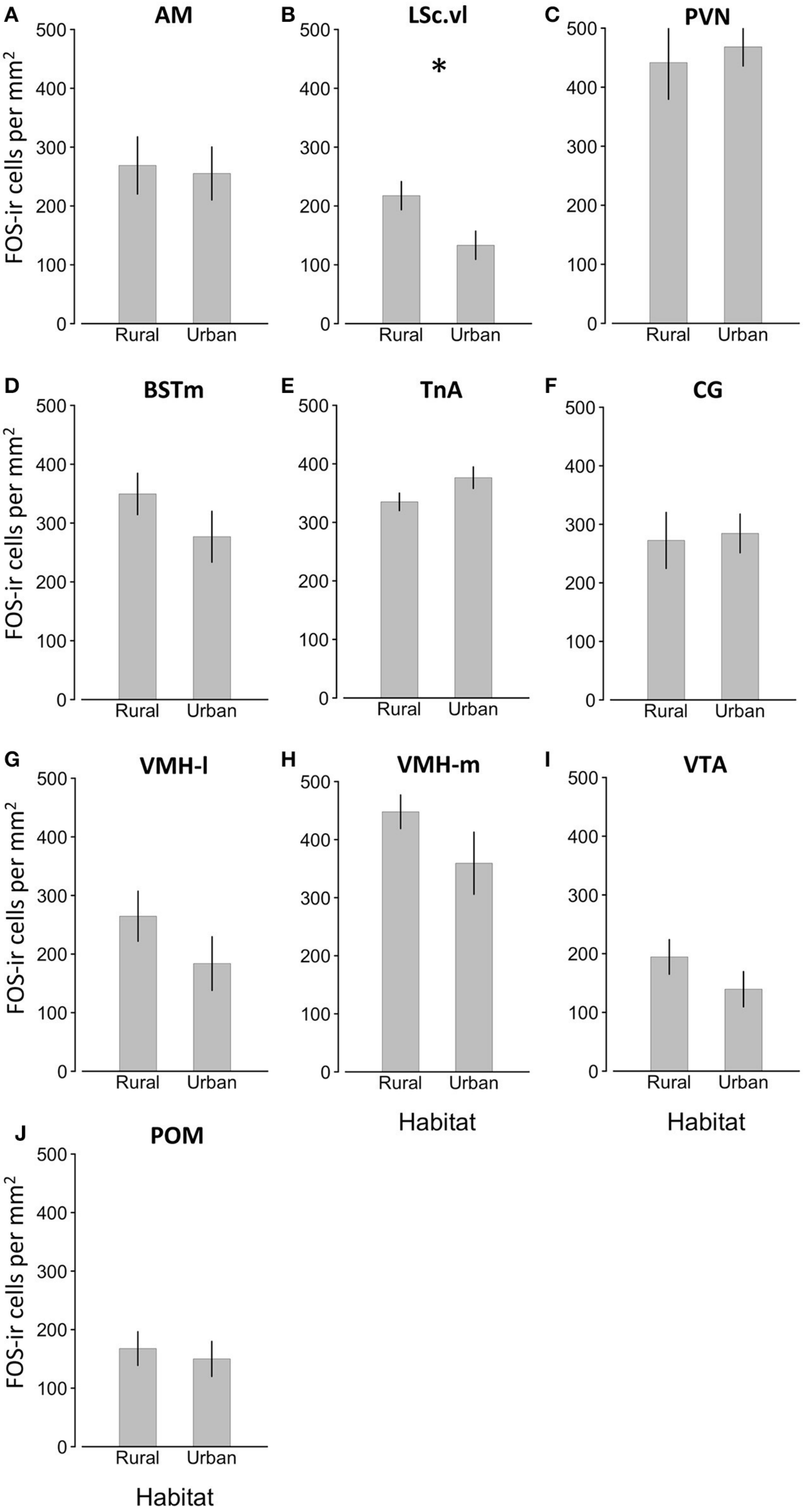

FIGURE 3 | Main effects of habitat type on FOS-ir ±SEM within nine brain regions of interest (A–J) Effects of day of year, duration of playback, relationship with plasma testosterone, and controls for the number of tissue sections in which we found the brain regions of interest were included in the model, but are not represented. Asterisk denotes significant difference between groups.

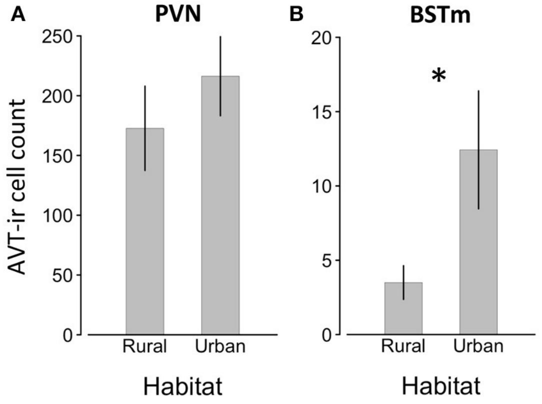

FIGURE 4 | Number of AVT positive cells ±SEM in the PVN (A) and BSTSm **(B)** in male song sparrows collected in rural and urban habitats. Asterisk denotes significant difference between groups.

who also used 10 min) or because capturing males in the wild using playback may unavoidably select the most aggressive birds from each population, minimizing our chances of detecting differences (though thoroughly sampling only two populations should have minimized this risk). Future studies should test the hypothesis that neural activity in the LSc.vl contributes to the modulation of territoriality across rural and urban habitats by comparing FOS expression within this brain region in passively caught subjects from each habitat, as well as by correlating expression with experimental subjects' behavioral responses to song challenge.

While FOS-ir in the LSc.vl is associated with differences in aggressive behavior, FOS-ir in the BSTm may reflect the perception of social challenge, but not the regulation of behavioral responses. It is difficult to disentangle neural responses induced by the perception of a social challenge from those induced by behavioral response to challenge, but previous studies in song sparrows have found correlations between IEG expression in the BSTm and exposure to song playback, but not behavioral response to challenge (Goodson and Evans, 2004; Goodson et al., 2005b). Working from the hypothesis that FOS-ir in the BSTm is positively associated with the perception of social challenge, our results suggest that rural and urban birds perceived playback differently. Specifically, the duration of playback did not impact FOS-ir in the BSTm (**Table 1**; Supplemental materials), yet there was a trend of elevated FOS-ir in the BSTm of rural birds relative to urban birds. This could be interpreted as rural birds perceiving the playback as a greater social challenge than did urban birds. This hypothesis could be tested by presenting birds from different habitats with social challenges of varying intensity such as familiar and unfamiliar conspecifics (Stoddard et al., 1990).

Habitat Differences in AVT-Like Immunoreactivity

Like FOS, the relationship between AVT within the BSTm and aggression can depend upon context and phenotype (Goodson et al., 2009a,b; Kabelik et al., 2009). However, generally, elevated AVT is associated with decreased territorial aggression in breeding, territorial male songbirds. Septal infusions of AVT reduce male-male aggression (Goodson, 1998; Kabelik et al., 2009). Further, studies using double labeling of cells with IEGs and AVT show that AVT neurons in the BSTm are activated by positive social stimuli, such as a potential mate (Goodson and Wang, 2006; Goodson et al., 2009a; Goodson and Kingsbury, 2011). Here, we found that urban birds, which are persistently more territorially aggressive, had more AVT-like immunoreactive neurons in the BSTm, but not the PVN. While AVT levels in the BSTm are associated with territoriality and aggression, AVT in the PVN is involved in maintaining homeostasis (Simon-Oppermann et al., 1980; Madison et al., 2008; Goodson et al., 2009b; Fokidis and Deviche, 2012). Thus, our finding is seemingly counter to studies that used septal manipulations of AVT and found decreased aggression (Goodson, 1998). However, immunoreactivity can reflect elevated production and secretion of the neuropeptide, or it can indicate an accumulation of peptide due to reduced secretion, making it difficult to interpret the functional significance of greater staining (Panzica et al., 2001; Goodson and Kabelik, 2009; Sewall et al., 2010). One interpretation is that urban male song sparrows were sequestering AVT within cell bodies in the BSTm, reducing bioavailable AVT, which would be consistent with lower AVT levels increasing territorial aggression. However, the present finding is counter to that of Fokidis and Deviche (2012), who reported that urban curve-billed thrashers, *Toxostoma curvirostre*, had significantly less AVT staining in the BSTm than desert thrashers and that AVT staining was also inversely related to territorial aggression. Overall, greater AVT-like staining in the BSTm of urban birds is counter to our predictions and the findings of a prior study, so resolving the contribution of the AVT system to behavioral differences associated with urbanization will require double-labeling cells for AVT and IEGs. The present results implicate AVT within socially-sensitive neurons of the BSTm in regulating well-described differences in territorial behavior between rural and urban male song sparrows, but more work is needed to resolve the direction of this relationship. Moreover, additional mechanisms, such as serotonin could contribute to the regulation of territorial aggression (Nelson and Trainor, 2007). Future work is needed to understand how multiple regulatory pathways are integrated to yield differences in territorial aggression in urban and rural song sparrows (Cohen et al., 2012). Further, given that species differ in their behavioral responses to urbanization and multiple physiological pathways could yield similar behavioral outcomes, our understanding of the proximate mechanisms that permit animals to cope with changing habitats is only in its infancy.

CONCLUSIONS

Urbanization impacts the physiology and behavior of free-living animals and is a particular concern for birds (Wingfield, 2008; Bonier, 2012; Sol et al., 2013; Wong and Candolin, 2015). Determining the mechanistic basis of behavioral responses to urbanization is essential to predicting the consequences and limitations of organismal reactions to habitat disturbance and will ultimately help us understand why some species adjust and others decline in the face of environmental change. Here, we found that, compared to rural male song sparrows, urban song sparrows had reduced expression of the IEG FOS within a node of the social behavior network of the brain (the LS), and also more AVT-like immunoreactive neurons within the BSTm. This study adds to the growing evidence that urbanization impacts the brain to influence behavior. We expect future work in this area will identify a vast number of behavioral responses to urbanization and also considerable diversity in the proximate mechanisms that permit such adjustments.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the US Geological Service's bird banding lab and the National Research Council's Guide for the care and use of laboratory animals. The protocol was approved by the Virginia Tech's Institutional Animal Care and Use Committee (protocol 13-074), the US Fish and Wildlife Service (permit MB08005B-0), the US Department of the Interior (permit 23818), the State of Virginia's Department of Game and Inland Fisheries (permit 053668).

AUTHOR CONTRIBUTIONS

KS conducted field work, sample collection, data analysis, and wrote the majority of the paper. SD completed all histology, enzyme immunoassay, immunohistochemistry, quantification, and assisted with editing the paper.

FUNDING

This work was supported by Start up funds from Virginia Tech and a Jeffress Memorial Trust award to KS.

ACKNOWLEDGMENTS

Thanks to T. Breeding and A. Wells for assistance with data collection, and Virginia Tech campus and StREAM lab for access to field sites.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00046/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 30 June 2017
doi: 10.3389/fevo.2017.00068

European Blackbirds Exposed to Aircraft Noise Advance Their Chorus, Modify Their Song and Spend More Time Singing

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Noise pollution has a strong impact on wildlife by disrupting vocal communication or inducing physiological stress. Songbirds are particularly reliant on vocal communication as they use song during territorial and sexual interactions. Birds living in noisy environments have been shown to change the acoustic and temporal parameters of their song presumably to maximize signal transmissibility. Also, research shows that birds advance their dawn chorus in urban environments to avoid the noisiest hours, but little is known on the consequences of these changes in the time they spent singing at dawn. Here we present a comprehensive view of the European blackbird singing behavior living next to a large airport in Madrid, using as a control a population living in a similar but silent forest. Blackbird song is composed of two parts: a series of loud low-frequency whistles (motif) and a final flourish (twitter). We found that airport blackbirds were more likely to sing songs without the twitter part. Also, when songs included a twitter part, airport blackbirds used a smaller proportion of song for the twitter than control blackbirds. Interestingly, our results show no differences in song frequency between airport and control populations. However airport blackbirds not only sang earlier but also increased the time they spent singing when chorus and aircraft traffic overlapped on time. This effect disappeared as the season progressed and the chorus and the aircraft traffic schedule were separated on time. We propose that the typical urban upshift in frequency might not be useful under the noise conditions and landscape structure found near airports. We suggest that the modifications in singing behavior induced by aircraft noise may be adaptive and that they are specific to airport acoustic habitat. Moreover, we found that adjustment of singing activity in relation to noise is plastic and possibly optimized to cope with aircraft traffic activity. In a soundscape characterized by intermittent and strong noise bursts, singing for longer could be more advantageous than modifying frequency parameters, although it is likely more costly.

Keywords: birdsong, anthropogenic noise, avian communication, urban ecology, behavioral plasticity

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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *23 February 2017*
Accepted: *13 June 2017*
Published: *30 June 2017*

Citation:
Sierro J, Schloesing E, Pavón I and Gil D (2017) European Blackbirds Exposed to Aircraft Noise Advance Their Chorus, Modify Their Song and Spend More Time Singing. Front. Ecol. Evol. 5:68. doi: 10.3389/fevo.2017.00068

INTRODUCTION

Birdsong is one of the most elaborate acoustic signals in the natural world. Like many other communication systems, birdsong is crucial for birds because it plays a key role in two very important aspects of reproduction: mate choice and territory defense (Catchpole and Slater, 2003). In temperate climates, males advertise their territory by singing from particular posts during the breeding season. Although it is common to hear birds singing throughout the day, many species concentrate their singing activity at dawn in what is known as dawn chorus (Kacelnik and Krebs, 1983; Catchpole and Slater, 2003). As a sound signal, birdsong has evolved under the selection pressures of the acoustic habitat in which the species thrives, such as the sound propagation properties of the habitat and its particular type of environmental noise (Morton, 1975; Ryan and Brenowitz, 1985; Hanna et al., 2011; Wiley, 2015). Environmental noise is present in every soundscape. Depending on its intensity it can distort the perception of the signal to a variable extent and reduce the number of receivers within the communication network (Hansen et al., 2005). Critically, worldwide urbanization leads to a remarkable increment of natural background noise together with other environmental factors that also hinder acoustic communication, such as the presence of vertical reflective surfaces or an increase in habitat fragmentation (Warren et al., 2006; Slabbekoorn and Ripmeester, 2008). Because of the important functions of song, as well as other vocal signals, increased environmental noise is likely to have a direct impact on fitness, posing a selection pressure over urban populations of birds (Slabbekoorn and Ripmeester, 2008).

Despite the altered conditions of the city soundscape, many bird species thrive in urban environments and cumulative evidence points out to the importance of behavioral plasticity in coping with noise (Ríos-Chelén et al., 2012; Shanahan et al., 2014). Regarding birdsong, studies have shown a general pattern of song modifications that appear in response to noise, such as upshifted frequency (Slabbekoorn and Peet, 2003; Nemeth and Brumm, 2009; Verzijden et al., 2010; Bermúdez-Cuamatzin et al., 2012), increased song amplitude (Cynx et al., 1998; Brumm, 2004; Brumm et al., 2009), modifications in temporal parameters of song (Slabbekoorn and den Boer-Visser, 2006; Díaz et al., 2011; Ríos-Chelén et al., 2013) or changes in the timing of the dawn chorus (Fuller et al., 2007; Arroyo Solís et al., 2013; Dominoni et al., 2016). In many bird species, it has been found that populations living near anthropogenic noise sources sing at higher frequencies, partly avoiding masking (Slabbekoorn and Peet, 2003; Nemeth and Brumm, 2009; Verzijden et al., 2010; Ríos-Chelén et al., 2012; Roca et al., 2016). Also, birds can increase the amplitude of their songs to counteract the detrimental effect of noise, thus improving the signal-to-noise ratio and the distance over which the signal can be perceived (Brumm and Todt, 2002; Brumm, 2004). On the other hand, those studies that have analyzed changes in the duration of songs in relation to increased background noise show inconclusive results: some species are found to sing shorter songs/syllables under noisy conditions (Slabbekoorn and den Boer-Visser, 2006; Verzijden et al., 2010; Francis et al., 2011), whereas other species increase song/syllable length (Nemeth and Brumm, 2009; Bermúdez-Cuamatzin et al., 2011; Ríos-Chelén et al., 2013). Thus, it is not yet clear the role that these changes of song duration play in noisy conditions, it may depend on the information that song length is coding in each species and the temporal characteristics of noise (intermittent vs. continuous; Gil and Brumm, 2013). A study with serins (*Serinus serinus*) showed an increase in time spent singing as noise levels increased but this relationship broke down at a 70 dBA threshold (Díaz et al., 2011). In contrast to direct song modifications, it is possible that increased time spent singing is a more efficient strategy to improve signal transmission without compromising the information coded by song length or syllable duration, although such a behavioral change is bound to be more costly in terms of energy balance (Thomas, 2002).

The high predictability of daily anthropogenic noise patterns allows birds to avoid temporal overlap with noisy hours by means of shifts in their singing activity. There is a growing body of evidence suggesting that birds advance the onset of dawn chorus when living in noisy conditions (Fuller et al., 2007; Gil et al., 2014; Dominoni et al., 2016; Dorado-Correa et al., 2016). The question remains open as to whether this shift of singing activity is a by-product of noise-induced perturbations on the birds' behavior, as suggested by some authors (Arroyo Solís et al., 2013), or an adaptive modification to counter act the effect of noise later on the day (Fuller et al., 2007; Dominoni et al., 2016). Taking an optimization approach and assuming that singing earlier has negative effects due to energy balance and predation (McNamara et al., 1987), it would be expected that birds should advance dawn chorus in order to avoid noisy conditions if this leads to improved signal transmission. In an earlier study (Gil et al., 2014), we suggested that the advance of singing activity at dawn was more relevant in those species whose dawn song was closer to the onset of aircraft traffic activity. On this respect, we should consider that the advance in dawn chorus depends on the amount of noise-chorus overlapping, which in turn is determined by at least three different factors: (1) human activity, which starts at a fixed time regardless of seasonal variations in daylight; (2) the circadian cycle of activity expressed by wild animals which, unlike humans, is synchronized with the natural cycles of sunlight; and (3) differences of the time of sunrise that varies in relation to latitude. Finally, it is yet to be confirmed whether or not an advance in the dawn chorus results in a prolonged singing activity during dawn, since such a change could carry heavier consequences in terms of time budgets.

European blackbirds (*Turdus merula*) belong to the thrushes family (Turdidae) and, although they are forest birds, they are also common urban dwellers (Evans et al., 2009). Blackbirds are among the earliest singers at dawn, with their first songs being heard 1 h before sunrise (Snow, 1958) although actual dawn chorus begins rather later around 30–45 min before sunrise (Ripmeester et al., 2010, pers. obs.), lasting for approximately 20–30 min (Snow, 1958). Males are territorial and will use several song posts to deliver their song (Cramp, 1992). Blackbird song comprises short phrases of few seconds that can be broke down in two parts, the *motif* and the *twitter*, although the twitter is not always present and some songs are built by entirely by motif notes. The motif is made of low frequency, high amplitude melodic notes (pure tones), whereas the twitter is a final flourish composed of a burst of short, higher frequency and low amplitude notes that cover a wider frequency spectrum (Dabelsteen, 1984). Previous studies of this species show that urban blackbirds sing higher frequency motif notes (Nemeth and Brumm, 2009; Ripmeester et al., 2010). Through an increase in the frequency of this typically low-frequency part, blackbirds may improve signal transmission by reducing masking with noise, although it has been argued that higher frequency motif notes are higher in amplitude too and that the later modification should be more relevant than frequency changes to improve transmission (Nemeth et al., 2013). Also, urban birds increase the proportion of their twitter within the song (Ripmeester et al., 2010). Even if this implies less spectral overlap with noise it might not be a functional noise-related modification since twitter elements are usually very weak in amplitude. In fact, because of the motivational role of the twitter during male-male interactions and its seasonal variation (increasing with date), it has been suggested that larger twitter proportions are due to the increased density of territories and the advanced breeding season in urban habitats (Dabelsteen, 1984; Ripmeester et al., 2010). Blackbirds have also been found to advance the onset of dawn song when living next to urban infrastructures such as cities or airports (Nordt and Klenke, 2013; Gil et al., 2014; Dominoni et al., 2016). In such cases, there is likely a compound effect of artificial lighting and anthropogenic noise, which are not always easy to tear apart. When acoustic and light pollution occur simultaneously, light seems to have a stronger impact over the advance of dawn song than noise (Nordt and Klenke, 2013; Da Silva et al., 2016), but birds also advance dawn chorus near large airports in places where artificial lighting is absent (Dominoni et al., 2014, 2016; Gil et al., 2014).

In the present study, we aimed to have a comprehensive view of the impact of aircraft traffic noise on the general singing behavior of the blackbird. Firstly, we focused on noise-induced modifications in the acoustic and temporal characteristics of song, comparing songs recorded from a blackbird population living near Madrid airport with songs of a population distant from the airport. It is important to underline the fact that both populations live in forest habitats with low urbanization of the landscape and that noise is the only important difference between sites as far as we could determine (Gil et al., 2014). We predicted that songs of airport blackbirds would be higher in frequency than those from the control population, following previous findings and to the extent that effects of road traffic noise are similar to aircraft noise. Although aircraft noise may show similar acoustic spectral characteristics, it has definitely different temporal characteristics. The temporal structure of aircraft noise events are usually louder, and more spaced over time, leaving silent intervals in between (Dominoni et al., 2016), in contrast to the continuous emission of car traffic noise (Arroyo Solís et al., 2013). Secondly, we investigated the onset and duration of dawn chorus. We predicted that, if the advance of dawn chorus allows the bird to avoid high levels of noise during rush hours, airport blackbirds should advance dawn chorus at the particular moment of the breeding season when natural singing time overlaps with aircraft traffic, whereas this advance would not be expected when the overlap is absent due to earlier sun rising hours. We believe that our set-up is suitable to investigate this prediction because strict aircraft traffic regulations and schedules lead to a predictable noise levels clearly separated on time of aircraft noise levels (http://sicaweb.cedex.es/ume-fase1.php?id=65) compared to the more diffuse and flexible dawn noise patterns in cities (cfr. Warren et al., 2006).

MATERIALS AND METHODS

Study Species and Study Site

The European blackbird is a widespread and abundant species all over the Western Palearctic. Thanks to its diverse habitat use, blackbird populations are found in high densities both in urban and non-urban environments making this species an excellent model to understand ecological and behavioral adaptations to the urban life (Partecke et al., 2006; Ripmeester et al., 2010; Mendes et al., 2011; Nemeth et al., 2013). In this study, we have focused on two blackbird populations living in a riparian forest along the Jarama river near Madrid. The "airport population" is located in the proximity of two runways of the Madrid-Barajas airport running parallel to the river forest. This population is affected by two airport runways; a distant one approximately 1.5 km away from the river forest (runway number 3), and a close-by runway, hardly 200 m from the edge of the forest (runway number 4) though this distance varies along the runway. The second population is located approx. 10 km northwards within the same strip of river forest, hereafter "control population" (see Figure 1 in Gil et al., 2014). The two habitats are similar (Gil et al., 2014), and consist of meadows and a gallery forest running along the river and ponds and composed mainly of willow trees, ash trees, alder trees and poplars. Although the control study site receives a small impact of aircraft traffic, it is negligible in comparison to the airport population (see below and Results for direct data on this difference). Moreover, a previous study in the same areas showed that there were no differences in light pollution between the two sites (Gil et al., 2014). For an aerial view of the study area please see Gil et al. (2014).

Noise Measurements

Noise maps provided by the airport authorities show a clear difference in average noise levels (2008 data: http://sicaweb.cedex.es/ume-fase1.php?id=65). The blackbird song posts near the airport that we included in this study were all located within the 65–75 L_{den} isophones, while the control area falls entirely inside the 55–60 L_{den} isophones. However, these maps do not show environmental noise levels from all sound sources -total sound-, only the equivalent noise contribution by aircraft events—specific sound—(International Organization for Standardization, 2016). To obtain direct information of the anthropogenic noise impact of both sites we collected our own data in the field.

We used recordings made by autonomous recording units (Song Meter^{TM}: model SM2 Wildlife Acoustics) to monitor the acoustic habitat at each site. The recorders were deployed in the field using a custom-made harness to hang them from a tree branch at a height of 4–5 m. We collected data during 6 days from the 12th to the 18th of March 2016 in the center of each study site. Song meters were programmed to record during 3 h (5:00 a.m.–8:00 a.m.) alternating periods of 10 min of recording and 10 min of inactivity. Aircraft traffic is restricted to two of the four runways during the night time (from 23 to 7 h a.m.), and it is reestablished to all runways during day time (from 7 a.m. to 23.h). On runway 3, aircraft activity is allowed during night time but it mostly takes place after 6 a.m. and is limited to certain aircraft which do not surpass a threshold of noise. For runway 4, the aircraft activity is allowed during day time with an onset time at 7 a.m. Hence, from 5 to 8 a.m. there is an increase in noise in three steps that represent the three characteristic noise conditions of the study site. For this description we defined three time intervals regarding the state of aircraft traffic; "*no traffic*" is the time interval from 5 to 6 a.m. when there is nearly no traffic activity, "*traffic runway 3*" defining the time interval from 6 to 7 a.m., when aircraft activity occurs only in the more distant runway and "*traffic runway 4*" when aircrafts can take off from both runways. In the lab, we used the methodology described by Merchant et al. (2015) to measure equivalent continuous sound levels from .wav recordings using PAMGuide functions in R software version 3.3.1 (Team, 2012). We used the following technical specifications for the calibration of microphone and recorder: sensitivity of transducer = 36 mV/Pa, zero-to-peak voltage of the analog-to-digital converter = 1.414 mV and a gain of 27 dB and we averaged the sound pressure level to a resolution of 15 s via the Welch method. Based on this calibration, we used the PAMGuide function to analyse the entire 3 h recordings of each day and obtain absolute decibel levels with zero weighting setting *atype* as "Broadband." We calculated the logarithmic average of broadband decibel levels per interval of aircraft activity (**Figure 2**). Furthermore we carried out a finer analysis per 1/3-octave band levels using the same function PAMGuide in the package but setting *atype* to "TOL" (Third Octave Levels) and later calculating the mean decibel levels of each frequency band of the 5 days sampled (**Figure 3**). We performed the description of the energy distribution of noise along the frequency spectrum in order to explore possible interactions of aircraft noise and vocal communication in birds.

High Quality Recordings and Song Analyses

By mid-February, we began to visit the study sites in order to map blackbird individuals' song-posts. We heard the first blackbirds singing during the first week of March (March the 2nd in the control site and March the 5th in the airport site). Full blackbird dawn chorus began a few days later around the 15th of March. During dawn, we identified the singing locations of individual male blackbirds by listening to neighboring males singing simultaneously. When detected, song posts were visited again over several days for confirmation. Once we mapped the song posts, we collected high quality song recordings during dawn chorus using a Marantz Professional PMD661 handheld solid state recorder and a Parabolic Microphone Telinga MK2 (sampling rate: 48 kHz, 24 bits). Each day, we targeted only one or two individuals and recorded around 15 min of song. We alternated daily between the control and the airport sites to avoid any seasonal effect in singing behavior interfering with potential population differences. In total we collected high quality recordings for 32 individuals, a balanced sample of 16 males per study site. In the lab, we selected 10 songs per male recorded with no aircraft noise in the background; songs that overlapped with aircraft noise were not amenable to acoustic analysis.

In the lab, the first step of song analysis was a visual inspection of the spectrogram using Audacity software (FFT-window length: 512, window type: Hanning, frequency limits: 0–10 kHz). During this step we took manual measurements using the cursor over the spectrogram for song length, singing effort (ratio between the sum song length of 10 songs divided time elapsed from the first to the last song), motif length, twitter length, twitter proportion (ratio between the length of the twitter part divided by song length), and note to silence ratio within song (the ratio between the sum of note durations and song length) (see **Figure 1**). Every note was categorized as a motif or a twitter note. After inspection of spectrograms, wav files were loaded into R for acoustic analysis using the "seewave" package (Sueur et al., 2006). First, we applied a high pass filter under 1 kHz (*ffilter* function) to remove background low frequency noise. Secondly, using previous manual measurements, we cut out every song from the original recording and exported them as individual wav files using the *savewav* function in seewave. By default, this function normalizes the amplitude of the wave to a numeric value ranging from −1 to 1. We then cut and saved separately every single note within each song (same procedure) and performed an acoustic analysis of each note extracting dominant frequency, which is the maximum amplitude frequency at each time point (*dfreq* function), FFT-window length: 512, window type: Hanning, window overlap: 50%, 5% amplitude threshold for signal detection. Dominant frequency function gives a frequency contour in the form of a two-column matrix corresponding to time and maximum amplitude frequency at each window point. From this contour we obtained mean frequency and the maximum and minimum dominant frequencies of each note. Note that these measures do not represent maximum and minimum frequencies as used in most studies. We decided to use this measurement because it is a more objective measurement than a manual procedure using the cursor where the presence of background noise may introduce biases and thus enables us to compare two study populations with differential noise levels. We developed a short script in R that allowed us to batch-process the notes semi automatically, plotting and inspecting the measurements over the original spectrogram for every analysis to ensure they were correct. If loud signals (commonly other bird's song or call) overlapped temporally but not spectrally with the blackbird note, we applied a band pass excluding this sound from signal detection. We dismissed any note if there was any sound artifact interfering with the detection of dominant frequency.

Automatic Recordings and Dawn Chorus Analyses

To understand how aircraft noise affects the start and the duration of dawn chorus, we deployed autonomous

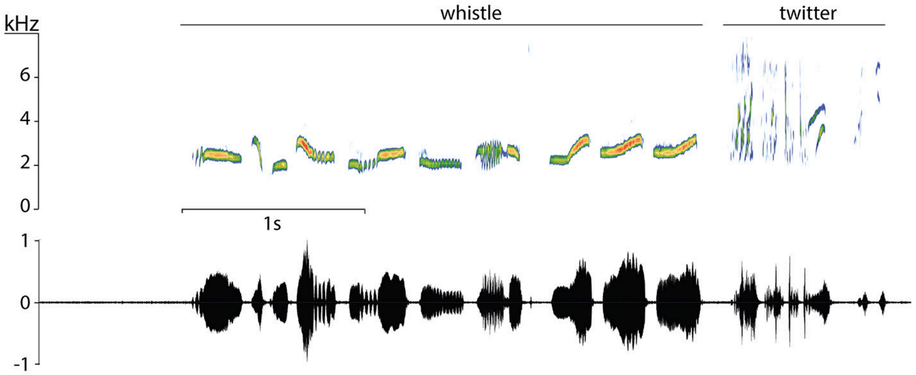

FIGURE 1 | Song of an European blackbird. In the spectrogram (Top) the motif and twitter notes are shown with their relative amplitude represented by differences in color (blue lowest, red highest). Below the spectrogram, the oscillogram of the same song shows the relative differences in amplitude.

recording devices in the field (Bioacoustic Audio Recorders^{TM} -BAR-, Frontier Labs). Each BAR was strapped onto a tree branch less than 5 m from the song post of a specific individual, recording its singing activity during 3 h of continuous recording from 1:30 h before sunrise to 1:30 h after sunrise (sampling rate: 48 kHz, 16 bits). The BARs were fixed in the same position for several days (3.18 ± 0.15 days per individual, mean ± SE) in order to get a representative sample of each individual's chorus characteristics. Afterwards, we retrieved the BAR and downloaded the data to an external hard drive before it was deployed back in the field to a new blackbird territory. We carried out a total of 105 automatic recordings of dawn chorus with 15 individuals in the airport and 18 in the control population from April to June.

In the lab, we monitored the automatic recordings manually using a set of Sennheiser HD 205 headphones. In every recording (1 individual, 1 day) we determined the start of dawn chorus, defined as the first blackbird song heard in the recording with high relative amplitude and continuity. Blackbird songs that presented very low relative amplitude in the recording were dismissed because they could be songs from neighboring territories. Continuity was validated by moving 5 min forward in the recording and checking whether the blackbird was still singing (Bruni et al., 2014). Note that this procedure is different to a previous study in which we took the absolute earliest song (Gil et al., 2014). If the blackbird was still singing 5 min later in the recording, we would confirm this song as the start of dawn chorus and if not, we would omit this song considering it an isolated song. Moving on in the recording we would repeat this procedure until determining the beginning of dawn chorus. Once we confirmed the start of the chorus, we continued the analysis by listening to 1 min of recording every 5 min starting from the beginning of the chorus until the end of the recording (1:30 h after sunrise). For every 5 min interval, we noted whether the blackbird was singing or not based on the 1 min that was analyzed. Dawn chorus duration was defined as the number of intervals in which a blackbird was singing uninterrupted multiplied by 5 min of interval duration. Over the 220.5 h of recording analyzed we identified a blackbird singing on 1,271 intervals and out of these, there were 196 intervals in which the blackbird that was singing was identified as the neighbor and not the territory holder, this represents 15.43% of occasions.

In order to understand how the temporal overlapping of dawn chorus with aircraft traffic activity affected song timing, we divided the season in two halves depending on whether chorus was overlapping or non-overlapping with day time traffic schedule. To this end we required a reference of what is the normal start time of blackbird dawn chorus living in a habitat with no noise impact. Therefore, we used the timing parameters of dawn chorus of our control population as the standard time of singing for blackbirds living under non-urban conditions. In the control site, blackbird chorus began 37.7 ± 1.22 min (mean ± SE) before sunrise lasting for 26.1 ± 1.98 min (mean ± SE), which is consistent with previous references (Snow, 1958; Blotzheim et al., 1993; Ripmeester et al., 2010). We estimated that natural blackbird chorus would overlap with the traffic when sunrise occurred at 7:11 a.m. or later. When sunrise occurred at 7:11, normal blackbird dawn chorus would begin at 6:34 on average, lasting for 26 min until 7:00 am and therefore not overlapping with aircraft traffic, according to our reference. Hence, when sunrise was later than 7:11, blackbird dawn chorus would overlap with aircraft traffic noise but later on the season when sunrise occurs before 7:11, blackbird dawn chorus would not overlap with high levels of aircraft traffic noise.

Statistics

All statistical analyses were carried out in R software, version 3.3.1 (Team, 2012). Firstly, we tested whether background noise differed between the study sites. We analyzed the differences of broadband dB levels between sites and its interaction with the three time intervals of aircraft traffic activity defined before. We fitted a Generalized Linear Mixed Model (glmm) to determine the effect of the study site, the time intervals (regarding the state of aircraft traffic) and its interaction on the dependent variable: dBz levels as an unbiased measure of noise. The model was fitted using a Gamma family distribution and a log link function. The diagnostic plots were visually inspected for possible deviations from heterogeneity and homoscedasticity assumptions. After this, we proceeded to model selection by computing all possible models using *dredge* function from the "MuMIn" package (Barton, 2011) and ranking all models by their Akaike's Information Criteria (AICc). Final model was the full model being the best model with a ΔAICc of 15.59 with the second best.

To investigate potential differences in the acoustic characteristics of the song between the two study populations, we compared the mean, the maximum and minimum dominant frequency of each note. For the analysis, we treated the motif and twitter data separately (Dabelsteen et al., 1993). The data for this analysis was nested using random factors: notes within songs, songs within individuals. Therefore, we fitted Linear Mixed Models (lmm). When the response variable was not normal, we fitted a Generalized Linear Mixed Models (glmm) with a Gamma family distribution since all frequency parameters are positive continuous variables.

Then, using song as unit, we compared several temporal parameters such as the note to silence ratio within song, song length, singing effort, motif length, twitter length and twitter proportion. The analysis of twitter length and twitter proportion was restricted to the songs that actually presented a twitter section. This subset of the data included 233 songs from all 16 individuals at each site. All models had study site as fixed term: a categorical variable of two levels: "airport" and "control" (see **Table 2** for model details). In this model the random term was only the individual because the measurements were taken per song and not per note. We also fitted a binomial Generalized Linear Model (glm) to investigate population differences in the number of songs with twitter vs. number of songs without twitter. For this purpose we built another data set summarizing the sum of songs with twitter and without twitter per individual. In this binomial model, because each individual was represented only once in the data set it was not necessary to fit a mixed effect model. We validated all models by visual inspection of diagnostic plots.

Finally we analyzed which characteristics explained the start and the duration of dawn chorus. For both parameters, we fitted a glmm with Poisson distributions and log link functions. The full model was similar in both cases with the following factors in the fixed term: study site ("airport" and "control") and traffic overlap with dawn chorus ("overlap," "no overlap") and their full interaction. Every individual territory was sampled for several days so individuals were included as random factor to avoid pseudo-replication and account for intra individual variation. We validated all models by visual inspection of diagnostic plots and proceeded to model selection using AICc. In the model analyzing start of dawn chorus the final model was the best model since the second best was at a distance of more than 2 AICc units. The final model in the case of dawn chorus duration was the model average of the best three models.

RESULTS

Noise

We tested differences in background noise over time, from 5 to 8 a.m., between the two study sites and the interaction between study site and time (see **Table 1** for model output). Between 5 and 6 a.m., noise levels are low at both sites but significantly higher in the airport. The estimated decibel levels are very low in both cases and the difference is small; airport 57.40 dB(Z) and control 54.05 dB(Z). Nevertheless, these levels of noise are still very silent conditions. In the interval "traffic runway 3" we find a significant increase in noise regardless of the study site. This increase is probably due to the impact of aircraft traffic noise coming from runway 3 and perhaps other abiotic factors such as increased wind speed. It is important to notice that noise conditions are not significantly different between sites during this interval. After 7 a.m., the increment in noise at the airport site is markedly abrupt and significantly higher than at the control site with an estimated average noise level of 77.47 dB(Z) in the airport and 63.43 dB(Z) at the control site (see **Figure 2**). It is the onset of aircraft traffic under the day-time schedule that brings clear and marked differences in between study sites and, most important, it is highly fixed and sudden as it always begins at 7 a.m. throughout the entire year.

Most of the energy resulting from the noise produced by aircraft traffic is concentrated on the lower frequency range but it also expands to the higher frequencies typical of birdsong (**Figure 3C**). In **Figure 3C** we can see how the noise contribution coming from biotic noise, mostly bird song and other vocalization, shows a clear peak between 3 and 6 kHz in the control study site (dashed line) (Brumm and Slabbekoorn, 2005; Nemeth and Brumm, 2009). In the airport site (continuous line) this contribution is concealed because aircraft noise partially overlaps with the lower frequencies used by birds. This is specially relevant for our model species since the typical frequencies

TABLE 1 | Results from the GLMMs to understand noise differences between sites.

Model Formula	Family	Fixed effects	Estimate	SE	*t*-value	Pr(>|t|)	Sig.
dBz ~ study site * time interval + (1|day)	Gamma (link = log)	Intercept	3.9904	0.0184	217.23	<0.001	***
		Airport	0.0577	0.0252	2.29	0.022	*
		Traffic runway 3	0.1318	0.0252	5.22	<0.001	***
		Traffic runway 4	0.1578	0.0252	6.26	<0.001	***
		Airport:Traffic runway 3	−0.0102	0.0357	−0.29	0.7743	
		Airport:Traffic runway 4	0.1427	0.0357	4.00	<0.001	***

used by blackbirds in their motif notes are concentrated in the frequency range from 1.5 to 3 kHz (Dabelsteen, 1984). Finally, we can observe that the curves of noise along the spectrum are very similar for the two study sites from 5 to 7 a.m. (**Figures 3A,B**) and this fact is obviously different after 7 a.m. when the day-time traffic schedule begins at the airport.

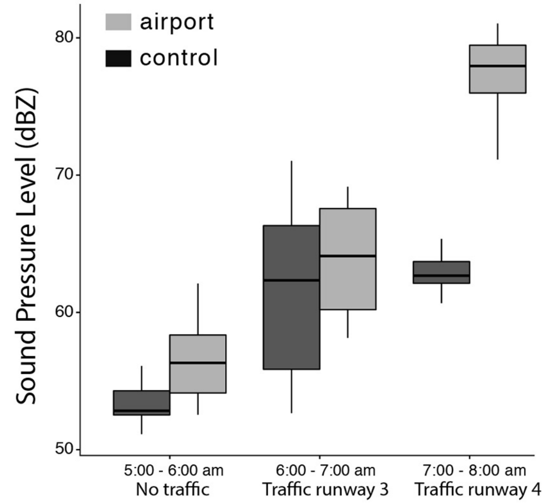

FIGURE 2 | Noise levels at both sites during early morning. Before 6 a.m. there are some small differences between sites that disappear at 6 am. However, obvious differences between study sites arise at 7 am with the onset of day-time traffic schedule.

Song Characteristics

We found no differences between the airport and the control populations in any of the song frequency parameters (see **Figure 4**). However, regarding the temporal characteristics of song, we found significant differences between the two study populations. In the airport populations, songs without twitter part (i.e., songs composed only by a motif), were sang significantly more often than in the control (see **Figure 5A**). Regarding the subset of songs that included twitter, blackbirds next to the airport used shorter twitter proportions (see **Figure 5B**). In the airport, blackbirds also used a shorter twitter in absolute duration but this difference was not statistically significant. Other temporal parameters analyzed such as the motif duration, note to silence ratio within song, song length or singing effort presented no differences between populations (see **Table 2** for model results).

Dawn Chorus

The final model investigating the factors that influence the onset of dawn chorus shows that airport blackbirds significantly advanced their chorus but only when it overlapped temporally with the day-time traffic schedule. Onset of dawn chorus in the control population was not different whether it overlapped or not with traffic activity. The model estimates a dawn chorus advance of 12.8 min in the airport when there was an overlap between the

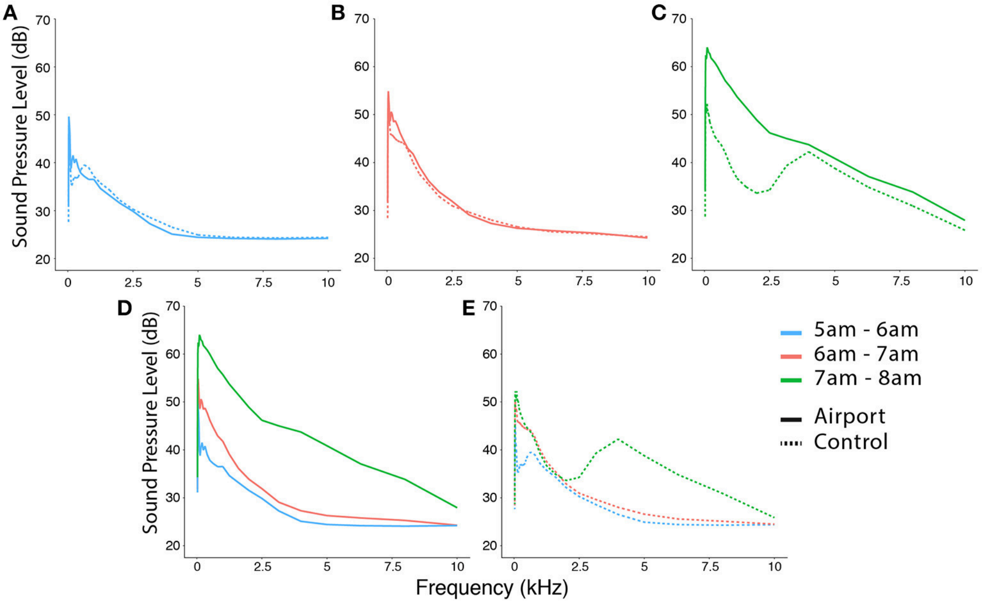

FIGURE 3 | (A–C) show the noise distribution along the spectrum during the three time intervals defined previously: "no traffic," "traffic runway 3," and "traffic runway 4." In each of these three plots, dashed lines represent the control site and continuous lines represent the airport site. We can see that both sites are very similar in **(A,B)** but this changes dramatically in **(C)** that shows the start of the noise produced by the day-time traffic. Here we can appreciate how aircraft noise overlaps at least partially with the frequencies typical of bird vocalizations whose normal representation in a quiet forest looks similar to the control curve. Finally, **(D,E)** show the three time intervals plotted together for each study site separately illustrating more clearly the increase of noise at dawn.

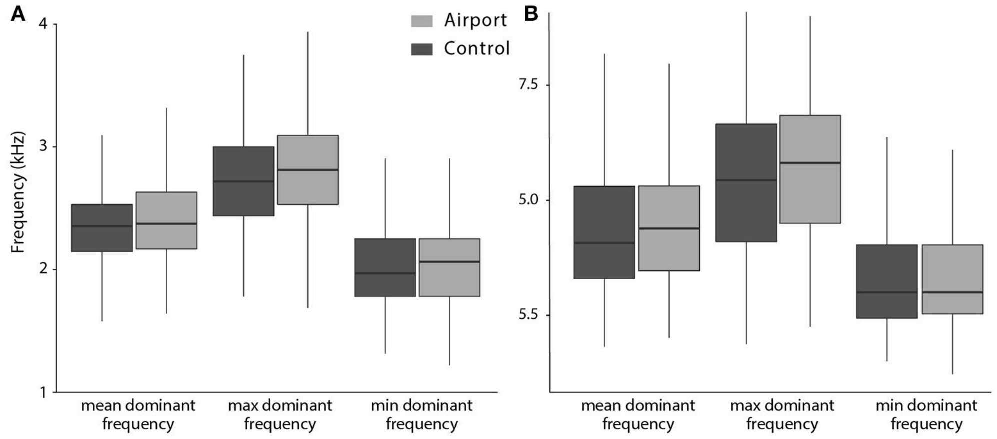

FIGURE 4 | Boxplots showing frequency characteristics of blackbird song in the two populations of the study. **(A)** Shows the mean, maximum and minimum dominant frequency of motif notes and **(B)** shows the mean, maximum and minimum dominant frequency of twitter notes.

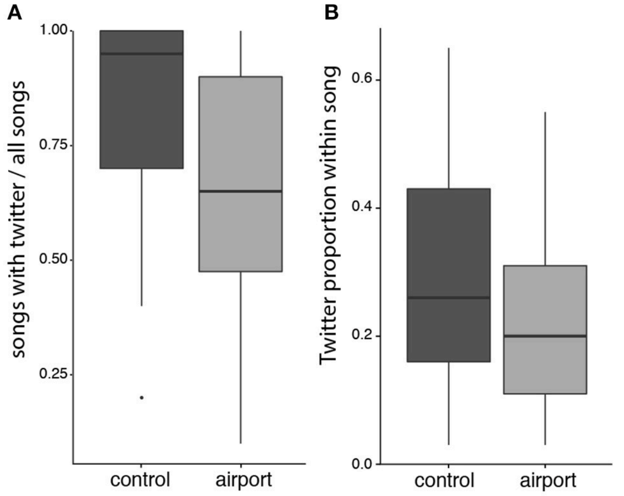

FIGURE 5 | Boxplots comparing **(A)** the proportion of songs with twitter out of the total number of songs analyzed per male, and **(B)** the proportion of twitter length within the song for the subset of songs that present twitter notes.

dawn chorus and aircraft traffic (see **Figure 6B** and **Table 3** for model results).

A visual inspection of the average probability of hearing a blackbird singing along the early morning showed a very clear peak of singing activity that corresponded to dawn chorus and this was followed by sporadic singing activity (see **Figure 6A**). We tested whether the duration of dawn chorus was dependent on aircraft noise and/or study site. Similar to the effect found on the onset of dawn chorus, the duration of the dawn chorus was not systematically longer at the airport but blackbirds enlarged the duration of their chorus when it overlapped with day time aircraft traffic. The model estimates a difference of 24.1 min between sites, airport birds singing a longer dawn chorus. When dawn chorus did not overlap with traffic activity, the duration was similar at both sites (see **Figure 6C** and **Table 3**).

DISCUSSION

We examined differences in song and singing behavior in two blackbird populations differing in the impact of aircraft noise perturbation. Our data show several differences in song design and timing that suggest adaptive changes to the local noise environment. Firstly, blackbirds living near the airport sang more often songs without twitter (only motif notes) and, furthermore, when they did include a twitter part in their songs, the twitter proportion was smaller. This may suggest that airport blackbirds emphasized the loudest part of their songs (motifs), disregarding the fainter part (twitter), in an effort to reduce masking by noise. However, airport blackbirds did not use higher frequencies than those in the control population. This is in contrast to previous findings on this and similar species living near other anthropogenic sources of noise (Slabbekoorn and Peet, 2003; Nemeth and Brumm, 2009; Ripmeester et al., 2010). Additionally, blackbirds living next to the airport advanced the timing of their singing activity at dawn when their dawn chorus overlapped temporally with aircraft activity (early on the season). Interestingly, this advance in chorus onset was coupled with a significant increase in chorus duration. Later on the season, when the timing of the blackbird chorus fell well before the onset of day-time traffic, we found no differences in timing between the control and airport populations. Therefore, blackbirds seem to optimize the timing of their singing activity by adjusting their chorus when it is indeed beneficial but this modification is not constant throughout the season. This may imply that selection for early singers in noisy environments does not lead to an invariant advance but rather favours a highly plastic response in their singing behavior.

A large body of evidence shows that increased frequencies and increased amplitude are two typical urban song modifications (Slabbekoorn and Peet, 2003; Ríos-Chelén et al., 2012; Brumm and Zollinger, 2013). Many authors have argued that these modifications enhance signal transmission in an urban environment and therefore that they are adaptive, but there is

TABLE 2 | Results of GLMMs modeling song characteristics.

Model	Family	Effects	Estimate	SE	t value	Pr(>|t|)	Sig.
Motif peak frequency ∼ study site + (1|id/song)	Gamma (inverse)	Intercept	0.4228	0.0046	91.04	<0.001	***
		Airport	−0.0072	0.0066	−1.09	0.274	
Twitter peak frequency ∼ study site + (1|id/song)	Gamma (inverse)	Intercept	0.2531	0.0105	24.086	<0.001	***
		Airport	−0.0082	0.0142	−0.576	0.565	
Motif maximum frequency ∼ study site + (1|id/song)	Gamma (inverse)	Intercept	0.3669	0.0054	68.22	<0.001	***
		Airport	−0.0114	0.0077	−1.49	0.136	
Twitter maximum frequency ∼ study site + (1|id/song)	Gaussian	Intercept	5.3709	0.1900	28.26	<0.001	***
		Airport	0.2454	0.2784	0.881	0.387	
Motif minimum frequency ∼ study site + (1|id/song)	Gamma (log)	Intercept	0.7125	0.0120	59.37	<0.001	***
		Airport	0.0036	0.0171	0.21	0.833	
Twitter minimum frequency ∼ study site + (1|id/song)	Gamma (inverse)	Intercept	0.3393	0.0135	25.06	<0.001	***
		Airport	−0.0037	0.0184	−0.202	0.84	
Note to silence ratio ∼ study site + (1|id)	Gaussian	Intercept	0.681	0.0091	75.10	<0.001	***
		Airport	0.0002	0.0128	0.019	0.985	
Song length ∼ study site + (1|id)	Gamma (inverse)	Intercept	0.4044	0.0197	20.531	<0.001	***
		Airport	−0.0102	0.0277	−0.369	0.712	
Singing effort ∼ study site + (1|id)	Gaussian	Intercept	0.4673	0.0260	17.958	<0.001	***
		Airport	−0.0088	0.0361	−0.243	0.81	
Motif length ∼ study site + (1|id)	Gamma (log)	Intercept	0.5618	0.0727	7.730	<0.001	***
		Airport	0.1596	0.1028	1.553	0.121	
Twitter length ∼ study site + (1|id)	Gamma (inverse)	Intercept	1.5672	0.1840	8.516	<0.001	***
		Airport	0.4941	0.2678	1.845	0.065	.
Twitter proportion ∼ study site + (1|id)	Gaussian	Intercept	3.9453	0.4201	9.391	<0.001	***
		Airport	1.2121	0.6119	1.981	0.0476	*
(twitter yes, twitter no) ∼ study site	Binomial	Intercept	0.6190	0.1657	3.735	<0.001	***
		Airport	0.8068	0.2598	3.106	0.002	**

some debate as to whether higher frequencies are a consequence of increases in amplitude (Brumm and Slabbekoorn, 2005; Slabbekoorn and den Boer-Visser, 2006; Warren et al., 2006; Nemeth and Brumm, 2010). Data show that in some species higher-frequency songs are louder (Nemeth et al., 2013), and simulations suggest that the benefit of singing higher is much smaller than that of singing louder (Nemeth and Brumm, 2010). Nevertheless, these patterns need not be universal to all species. Adding a new layer of complexity, experimental studies of signal degradation under urban and rural environments have shown that great tit (*Parus major*) urban songs suffered less degradation in both habitats contradicting the Acoustic Adaptation Hypothesis (Mockford et al., 2011). Regardless of the reduced signal degradation of urban songs, that study showed that rural songs prevail in rural environments suggesting that urban modifications may compromise important information of song (Mockford et al., 2011). In contrast to these findings, we found no differences in song frequency in blackbirds living near an airport. A main difference with the previous literature is that we focused on the impact of noise produced by aircraft traffic near a large airport where noise bursts during aircraft events reach very high dB levels, up to 110 dB. Unlike the continuous noise levels of noise found in the city (Dooling and Popper, 2007; Arroyo Solís et al., 2013) in the airport environment that we studied we commonly encounter silent intervals between aircraft events when noise levels may drop below 60 dB(Z) resembling those of a quiet forest (personal observation). It is possible that blackbirds do indeed increase song frequency during noise bursts produced by aircraft and return to the use of normal frequencies soon afterwards. Unfortunately, since we could only conduct song analyses when background noise levels were low, we cannot test this hypothesis. But our data do show a lack of differences in song frequency between areas when aircraft noise is absent. Alternatively, it may be argued that there is a particular noise amplitude threshold over which any modification of song becomes irrelevant (Brumm et al., 2009; Díaz et al., 2011). In addition, during silent conditions, it is likely that higher frequency songs typical of the cities entail other costs because forest songs of lower frequency may provide relevant information and perhaps better sound transmission through the foliage of the forest (Gil and Gahr, 2002; Slabbekoorn et al., 2007; Mockford et al., 2011).

Regarding the temporal parameters of song, we found that blackbirds in the airport population were more likely to drop the twitter of their songs than control blackbirds and, when they did sing twitters, the twitter proportion of these songs was significantly smaller. In contrast, a previous study in cities has shown that urban blackbirds use a larger twitter proportions than rural birds (Ripmeester et al., 2010). It has been argued that these differences could be due to an advanced breeding season and increased territory density in the city and not related to noise (Ripmeester et al., 2010). These confounding factors are unlikely to be a bias in our study because: (1) breeding season is improbably different between the two sites (see Results section) plus we alternated recording days to avoid biases due to date. In addition, given the similarity between both study sites in habitat and vegetation (Gil et al., 2014), we do not expect to find differences in density, although we do not have the data to support this claim.

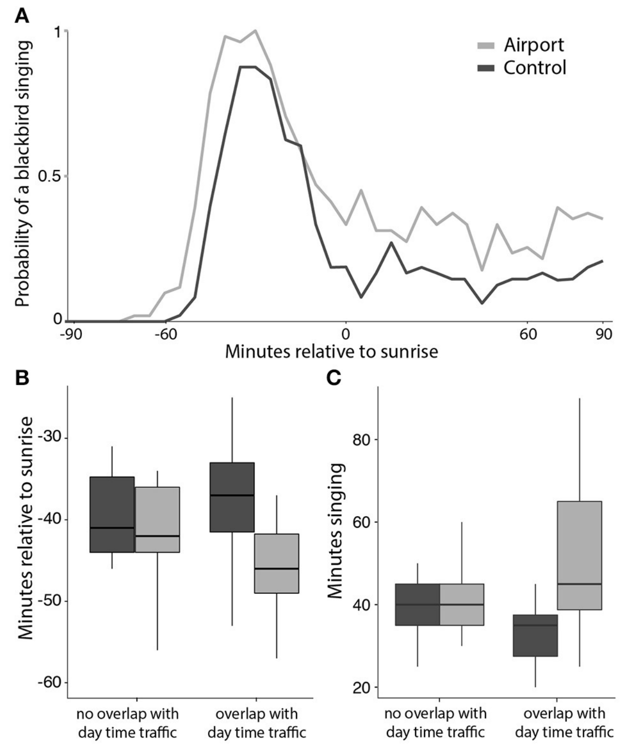

FIGURE 6 | Characterization of the temporal parameters of dawn chorus. Curves in **(A)** show the probability of hearing a blackbird singing at dawn. All recordings were standardized in relation to sunrise time = 0, negative numbers meaning minutes before sunrise. Boxplots in show the differences in onset of dawn chorus in minutes relative to sunrise **(B)** and the duration of the chorus **(C)**, depending on whether the chorus overlapped with day time traffic or not.

A possible adaptive acoustic explanation for the patterns found may be in relation to the amplitude of the different parts that make up the blackbird song. Although twitter notes are higher in frequency than motif notes and therefore suffer less spectral masking by anthropogenic noise they are much weaker in amplitude and would present a much lower signal to noise ratio under aircraft noise than motif notes (Dabelsteen, 1984). In fact, we were obliged to drop analyses of blackbird song under aircraft noise conditions because twitter notes were nearly fully concealed by noise in the spectrogram. On the other hand, some researchers have previously suggested that increased tonality of sound elements is adaptive under noisy conditions (Dubois and Martens, 1984; Hanna et al., 2011; Mockford et al., 2011). Following this argument and given that the twitter part of the song is nearly lost completely it may pay off for blackbirds to favor the motif rather than the twitter of song.

We also found that birds living near the airport advanced the onset of dawn chorus and increased the duration of the chorus in comparison with the control population. Our results suggest that these changes in dawn chorus timing are related to the increased noise levels due to the onset of aircraft traffic in the runway closest to the forest at 7 a.m. The reason why we find aircraft traffic as a likely cause of this modifications is because (1) noise increases to significant higher levels after 7 a.m. compared to the control population and (2) blackbirds near the airport do not show this modifications later on the season when the blackbird dawn chorus does not overlap with day-time aircraft traffic. When blackbird dawn chorus occurs entirely before 7 a.m. both populations show similar chorus characteristics. Although the start and duration of dawn chorus may vary along the season in relation to their breeding stage (Thomas, 1999; Bruni et al., 2014) this should not be a confounding factor in this study since recordings were made simultaneously at both sites. In addition, the distance between study sites is small and the habitat is nearly identical within the same strip of forest so we find unlikely that breeding stage could be a bias in these results.

TABLE 3 | Results of blackbird dawn chorus GLMMs.

Final model	Family	Fixed effects	Estimate	SE	*z*-value	Pr(>|z|)	Sig.
chorus onset ∼ study site * traffic overlap + (1|id)	Poisson (link = log)	Intercept	3.6780	0.0398	92.48	< 0.001	***
		Airport	0.0450	0.0546	0.83	0.41	
		Traffic overlap-yes	−0.0746	0.0524	−1.42	0.16	
		Airport : traffic overlap	0.1972	0.0729	2.71	0.0068	**
chorus duration ∼ study site * traffic overlap + (1|id)	Poisson (link = log)	Intercept	3.2593	0.0969	33.27	< 0.001	***
		Airport	0.2202	0.1616	1.352	0.18	
		Traffic overlap-yes	−0.0508	0.1438	0.350	0.73	
		Airport: traffic overlap	0.3904	0.1910	2.018	0.044	*

We present the first evidence that the advance of dawn chorus in this context is not systemic but plastic as the difference with the control population disappears when dawn chorus does not interfere with aircraft traffic noise. Moreover, this advance is possibly not due to an early awakening of the birds by noise since the advance occurs mostly when dawn chorus begins during the interval from 6 to 7 a.m. in the morning when noise levels are similar at both sites. Our model shows that noise levels during this interval are not significantly different between airport and control sites because airport traffic is still restricted by the night schedule. Therefore, both airport and control blackbirds are under similar noise levels but still the onset of dawn chorus at the airport is estimated to start 12.8 min earlier.

Perhaps more relevant than singing earlier is the finding that blackbirds increased the duration of dawn chorus near the airport when dawn chorus overlapped temporally with aircraft traffic. The model estimates that airport blackbirds sing an average of 24.1 min more than control blackbirds, almost twice as long. This novel finding further supports the adaptive perspective of the modifications of dawn chorus under noisy conditions because, in this case, the debate of whether birds are awaken or not by noise is no longer relevant. It seems intuitive that singing for longer increases the communicative efficiency of song as suggested by previous evidence (Díaz et al., 2011; Gil et al., 2014). This increase in the duration of dawn chorus under intermittent noise conditions may provide a compensation for those songs that have been masked during strong bursts of aircraft noise, ensuring that songs are being heard without compromising information coded on other temporal parameters such as song rate or song length (Wasserman and Cigliano, 1991; Galeotti et al., 1997; Linhart et al., 2012). However, it also seems plausible that doubling the normal time allocated for song at dawn may carry higher costs than the advance of dawn chorus. Spending more time singing will likely have negative effects on fitness by increased metabolic costs associated with song or by taking this time away from other vital tasks (e.g., foraging; Thomas, 1999, 2002).

Until now, there have been several attempts to explain the mechanism underlying noise-dependent advance of dawn chorus onset. An experimental study (Arroyo Solís et al., 2013) suggested that the advance of dawn chorus could be due to a short-term response (e.g., birds are waken up by increased noise levels) but two other correlative studies (Gil et al., 2014; Dominoni et al., 2016) implied that there must be another mechanism underlying the advance of chorus because this modification happens before noise levels increase at dawn. Our results support the second hypothesis since the advance of dawn chorus in the airport occurred mostly when noise levels were similar to control at the onset. Perhaps the two mechanisms, a direct awakening by noise or a plastic adaptive response to anthropogenic noise, may be associated within the same process but at different steps. Birds may respond with short-term advances of their singing activity when suddenly noise levels increase at dawn. In such cases, there could be selection for those individuals that sing earlier than usual but, if noise levels follow a strict and periodic daily pattern, birds may develop a long-term response that optimizes their singing activity advancing their chorus in order to avoid noisy hours.

In conclusion, short-term noise-induced modifications of song regarding spectral and temporal characteristics may not follow a linear response but depend on the amplitude and temporal features of anthropogenic noise, as suggested by some authors (Brumm et al., 2009; Díaz et al., 2011; Gil et al., 2014). Anthropogenic noise includes an ample array of sources that may lead to different adaptive responses of song depending on the acoustic features and the temporal regime of noise. This is supported by our results that found airport blackbirds showing different song modifications than those found on other studies for their city counterparts (Nemeth and Brumm, 2009; Ripmeester et al., 2010). In relation to long-term noise-induced changes in singing behavior, our results agree with former evidence that birds advance the onset of chorus in locations where background levels rise at dawn. Finally, we provide evidence that anthropogenic noise may induce birds to increase the time singing at dawn, suggesting higher fitness costs in relation to daily energy expenditure.

AUTHOR CONTRIBUTIONS

DG directed the research and provided most of the conceptual framework behind this study. JS participated in the development of hypotheses and predictions, carried out the field work and data collection, performed acoustic and statistical analyses and wrote the first draft. ES helped out during field work and IP provided technical knowledge on acoustics and noise measurements.

FUNDING

This study has been funded by a research grant from the Spanish Ministerio de Economía y Competitividad (CGL2014-55577R) to DG.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 18 July 2017
doi: 10.3389/fevo.2017.00077

Reproductive Contributions of Cardinals Are Consistent with a Hypothesis of Relaxed Selection in Urban Landscapes

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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *08 January 2017*
Accepted: *29 June 2017*
Published: *18 July 2017*

Citation:
Rodewald AD and Arcese P (2017)
Reproductive Contributions of
Cardinals Are Consistent with a
Hypothesis of Relaxed Selection in
Urban Landscapes.
Front. Ecol. Evol. 5:77.
doi: 10.3389/fevo.2017.00077

Human activities are leading to rapid environmental change globally and may affect the eco-evolutionary dynamics of species inhabiting human-dominated landscapes. Theory suggests that increases in environmental heterogeneity should promote variation in reproductive performance among individuals. At the same time, we know that novel environments, such as our urbanizing study system, may represent more benign or predictable environments due to resource subsidies and ecological changes. We tested the hypothesis that reduced environmental heterogeneity and enhanced resource availability in cities relax selective pressures on birds by testing if urban females vary less than rural females in their demographic contributions to local populations. From 2004 to 2014, we monitored local population densities and annual reproductive output of 470 female Northern Cardinals (*Cardinalis cardinalis*) breeding at 14 forested sites distributed across a rural-to-urban landscape gradient in Ohio, USA. Reproductive contribution was measured as the difference between individual and site-averaged annual reproductive output across all nesting attempts, divided by the annual density at each site. We show that among-individual variation in reproductive contribution to the next year's population declined with increasing urbanization, despite similar variability in body condition across the rural-urban gradient. Thus, female cardinals that bred in urban habitats within our study area were more similar in their contribution to the next generation than rural breeders, where a pattern of winners and losers was more evident. Within-individual variation in annual reproductive contribution also declined with increasing urbanization, indicating that performance of females was also more consistent among years in urban than rural landscapes. These findings are consistent with the hypothesis that urbanized environments offer more homogeneous or predictable conditions that may buffer individuals from environmental heterogeneity and relax natural selection.

Keywords: birds, body condition, rural, urban, natural selection, variance in reproduction

INTRODUCTION

Human activities are causing rapid and novel environmental change around the planet, highlighting a need to understand how non-human species adapt to such changes. Although, evolutionary and ecological processes are often characterized as operating on different time scales, rapid environmental change can lead to strong natural selection and rapid adaptive evolution (Thompson, 1998; Reznick and Ghalambor, 2001; Hendry et al., 2008). Examples of rapid adaptation to pollutants (Berry, 1964; McNeilly and Bradshaw, 1968; Kettlewell, 1973), pesticides (Georghiou, 1972), and harvest (Handford et al., 1977) are well-known, but more recent examples (e.g., Olsen et al., 2004; Phillips and Shine, 2004; Law and Salick, 2005) highlight the key role of eco-evolutionary dynamics on species persistence and distribution (e.g., Davis and Shaw, 2001; Stuart et al., 2014).

Perhaps nowhere is an understanding of the interplay between ecological and evolutionary processes more urgently needed than in human-altered systems, where anthropogenic disturbance can lead to strong selection on fitness traits (Stockwell et al., 2003; Kinnison and Hairston, 2007; Hendry et al., 2011). Urbanizing landscapes therefore offer excellent opportunities to study spatial and temporal heterogeneity in selection across rural to urban gradients (Siepielski et al., 2009; Cornwallis and Uller, 2010; Safran et al., 2010). Urban development alters biological (e.g., types of food), physical (e.g., temperature, light), chemical (e.g., pollutants), and ecological (e.g., densities of competitors or predators) factors known in other systems to strongly influence selection on phenotype (Reznick and Ghalambor, 2001). However, relatively few studies of eco-evolutionary dynamics in urban systems exist (Alberti, 2015). Badyaev et al. (2008) found evidence that urbanization via access to human-provided birdseed led to adaptive divergence in bill shape in urban and desert house finches (*Carpodacus mexicanus*). Atwell et al. (2014) report correlated changes in life history traits in an urbanizing junco population (*Junco hyemalis*). Likewise, Marnocha et al. (2011) provided evidence that morphological changes in brown anoles (*Anolis sagrei*) represented an adaptive response to human-induced habitat alteration via residential development. A recent global meta-analysis of 1,600 phenotypic changes across species, regions, and ecosystems showed that rates of phenotypic change were also greater urban than non-urban systems, consistent with an hypothesis of strong selection (Alberti et al., 2017).

In contrast, urban breeders might also be expected to experience relaxed selection pressures if urban environments are buffered from the deleterious effects of environmental variation experienced by rural breeders. For example, buffering in urban habitats might occur via increased predictability or homogenization of resources and the subsequent decoupling of urban breeders from naturally-occurring cycles of nutrients and water (Shochat et al., 2006; Buyantuyev and Wu, 2009, 2012; Groffman et al., 2014). Although, some work suggests that even abundant urban birds may reproduce less well in cities than rural areas (Meyrier et al., 2017), our prior results show that urban forests provide cardinals with more reliable and predictable food subsidies, including birdfeeders and the fruits of exotic shrubs, than those available to rural breeders (Atchison and Rodewald, 2006; Leston and Rodewald, 2006). Urban areas also offer warmer winter temperatures (Atchison and Rodewald, 2006; Shustack et al., 2009) and preferred nesting substrates (Leston and Rodewald, 2006; Rodewald et al., 2010). Overall, these factors promote high densities of cardinals in urban forests in patterns that are consistent with resource-matching (Rodewald and Shustack, 2008). However, despite nesting earlier than rural cardinals (Shustack and Rodewald, 2011) and experiencing similar rates of brood parasitism and predation on nests (Rodewald et al., 2013), fledglings (Ausprey and Rodewald, 2011), and adults (Rodewald and Shustack, 2008), urban and rural cardinals produced similar numbers of offspring each year (Rodewald and Shustack, 2008; Rodewald et al., 2013). Our prior results are also consistent with the hypothesis that abundant, accessible resources in urban habitats has relaxed selection on male coloration in Northern Cardinals (*Cardinalis cardinalis*) by disassociating color, condition, and reproductive performance (Rodewald et al., 2011). Overall, therefore, we expected to observe evidence of relaxed selection on breeding cardinals in urban vs. rural habitats.

Specifically, we estimated individual variation in the reproductive contributions of female cardinals to local population growth following Ezard et al. (2009) and Coulson et al. (2006). These methods allowed us to test the prediction that variation in female reproductive contributions to the next generation should be more similar in urban than rural habitats due to environmental buffering, consistent with an hypothesis of relaxed selection in urban vs. rural habitats, while accounting for variation in local population density (Coulson et al., 2006; Pelletier et al., 2007, 2009). We also tested two corollaries of the hypothesis that urban areas represent more benign selective environments than rural areas, by testing if urban females were in better condition and had greater reproductive success than rural females.

MATERIALS AND METHODS

Study System

From 2004 to 2014, we studied a common synanthropic bird, the Northern Cardinal (*C. cardinalis*). Cardinals are year-round residents that nest in understory and midstory vegetation and defend territories during the breeding season (Halkin and Linville, 1999). Breeding densities of cardinals are highest in sites with dense understory shrubs (Leston and Rodewald, 2006), particularly Amur honeysuckle (*Lonierca mackii*), an exotic shrub that is preferred as a nesting substrate and can act as an ecological trap (Rodewald et al., 2010). Cardinals are multi-brooded, can quickly re-nest after failure, and often make 3–5 nest attempts annually. Nest predation is overwhelmingly the most common cause of nest failure in our system, and the nest predator community is diverse with 21 species documented to depredate cardinal nests (Rodewald and Kearns, 2011).

We studied cardinals at 14 sites located in mature riparian forests distributed across a rural-to-urban landscape in Ohio, USA (ca. 40N 00′ 83W 00′). Forest patches varied in size (mean width 163 m ± 2 SE) but were comparable in urban and rural landscapes. Building densities in our landscapes ranged from 0.1 to 7.3 buildings per ha (10–727 buildings/km^2), and agriculture was the most common non-urban land use in rural landscapes. To quantify the matrix, we derived an urban index based on a principal components analysis (PCA) of landscape composition (number of buildings, percentages of agriculture, pavement, lawn, roads) within a 1-km radius area centered on each study site. We used the first principal component as an "urban index" because it explained most of the variation among sites and correlated strongly with urban land uses, being positively correlated with buildings, roads, pavement, and lawn, and negatively related to agriculture. Our measurements of landscape composition at our sites in 2001 and 2006 indicated that study sites changed little in the amount of urbanization over the course of our study.

Surveys of Avian Communities

Density of breeding cardinals within a 2-ha grid at each site was determined using spot-mapping (Bibby et al., 2000), noting the location, sex, and behavior of birds on detailed maps. This allowed us to estimate of the density of birds and number of territories in a specified area based on territorial behavior. Each grid was traversed at 50-m intervals 8–10 times from mid-April to June of each year.

Banding and Nest-Searching

To estimate annual reproduction, female cardinals were target-banded and individually-marked with a numbered aluminum metal (United States Geological Survey) and unique combination of colored plastic bands. Birds were captured and measured early in the breeding season, typically during territory establishment or nest building. Morphometric measurements (mass ± 0.5 g, wing ± 0.5 mm, and tarsus length ± 0.1 mm) were collected for each individual and used to calculate an index of body condition for each female at the time of first capture. While no single metric can fully capture body condition of an organism as is relevant to fitness, we used an approach commonly used in ornithological studies. To do so, we first used a PCA to estimate frame-size from wing and tarsus length. PC1 was positively correlated to wing and tarsus length (0.76 for each), explained 59% of variation in size (eigenvalue = 1.16), and used thereafter to indicate frame-size. We then regressed mass on frame-size and used residuals as a body condition index: females heavier or lighter than expected given frame-size were thus assumed to be in higher or lower condition than expected on average. Previous research in our system indicates that male brightness, timing of breeding, and ability to secure a preferred territory were all positively related to our condition index in rural birds (Rodewald et al., 2011), suggesting that it is linked positively to potentially influential reproductive traits. We examined relationships between urbanization and (a) body condition and (b) variance in body condition (i.e., among females breeding at the same site) separately using mixed models using site as a random factor and urban index as a fixed effect (predictor).

Field teams monitored all nesting attempts of known individuals from late March to September. Most nests were located early in the nesting stage, usually during building or egg-laying, and then checked at 1–3 day intervals. For nests that successfully fledged young, numbers of young were determined by either counting the number of nestlings immediately prior to fledging and/or by observing parents and young for extended periods near the time of fledging. Numbers of young that successfully fledged were summed across all nesting attempts for a given female in each year. Number of fledglings was then used as a response variable in a mixed model that included urban index as a fixed effect and site as a random effect.

Calculation of Reproductive Contributions of Individuals to Local Populations

Because natural selection and population dynamics are each driven by the birth and death of individuals, a demographic signature of selection can be measured on ecological time scales by examining the differential contributions of individuals to populations in a next generation (Coulson et al., 2006; Pelletier et al., 2007, 2009). Many studies of evolutionary change and selection estimate the fitness of alleles or phenotypes by measuring their representation in populations in future (Hamilton, 1964; Dawkins, 1982; Metz et al., 1992; Benton and Grant, 2000). Fitness can also be estimated as the relative reproductive performance of individuals within populations (Fisher, 1930; Lande, 1982), which is a function of population size and individual reproductive contributions to future generations (Coulson et al., 2006), and can be estimated annually to avoid complications related to among-individual variation in generation length, and helps control for the potential effects of environmental and ecological variation over an individual's lifetime.

We followed Coulson et al. (2006) to estimate individual contributions to population growth annually rather than by generation. Because our previous work showed that survival in rural and urban sites was similar in adults ($\varphi = 0.57 + 0.04$ SE; Rodewald and Shustack, 2008) and juveniles ($\varphi = 0.44$; 71 days, $n = 45$ birds; Ausprey and Rodewald, 2011), we simplified Coulson's equation by treating survival in each site as constant and calculated an individual's annual contribution to population growth, $p_{t(\mathrm{i})}$, as the number of offspring produced by female i in year t [$f_{t(i)}$] minus the mean f_t for that site and year, divided by the population density (per 10 ha) for that site and year minus one.

Reproductive contributions were calculated for 470 females in 14 study sites. In order to compare across populations of different densities, we summed the squared reproductive contributions across all females breeding within a given site and year [$\Sigma = p_{t(\mathrm{i})}{}^2$]. We then used this sum as a measure of variation in contribution and also as a response variable in a mixed model that included urban index as a fixed effect and site as a random effect. We also calculated variance in the reproductive contributions of individual females across years, and used that as a response variable in a mixed model that included urban index as a fixed effect and site as a random effect. Two *post-hoc* tests were used to

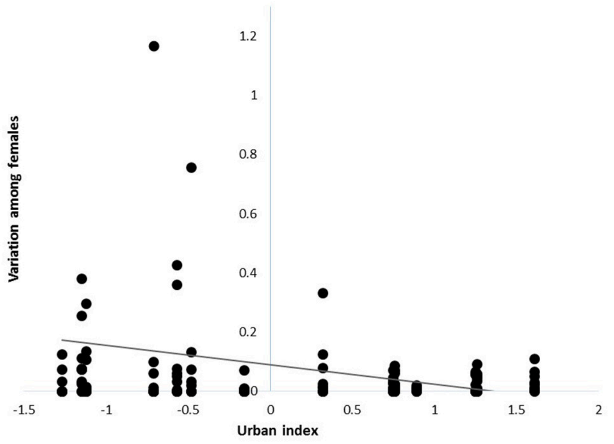

FIGURE 1 | Variance in reproductive contributions of female cardinals breeding across a rural-to-urban landscape gradient in Ohio, USA from 2004 - 2014. Each point reflects the variation among females breeding within a given site and year (i.e., sum of squared reproductive contributions).

TABLE 1 | The urban index and mean (SE) for density, mean number of fledglings per year, and body condition of female cardinals distributed across 14 forested sites in central Ohio, 2004–2014. Body condition is the residual from a regression of mass against a principle component of frame size (i.e., wing and tarsus).

Site	Urban index	Density (2 ha)	Number of fledglings	Body condition
Ngalena	−1.27	1.2 (0.23)	1.3 (0.37)	2.07 (1.83)
Pubhunt	−1.15	1.9 (0.17)	2.0 (0.26)	−0.41 (0.66)
Prairie	−1.12	1.8 (0.23)	2.0 (0.54)	1.31 (0.68)
Creeks	−0.71	1.6 (0.28)	1.8 (0.42)	−1.04 (1.18)
Sgalena	−0.57	2.1 (0.15)	1.1 (0.27)	1.22 (1.02)
Galena	−0.48	1.9 (0.21)	2.3 (0.34)	0.12 (1.40)
Elkrun	−0.16	4.2 (0.31)	2.1 (0.35)	1.83 (1.62)
Woodside	0.32	2.3 (0.36)	2.0 (0.47)	1.31 (1.04)
Rushrun	0.75	4.4 (0.22)	1.8 (0.17)	−1.39 (0.53)
Cherry	0.76	3.3 (0.28)	1.6 (0.33)	−0.37 (1.05)
Kenny	0.89	7.3 (0.39)	1.8 (0.35)	0.31 (0.64)
Casto	1.25	3.7 (0.27)	1.9 (0.22)	−1.44 (0.91)
Lou	1.26	4.1 (0.33)	1.5 (0.42)	0.94 (1.10)
Tuttle	1.61	4.1 (0.41)	2.1 (0.35)	−0.63 (0.62)

examine the relationships between numbers of young produced (response variable) and either body condition of females or population density, using mixed models with site as a random effect variable. Sample sizes differ among the tests (see Section Results) because we did not have morphometric measurements for every banded female in every year for which we had annual reproduction, nor reproductive data for every banded female.

RESULTS

Variation in individual contributions to local populations declined with increasing urbanization [$\beta = -0.08 \pm 0.03$ SE, $F_{(1,\,90)} = 5.41$, $P = 0.02$; $n = 113$; **Figure 1**], indicating that the reproductive contributions of females to local populations were more homogenous in urban than rural sites. However, despite marked variation in the reproductive contributions of individuals, the mean number of fledglings produced by females at a site in a given year was similar across the rural-urban landscape gradient [$F_{(1,\,99)} = 0.06$; $P = 0.81$, $n = 113$; **Table 1**].

Body condition declined as urbanization increased [$\beta = -0.49 \pm 0.22$ SE; $F_{(1,\,317)} = 5.14$, $P = 0.02$; $n = 341$], but variation in body condition among females at the same site was similar across the rural to urban gradient [$F_{(1,\,64)} = 0.03$, $P = 0.86$; $n = 78$]. Thus, despite heterogeneity in the reproductive contributions of females across the rural-urban gradient, similar patterns did not emerge with respect to body condition. In contrast, urban females were more similar in their reproductive contributions among years than were females in rural sites [$\beta = -0.03 \pm 0.01$ SE, $F_{(1,\,108)} = 7.91$, $P = 0.01$; $n = 122$; **Figure 2**]. Our two *post-hoc* analyses showed that (1) body condition was negatively related to number of fledglings produced in a given year [$\beta = -0.08 + 0.03$; $F_{(1,\,182)} = 5.54$; $P = 0.02$, $n = 204$] and (2) density at a site was not significantly related to fledgling production [$F_{(1,\,446)} = 0.70$; $P = 0.40$, $n = 470$].

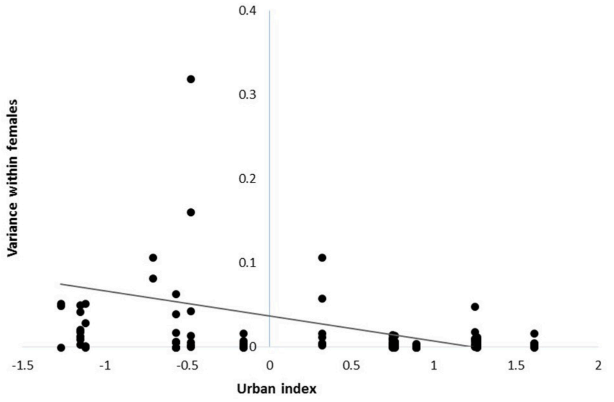

FIGURE 2 | Within-individual variance in reproductive contributions for female cardinals across years at sites distributed along a rural-to-urban landscape gradient in Ohio, USA from 2004 - 2014. Each point represents the variance within an individual across years.

DISCUSSION

We found that the annual reproductive contributions of female cardinals breeding in cities were less variable—both within and among individuals—than observed among rural females. The comparatively lower variation in contributions among females at urban than rural sites was observed despite similar patterns of among-individual variation in body condition. This finding implies that the higher variation in reproductive contribution in rural as compared to urban females was not a consequence of higher variation in female condition.

Theory suggests that environmental heterogeneity, either spatially or temporally, should promote variation in reproductive performance among individuals and the intensity of natural selection on phenotype (Wade and Kalisz, 1990; Byers, 2005; Siepielski et al., 2009, 2011, 2013; MacColl, 2011). Conversely, in more homogeneous or benign environments, we might expect reduced variation in the reproductive contributions of individuals and intensity of natural selection. Although, we did not measure selection directly, our results are consistent with the hypothesis that urban environments are more spatially and temporally homogeneous and/or resource-rich than rural environments and thereby buffer individuals from environmental variation and resource shortage in ways that relax natural selection. Indeed, our prior results in this system show that urban forests contained 2.5 times more fruit, 2.7 times more birdfeeders, and 2.5 times more preferred nesting substrate for cardinals than rural forests (Leston and Rodewald, 2006). High resource availability in urban as compared to rural forests is also consistent with our earlier observation that cardinal territories in urban forests were about one-third the size of those in rural forests (Rodewald and Shustack, 2008), as expected if territory size and resource abundance were negatively related (Hixon, 1980; Norton et al., 1982; Smith and Shugart, 1987). Access to urban-associated resources was also suggested to have reduced selection on male plumage color in urban vs. rural cardinals (Rodewald et al., 2011).

In contrast, we failed to support two assumptions about how resource-rich environments might affect urban populations. First, although high resource abundance or predictability in urban areas might be expected to enhance female condition, we found that female body condition declined as urbanization increased and that body condition was negatively related to number of fledglings produced. Such a pattern might arise if females in resource-rich environments invest more heavily in reproduction at the expense of future survival or reproduction in order to take advantage of temporal peaks in resource abundance (Williams, 1966; Wilson et al., 2007; Tarwater and Arcese, 2017). Indeed, many birds adjust body condition to balance the risks of food deprivation and depredation (Rogers, 1987) and when trading-off future reproduction or survival to invest in current reproduction (Arcese and Smith, 1988; Tarwater and Arcese, 2017). Whether cardinals engage in such trade-offs remains uncertain, but previous work in our system shows that urban cardinals bred earlier than rural birds, and although individuals that bred early attempted a greater number of nests, there was no measurable increase in numbers of fledglings due to the high rates of predation early in the breeding season (Rodewald et al., 2010; Shustack and Rodewald, 2011). Previous work in our system also provided evidence that cardinals distribute themselves in an ideal-free, or "resource-matching" manner, whereby resource-rich urban sites attain higher densities and support smaller territories, but perform similarly to individuals at lower density site in terms of condition, survival and reproduction (Rodewald and Shustack, 2008). If "faster" life histories are favored in predictable and/or resource-rich sites, which we have shown previously occur mainly in our urban study sites, the high variance in annual reproductive contribution observed among females in rural sites may simply reflect a wider range of reproductive tactics employed by females faced with higher spatial and temporal variation in resources (Williams, 1966; Wilson et al., 2007; Tarwater and Arcese, 2017). However, there is also the possibility that there is an advantage to being lean in urban environments. For example, in resource-rich environments, the relative benefit of fat reserves may be small compared to the potential costs of doing so via reduced agility or ability to evade predators (Rogers, 1987, 2015; Rogers and Smith, 1993).

In contrast to the assumption that resource-rich urban environments might enhance reproductive success, we found that urban and rural females had similar reproductive success. This result may be due to the fact that cardinal density increased in resource-rich urban areas, but territory size declined (Rodewald and Shustack, 2008), suggesting an ideal-free distribution of cardinal territories and reproductive success overall (Fretwell and Lucas, 1969). Similarly, the resource matching hypothesis predicts that individual fitness will not differ in rural and urban sites and is consistent with our finding no effect of site density on the number of young fledged annually. An ideal-free distribution of territories and annual reproductive success via resource matching is also consistent with our earlier results showing no difference in survival, condition, or reproductive output between urban and rural environments (Rodewald and Shustack, 2008). Similar patterns of resource matching have been demonstrated in many taxa, including birds (Harper, 1982; Recer et al., 1987; Diaz et al., 1998; Telleria and Perez-Tris, 2003), mammals (Morris, 1994), and fish (Milinski, 1984, 1988; Abrahams, 1989; Gotceitas and Colgan, 1991).

By driving change in global climate and land cover, humans create novel ecological conditions that are likely to drive evolutionary change in species capable of taking advantage of these "ecological opportunities" (Schluter, 2000; Badyaev et al., 2008; Atwell et al., 2014; Norman and Christidis, 2016). In particular, to the degree that urbanization increases environmental heterogeneity, we might expect populations occupying urban areas to experience increased variation in reproductive performance, their contributions to future generations, and the intensity of natural selection on individual phenotype. In contrast, our results are consistent with the hypothesis that urban areas represent more benign or predictable environments than rural areas due to resource subsidies and habitat homogenization, leading to a reduction in individual variance in the annual reproductive contributions among urban as compared to rural females. These and other results from urbanizing bird populations (e.g., Badyaev et al., 2008; Atwell et al., 2014; Alberti et al., 2017) suggest that comparative studies of life history and morphological evolution in populations distributed across more and less human-dominated landscapes offer outstanding opportunities to test for temporal and spatial variation in the intensity of natural selection and evolution of novel phenotypes.

AUTHOR CONTRIBUTIONS

Both authors contributed to developing research questions, analyzing the data, interpreting the results, and writing the paper. AR oversaw the field research as part of her long term study system.

FUNDING

This work was supported by funding from the National Science Foundation (DEB-0340879 and DEB- 0639429), US Fish and Wildlife Service, Ohio Division of Wildlife, Ohio State University, and the Ohio Agricultural Research and Development Center.

ACKNOWLEDGMENTS

Our deep gratitude goes to the many graduate students, especially D. Shustack, L. Kearns, I. Ausprey, M. Bakermans, K. Borgmann, L. Leston, J. Malpass, D. Narango, B. Padilla, S. Rose, L. Rowse, J. Smith-Castro, and others who have spent countless hours collecting field data. We thank Franklin County Metro Parks, Columbus Recreation, and Parks, Ohio Division of Wildlife, The Nature Conservancy, City of Bexley, Gahanna Parks, and Recreation and private landowners for access to study sites. We appreciate the thoughtful comments from reviewers of this manuscript, particularly T. Coulson. All research was conducted in accordance with approved protocol by Ohio State University's Institutional Animal Use and Care Committee (2010A0003, 2007A0015, 2004A0047, 00A0167) and banding was conducted under US Federal Bird Banding Permit 22272.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 29 May 2017
doi: 10.3389/fevo.2017.00053

Urbanization Is Associated with Divergence in Pace-of-Life in Great Tits

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As an extension of the classic life history theory, the recently highlighted pace-of-life syndrome hypothesis predicts the coevolution of behavioral, physiological and life-history traits. For instance, bolder and shyer individuals do not only differ in personality profiles, but also in neuro-endocrinology and breeding patterns. While theory predicts that bolder (i.e., proactive), more aggressive individuals should colonize more rapidly urbanized habitats than shyer (i.e., reactive), less aggressive individuals, it is also predicted that across generations, adaptive selection processes could favor shyer individuals that are more sensitive to novel environmental cues. Here we compared two personality traits (handling aggression, exploration score in a novel environment), one physiological trait related to stress response (breath rate) and four breeding traits (lay date, clutch size, hatching success and fledging success) in a rural and an urban study population of Mediterranean great tits *Parus major*. Mixed models revealed strong phenotypic divergence between forest and city in most traits explored, in particular in personality, whereby urban great tits were more reactive to stress and faster explorers compared to rural birds (yet not more aggressive). Urban birds also laid smaller broods earlier in spring compared to their rural conspecifics, and city broods resulted in lower hatching success yet interestingly fledging success was similar. Nest-box centered measures of anthropogenic (artificial light, pedestrians, and cars) perturbation and resource abundance allowed us to go beyond the classical forest/city comparison by exploring the phenotypic variation across an urbanization gradient. This revealed that high urbanization in nest-box surroundings was associated overall with earlier breeding and smaller clutches, but also with faster breath rate, although these trends showed strong annual variation. Ongoing rapid urbanization and non-random gene flow between rural and urban great tits could both contribute to the high prevalence of bold breeders in the city. Our study suggests the existence of urban and rural great tit ecotypes with different pace-of-life, but also a finer-scale divergence along the degree of urbanization within the city. Future studies are required to determine whether this phenotypic variation at different spatiotemporal scales is adaptive and whether it has a genetic basis or results from phenotypic plasticity.

Keywords: urban pace-of-life, great tit, personality traits, breath rate, breeding performance, urbanization gradient

Edited by:
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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *01 March 2017*
Accepted: *10 May 2017*
Published: *29 May 2017*

Citation:
Charmantier A, Demeyrier V, Lambrechts M, Perret S and Grégoire A (2017) Urbanization Is Associated with Divergence in Pace-of-Life in Great Tits.
Front. Ecol. Evol. 5:53.
doi: 10.3389/fevo.2017.00053

INTRODUCTION

Understanding the mechanisms by which organisms adapt to spatiotemporal environmental heterogeneity remains a fundamental goal in evolutionary ecology. It is tightly linked to another major goal in the field, namely understanding the maintenance of the wide phenotypic (and genetic) variation we observe in nature. As an extension of the classic life history theory (Roff, 1992, 2002; Stearns, 1992), the recently highlighted pace-of-life syndrome (POLS) hypothesis (Ricklefs and Wikelski, 2002; Martin et al., 2006; Réale et al., 2010), predicts the coevolution of life histories with a suite of ecologically relevant co-varying traits in metabolism, immunology, and behavior. The POLS hypothesis provides testable predictions concerning the association between the slow-fast life history continuum and behavioral, physiological, and immunological traits. In short, the pace-of-life continuum differentiates between, on the one hand *slow* individuals with slow life-histories (e.g., high survival, delayed reproduction), reactive behaviors (e.g., shyness, low aggressiveness, and low activity) and low metabolism, and on the other hand *fast* individuals with fast life-histories, proactive behaviors and high metabolism (Réale et al., 2010). Although the POLS concept has attracted paramount attention in behavior ecology and eco-physiology, empirical support for the POLS hypothesis is currently mixed (Hille and Cooper, 2015), perhaps because POLS theory still lacks a conceptual framework to predict which ecological conditions will favor which syndromes. Most notably, many studies in the last decade have tested adaptive explanations for the evolution of repeatable individual differences in behavior (aka *personality*) (Wolf et al., 2007) and a number of theoretical studies have attempted to predict the conditions favoring the evolution of personalities (Dingemanse and Wolf, 2010).

The urban matrix and its progression across rural landscapes represent for many organisms a drastic and very rapid change in environmental conditions compared to the natural habitats where they have evolved (Sih et al., 2011). These urban conditions such as novel social interactions, pollution, or altered plant-animal interactions, induce novel selection pressures requiring adaptive changes from native organisms (Atwell et al., 2012). Moreover, the urban matrix is often highly heterogeneous, offering little connectivity between favorable habitats (Lindenmayer and Fischer, 2006). This radical environmental shift causing the fragmentation, isolation and degradation of natural habitats represents an acute global biodiversity conservation issue (e.g., McKinney, 2002; Aronson et al., 2014). However, urban development can also be viewed as a unique opportunity to investigate eco-evolutionary processes in a rapidly changing environment (Alberti, 2015; McDonnell and Hahs, 2015). Indeed, cities have recently been highlighted as hotspots of contemporary evolution, where rates of phenotypic changes are greater in urbanized areas compared to natural but also to non-urban anthropogenic habitats (Hendry et al., 2008; Alberti et al., 2017). In the context of the evolution of pace-of-life syndromes as outlined above, urban landscapes can be viewed as an ideal setting to test whether animals display differential phenotypes for physiological, life history and behavioral traits across the urban/natural landscape, but also within the heterogeneous urban matrix.

It is widely recognized that urbanization can act as an ecological filter favoring certain species that are more urban tolerant (Williams et al., 2009; Silva et al., 2016), thus leading to differences in species compositions across urbanization gradients (e.g., for avian communities, Sol et al., 2014; Clucas and Marzluff, 2015). For instance, in such inter-specific context, the "cognitive buffer hypothesis" predicts that larger brained animals have higher adaptive abilities in novel and challenging environmental conditions such as urban habitats (Sol et al., 2005). Since individuals within a species can show strong persistent variation in a large set of traits related to adaptation, a similar process of filtering can be predicted to occur between conspecifics, whereby individuals with particular phenotypes will be favored in the urban landscape. Although there are presently no formal theoretical predictions on POLS in urban environments, the general expectation is that urban habitats will be more favorable to fast living individuals (Evans et al., 2010; Atwell et al., 2012) which are often higher dispersers hence more likely colonizers (Cote et al., 2010). The ornithological literature already offers many examples of adjustments in urban birds compared to rural conspecifics that are concordant with this expectation (see recent review in Marzluff, 2017). Urban birds are usually more aggressive (e.g., Minias, 2015; Davies and Sewall, 2016), bolder (e.g., Ducatez et al., 2017), can be approached more closely by humans (e.g., Lin et al., 2012; Møller et al., 2015), are less neophobic (e.g., Tryjanowski et al., 2016), have higher levels of disturbance tolerance (Lowry et al., 2013) and reduced levels of physiological stress response (e.g., Partecke et al., 2006; Minias, 2015). Other empirical comparisons between urban and rural birds have also suggested shifts in breeding strategies: urban birds usually have earlier laying dates, smaller clutch sizes, lower reproductive success (e.g., Peach et al., 2008; review in Chamberlain et al., 2009), and higher adult survival rates (e.g., Rebolo-Ifran et al., 2015). Hence overall, the empirical literature so far seems to suggest that urban birds tend to have slow life histories yet fast behavioral syndromes (Figure 1 in Réale et al., 2010). This discrepancy could result from the lack of integrative study exploring all types of traits simultaneously in the same urban context, but it could also be indicative of the emergence of novel covariances between life history and behavioral traits. In order to test whether urban birds occupy a particular position on the POLS continuum, we now need multivariate studies that investigate breeding, physiological and behavioral traits in concert. Also, the heterogeneity of the urban matrix providing a variety of environments from forested parks to industrial areas could be exploited in order to go beyond the urban/rural dichotomy and test whether the fast-slow continuum matches the urbanization gradient.

Using longitudinal data across 6 years from a great tit *Parus major* population monitoring in the city of Montpellier (France) and in a nearby rural oak forest (La Rouvière), we address in this study the following main questions:

(i) Do great tits show divergent urban versus forest ecotypes for personality, physiological, and breeding traits?

(ii) Do these traits display gradual phenotypic variation across an urbanization gradient?
(iii) Is the phenotypic variation observed in these traits concordant with the pace-of-life syndrome hypothesis, whereby individuals in more constraining and novel environments will have faster life histories, will be bolder and more susceptible to acute stress?

The comparative approaches between urban and forest birds and across the urban gradient include two personality traits (handling aggression, exploration score in a novel environment), one physiological trait related to stress response (breath rate under constraint) and four breeding traits (laying date, clutch size, hatching success, and fledging success).

MATERIALS AND METHODS

Study Area and Monitoring

Sites and Monitoring

Breeding great tits *Parus major* were monitored and captured in nest-boxes in two main study areas in the south of France: a rural oak forest and an urban environment (Figure S1). First, captures and measures were done in the La Rouvière forest dominated by downy oaks (*Quercus humilis*), located 20 km North-West of the city of Montpellier. Great tits (and blue tits) have been monitored in this forest since 1991 with weekly visits to all nest-boxes and all individuals marked with unique leg rings (see details on monitoring in following paragraph and in Doutrelant et al., 2000; Blondel et al., 2006). Second, captures were also done in nest-boxes within the city of Montpellier (area 56.88 km^2, human population *ca* 272,000 in 2013, source: Wikipedia), where a similar breeding monitoring is performed in areas containing both parks and streets (Demeyrier et al., 2016). The monitoring in the city of Montpellier started in 2011, providing information on brood-specific life history (exact lay date, clutch size, hatching, and fledgling success) yet capture of breeding parents started in 2013 and behavioral assays (see below) in 2014. In this study we used datasets of varying sizes depending on the variables studied, with data collected between 2011 and 2016. Number of nest-boxes fluctuated over the years because of theft and changes in entrance-hole size. The number of nest-boxes with large entrance holes (32 mm) allowing great tits to enter and breed, fluctuated between 51 (in 2011) and 92 (in 2016) in La Rouvière forest, and between 163 (in 2012) and 183 (in 2013) in the city of Montpellier.

Breeding Data Collection

Monitoring of broods started before nest construction and ended after the last nestling had fledged. Weekly nest-box field visits to each brood resulted in the observation of the exact date when the first egg was laid (lay date, March 1st = 1), the number of eggs laid (clutch size), the number of hatchlings, and the number of fledglings (nests were visited at 15 days, and then 21 days after hatching to check fledging). In order to compare with previous studies (e.g., Vaugoyeau et al., 2016), only data collected on first broods were considered in the analyses. Parents were captured and measured when their nestlings were 9–15 days old and uniquely identified with metal rings provided by the CRBPO (Centre de Recherches sur la Biologie des Populations d'Oiseaux, Museum National d'Histoire Naturelle, Paris). Nestlings were also uniquely ringed and measured at 14–15 days old.

Urbanization Measures

As previously described in Demeyrier et al. (2016), four features of the urban matrix were measured within a 50 m radius around each nest-box: vegetation cover, car traffic, pedestrian count, and intensity of artificial light. Demeyrier et al. (2016) provide all the details on how these measures were performed, but also how they were analyzed in a principal component analysis (PCA, see Figure 4 in Demeyrier et al., 2016). From this PCA, we extracted the two major axes: PC1 reflecting habitat naturalness and hereafter abbreviated "naturalness" (positively related to vegetation cover and negatively correlated with car traffic and artificial lights, explaining 56.6% of the variance), and PC2 reflecting the pedestrian frequency, hereafter abbreviated "pedestrian" (explaining 16.7% of the variance in the urban environmental features).

Behavioral Assays

The behavioral trials were performed in both La Rouvière forest and Montpellier in 2014, 2015, and 2016. Great tit parents were caught in their breeding nest-boxes while feeding their 9–15 days old nestlings. A captured bird was kept for around 20 min in total, during which time it was submitted to the following sequence of events: capture in the nest-box, handling aggression scoring, resting phase in cloth bag for 5 min, measure of breath rate during handling, resting phase of 2 min in small acclimatization compartment adjacent but separated from the main open-field cage (**Figure 1**), exploratory scoring in open-field trial during 4 min, ringing (if not already ringed), morphological measurements (body mass, tarsus, tail and wing lengths), and blood sampling.

First, immediately after its capture, a bird was tested for handling aggression by the capturer. The handler held the bird high up in the legs with one hand and nagged the bird with one finger from his/her other hand following a standardized procedure where the finger approaches the bird but does not touch it. After *ca.* 15 s of test and observation, the handler attributed a handling aggression score (HA, following the standardized criteria list in Table S4 of Dubuc-Messier et al., 2016, see also Brommer and Kluen, 2012; Class et al., 2014), ranging from 0 (completely unreactive bird) to 3 (bird aiming for the handler's finger all the time, spreading its wings and tail), with 0.5 increments. Following this first behavioral assay, the bird was placed in a bag for 5 min and brought to the open-field apparatus which was less than 5 min walking distance from the nest-box. Second, right after it was removed from the cloth bag, the bird was held still on its back by the handler who counted twice in a row the number of breaths during 30 s (breaths were visually expressed via fast movements of the chest). Handling breath rate (HBR) was the number of breaths per minute, averaged over the two measures (Carere and van Oers, 2004; Fucikova et al., 2009; Brommer and Kluen, 2012; Kluen et al., 2014). HBR has been shown in other tit populations to be

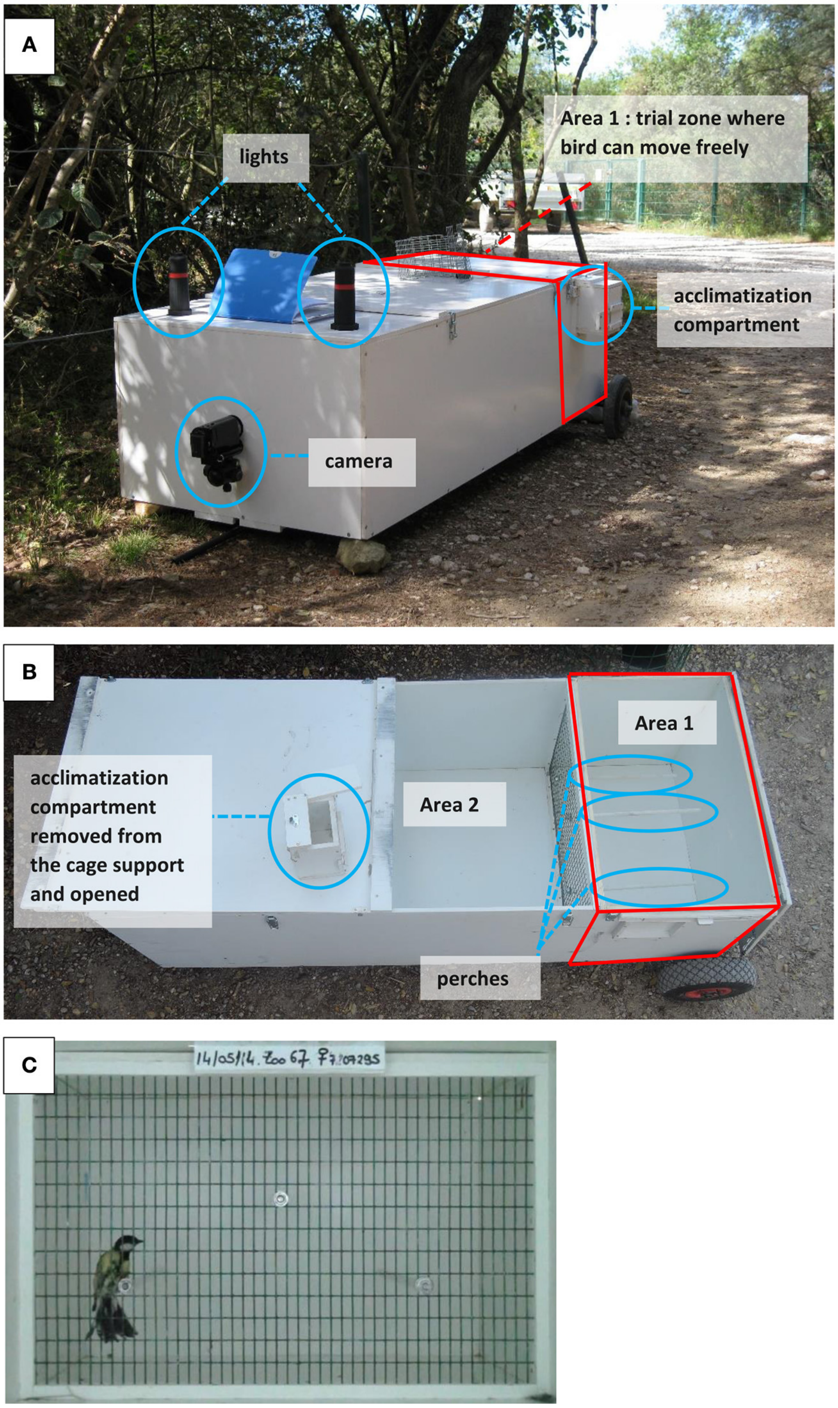

FIGURE 1 | Pictures of the experimental open-field cage for the novel environment test. **(A)** View of the operational cage at the zoo of Lunaret (Montpellier). **(B)** View from above to see the division between area 1 of the cage where the bird is released during the trial and area 2 (the rest of the cage) which serves as an empty compartment to control for distance with the camera and light conditions. **(C)** Picture of the movie view during a trial.

positively related to stress response (Carere and van Oers, 2004). Third, a "novel environment test" was performed in a unique experimental open-field cage built following the exact set up and dimensions described in Stuber et al. (2013) (**Figure 1**). This kind of set-up has been classically used to measure exploration behavior in great tits (e.g., Stuber et al., 2013) and blue tits (e.g., Mutzel et al., 2013; Dubuc-Messier et al., 2016), and provides exploratory scores that are repeatable and well correlated to those performed in the laboratory (e.g., in Dutch and German great tits, Dingemanse et al., 2002; Stuber et al., 2013). Right after the HBR measure, the bird was placed in a double door small compartment on the side of the open-field cage for acclimatization during 2 min with no visual cue other than the small cage of dimensions 11 × 12 × 11 cm. Following this resting time, the bird was released through a sliding door into the exploration cage of 61 × 39 × 40 cm dimensions, where it was filmed during 4 min by a camcorder (CH4 Sony Handycam) fixed 110 cm away from the wired side of the open-field cage. The bird was free to move around the cage and/or to settle on three perches. The light was standardized since the open-field cage was placed in a wider cage of dimension 61 × 150 × 40 cm, lighted by two led lights. Following previous studies on great tits, the total number of flights and hops during the open-field trial was used as a proxy of exploratory behavior (e.g. Dingemanse et al., 2002). This number was square root transformed (to minimize deviation from normality and following Nicolaus et al., 2016) and hereafter called exploration score. VD analyzed all videos using the software JWatcher (Blumstein et al., 2006) and scored this behavior.

TABLE 1 | Phenotypic correlations (above the diagonal) among **(A,B)** breeding traits (in forest and city) and **(C,D)** behavior and physiological traits (in forest and city), and their associated *P*-values (below the diagonal).

(A)

Forest	Lay date	Clutch size	Nb hatchlings	Nb fledglings
Lay date		−0.105	**−0.19**	**−0.24**
Clutch size	0.21		**0.58**	0.13
Nb hatchlings	0.02	4.E-14		**0.44**
Nb fledglings	0.003	0.13	1.E-08	

(B)

City	Lay date	Clutch size	Nb hatchlings	Nb fledglings
Lay date		**−0.182**	0.017	**0.12**
Clutch size	6.E-05		**0.441**	**0.29**
Nb hatchlings	0.75	<2e-16		**0.62**
Nb fledglings	0.03	3E-08	<2e-16	

(C)

Forest	HA	Exploration	HBR
HA		0.12	−3.E-04
Exploration	0.22		−0.05
HBR	1.00	0.62	

(D)

City	HA	Exploration	HBR
HA		−0.05	0.07
Exploration	0.38		−0.05
HBR	0.24	0.47	

Lay date is calendar date of first egg laid; clutch size is the number of eggs laid; nb hatchlings is the number of nestlings hatched; nb fledglings is the number of nestlings that survived until fledging; HA is handling aggression; exploration is the exploration score from open-field trials and HBR is handling breath rate. Pearson's correlation coefficients are provided above the diagonal and associated P-values below the diagonal. In bold are highlighted significant coefficients ($P < 0.05$). For values of the t statistics and degrees of freedom, see Table S1.

Data Analysis

All statistical analyses were run with the software R (version 3.3.2, R Core Team, 2016). We used several datasets to restrict the limitations of missing data in the various variables. In a first step of the analyses, we compared breeding, personality and physiological traits between the forest and the urban habitats using two-sided *t*-tests on the entire dataset available (considering the data from both known and unknown parents), hence not restricting to observations where the identity of one parent was known. For this and all other analyses, we removed estimations of number of hatchlings and number of fledglings for all broods that had been (experimentally) manipulated after the egg-laying stage, which explains smaller sample sizes for these two variables compared to lay date and clutch size.

The first step in the statistical analysis compared population means using a dataset where many female identities were unknown (since parents were not captured during the first 2 years of monitoring) providing an idea of results that can be obtained without comprehensive monitoring and individual ringing.

In a second step, we more formally tested for differences between forest and urban birds with univariate linear mixed models (LMM, package lme4, Bates et al., 2015) which included habitat type (forest *versus* city) as a fixed effect and female identity as a random effect. All LMMs initially included as fixed effects habitat (forest *versus* urban), year, a habitat × year interaction and other trait-specific fixed effects (see below). The fixed effect model was selected in a backward stepwise procedure starting with a model including all variables. The significance of each fixed effect was tested using likelihood-ratio tests (Bates et al., 2015). While all behavioral and physiological traits showed no significant correlations (**Tables 1C,D**), the three reproductive performance traits were highly correlated (**Tables 1A,B** and Table S1). Hence clutch size was included as a fixed effect in brood size models (hereafter called hatching success), and brood size was included as a fixed effect in number of fledglings models (hereafter called fledgling success). For handling aggression and breath rate, the initial full model included fixed effects for sex (and sex × habitat interaction), age, individual life-long capture rank (from 1 to 3, for first to third capture of the same individual) and ambient temperature, and observer/manipulator identity as a random effect. For exploration score, we included sex (and sex × habitat interaction), age, test rank (from 1 to 3), and temperature as fixed effects. As our factor of interest, habitat was kept in all models, even if not significant, to provide the effect size and statistics associated with its fit in the model. Since these LMMs included a female random effect, the dataset used in this second step included breeding parameters that had been recorded for broods where the female was captured only, hence the number of observations was reduced compared to the first dataset.

Based on the best models retained in this step 2, we estimated an adjusted repeatability with the package rptR (Nakagawa and Schielzeth, 2010) for all focal traits. We estimated overall repeatabilities across the whole dataset but also sex-specific and habitat-specific repeatabilities.

In a third and final step, we used only data collected in the city of Montpellier to contrast the measured traits against the two urbanization gradients revealed by the PCA of environmental features in the city: habitat naturalness, abbreviated "naturalness" (PC1) and pedestrian frequency, abbreviated "pedestrian" (PC2). Model selection was similar to that in step 2 (with similar fixed and random effects structure), apart from the use of naturalness and pedestrian instead of the habitat variable which previously was used to compare forest and city. As previously, naturalness was kept in all models since it was our main variable of interest.

Sample sizes are indicated in **Table 2** (for step 1), **Table 3** (for step 2), and **Table 4** (for step 3). While models for phenology and reproductive success were female-specific, models on behavior and physiology pooled data on both sexes, with balanced sampling across sexes since 47–48% of observations were on males (detailed sex-specific sample sizes are in Table S3).

RESULTS

Comparison of mean traits between forest and city using the largest dataset (2011–2016) without controlling for the identity of individuals showed strong differences in mean lay dates, clutch sizes, number of hatchlings, and exploration score, but no significant differences in number of fledglings, handling aggression, and breath rate during constraint (**Table 2**, Figure S2).

An interesting result from this investigation on all monitoring data collected (after removing experimental broods) was a lower percentage of complete brood failure (i.e., no fledgling) in the city: 31% of complete brood failure in the forest *versus* 21% in the city ($t = -2.167$, $df = 253.82$, $P = 0.03$). Unfortunately, this data could not be analyzed using Generalized Linear Mixed Models with individual identity as random effect since most failed broods did not allow parent capture and identification. In the latter dataset used in the LMM for breeding traits, brood failure was 32% in the forest vs. 29% in the city. Unfortunately, the large majority of these failures were of unknown cause, but we can hypothesize predation on a parent, catastrophic climatic event or abandonment by parents because of high levels of stress and parental care. Future research should investigate whether some of these drivers of brood failure differ between the forest and city environments.

Hence at first glance when comparing mean traits without controlling for fixed effects that may be important or the non-independence of data collected on the same individuals, city birds laid earlier, and smaller first clutches, with decreased hatching success yet decreased occurrence of complete brood failure, and they did not fledge a lower number of offspring (see statistical details in **Table 1**). They also presented significantly higher exploration activity as well as slightly higher handling aggression and breath rate, although not significantly so.

When accounting in step 2 for the non-independence of data collected on the same individuals, restricting to records from known individuals (2013–2016, sample sizes in **Table 3**), and controlling for fixed effects, LMMs confirmed strong differences between forest and city great tits in their lay date, clutch size, hatching success (number of hatchlings when controlling for clutch size) (**Table 3** and **Figure 2**), and interestingly still no significant difference between forest and city birds in their fledging success (number of fledglings controlling for number of hatchlings, **Table 3** and **Figure 2**). Note that this non-significant result remained when modeling number of fledglings without controlling for the strong positive correlation with number of hatchlings, that is when comparing absolute fledgling number (Table S2). As in step 1, step 2 confirmed that city birds laid earlier and smaller first clutches, with decreased hatching success (**Table 3**). Urban great tits also displayed faster exploration scores and faster breath rates, but did not differ from their forest conspecifics in handling aggression (see statistical details in **Table 3** and **Figure 2**).

Both estimations of reproductive success were not repeatable among females (adjusted repeatabilities of 0.31 ($SE = 0.14$) for hatching success and 0.16 ($SE = 0.14$) for fledgling success, $P = 1$, Nakagawa and Schielzeth, 2010). All other traits were significantly repeatable with adjusted repeatabilities of 0.43 (SE $= 0.10$, $P = 2.3\text{e-}5$) for lay date, 0.55 ($SE = 0.08$, $P = 4.8\text{e-}3$) for clutch size, 0.34 ($SE = 0.09$, $P = 2.6\text{e-}4$) for handling aggression, 0.55 ($SE = 0.07$, $P = 5.2\text{e-}7$) for exploration score and 0.35 ($SE = 0.08$, $P = 8.6\text{e-}6$) for breath rate during constraint. While sex- and habitat-specific repeatabilities were based on small sample sizes, they suggested that behavioral and physiological traits might be more repeatable in females than in males and more repeatable in forest than urban habitats (see Table S3). In contrast, laying date

TABLE 2 | Comparison of mean traits between forest and city great tits based on the full monitoring dataset (hence including broods with unknown parents).

	Forest			City			*T*-test	
	Mean	***SD***	***n***	***Mean***	***SD***	***n***	***t***	***P***
Lay date	**42.45**	**6.8**	**150**	**38.42**	**8.11**	**480**	**-6.03**	**5e-9**
Clutch size	**9.19**	**1.84**	**144**	**7.59**	**1.53**	**487**	**-1.93**	**2e-16**
Number of hatchlings	**7.55**	**3.43**	**150**	**5.89**	**2.74**	**351**	**-5.26**	**3e-7**
Number of fledglings	4.52	3.64	150	4.38	2.88	349	-0.41	0.68
Handling aggression	1.75	1.01	106	1.81	1.04	310	0.56	0.57
Exploration score	**3.49**	**3.83**	**114**	**6.45**	**4.23**	**320**	**6.89**	**6e-11**
Breath rate	127.29	18.42	114	130.13	16.86	318	1.44	0.15

Boxplots for these comparisons are shown in Figure S2. In bold, traits showing significant differences between forest and city ($P < 0.05$).

TABLE 3 | Contrasting phenology, reproductive success, behavior, and physiology between forest and city birds (Habitat) using linear mixed models with female/individual identity and manipulator identity (for handling aggression and breath rate) as random effects.

	Lay date				Clutch size				Hatching success				Fledging success			
Fixed Effects	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***
Intercept	46.95	1.15	40.88	<2e-16	10.07	0.66	15.25	<2e-16	0.51	0.640	0.80	0.42	2.56	0.91	2.82	5e-03
Habitat-urban	**−3.97**	**0.99**	**−4.01**	**9e-05**	**−1.49**	**0.23**	**−6.53**	**4e-10**	**−0.68**	**0.24**	**−2.89**	**4e-3**	0.24	0.44	0.54	0.59
Year-2014	−10.50	1.09	−9.64	<2e-16	−0.17	0.27	−0.62	0.53	−0.33	0.27	−1.22	0.22	−0.11	0.53	−0.21	0.83
Year-2015	−2.90	1.14	−2.54	0.02	−0.63	0.24	−2.59	0.01	0.56	0.29	1.97	0.05	0.28	0.55	0.50	0.62
Year-2016	−5.78	1.13	−5.11	6e-07	−0.34	0.25	−1.33	0.18	−0.68	0.24	−2.89	0.04	−1.07	0.52	−2.04	0.04
Lay date	–	–	–	–	−0.01	0.01	−1.08	0.28	–	–	–	–	–	–	–	–
Clutch size	–	–	–	–	–	–	–	–	0.86	0.06	13.96	<2e-16	–	–	–	–
Brood size	–	–	–	–	–	–	–	–	–	–	–	–	0.34	0.09	3.79	2e-04
Random effects	**Variance**	***SD***	**Sample size**		**Variance**	***SD***	**Sample size**		**Variance**	***SD***	**Sample size**		**Variance**	***SD***	**Sample size**	
Female ID	19.88	4.46	Nb ♀ = 217		1.25	1.12	Nb ♀ = 217		0.65	0.81	Nb ♀ = 188		1.19	1.09	Nb ♀ = 187	
Residual	26.30	5.13	Nb obs. = 278		1.00	1.00	Nb obs. = 278		1.44	1.20	Nb obs. = 223		6.30	2.51	Nb obs. = 223	

	Handling aggression				Exploration score				Breath rate			
Fixed effects	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***
Intercept	1.97	0.15	13.08	6e-13	3.66	0.42	8.62	4e-16	133.04	4.35	30.55	<2e-16
Habitat - urban	0.07	0.12	0.60	0.55	**2.78**	**0.49**	**5.65**	**3e-8**	**4.02**	**1.95**	**2.06**	**0.04**
Sex-Female	−0.43	0.10	−4.17	4e-05	ns	ns	ns	ns	ns	ns	ns	ns
Ambient temperature	ns	ns	ns	ns	ns	ns	ns	ns	−0.36	0.16	−2.16	0.03
Random effects	**Variance**	***SD***	**Sample size**		**Variance**	***SD***	**Sample size**		**Variance**	***SD***	**Sample size**	
Individual ID	0.34	0.59	Nb ind. = 332		9.87	3.14	Nb ind. = 346		104.88	10.24	Nb ind. = 337	
Manipulator ID	0.04	0.21	Nb manipul. = 6		–	–			24.12	4.91	Nb manipul. = 4	
Residual	0.62	0.79	Nb obs. = 413		7.53	2.74	Nb obs. = 432		162.51	12.75	Nb obs. = 423	

Gray areas highlight the focal effect of habitat type (forest vs. city), with significant results in bold (P < 0.05). ns: non-significant (P > 0.05). Sample sizes are provided for the number of females (Nb ♀) or individuals (Nb ind.), the number of observations (Nb obs.) and the number of manipulators (Nb manipul.).

and clutch size were repeatable in urban birds but not in forest birds (where again, sample sizes were small, Table S3).

The step 3 analysis along the gradients of naturalness and pedestrian pressure showed no influence of pedestrian frequency on any of the focal traits (**Table 4**). Three out of the seven traits studied showed variation across the naturalness gradient: in more urbanized areas great tit females laid earlier and smaller broods and birds had generally (see below) faster breath rates during handling (i.e., similar trends as found when contrasting urban and forest birds). In all three cases however, the strength (and even direction in the case of breath rate) of the urbanization effect showed significant variation across years (**Table 4**, **Figure 3**): for example, later laying in more natural habitats was true for 3 years out of four (**Figure 3A**) and breath rate showed inconsistent changes along the urban gradient across the 3 years of study (**Figure 3C**).

DISCUSSION

Based on 6 years of great tit monitoring in an oak forest and the nearby city of Montpellier in southern France, our study reveals strong phenotypic divergence for a suite of breeding, behavioral and physiological traits, both when comparing forest and city birds, but also for some of the traits, within the urban gradient. We discuss below how these results fit with the pace-of-life syndrome hypothesis, and what processes could be involved in these divergences.

Phenotypic Divergence between Forest and City and along the Urbanization Gradient

We confirm here a commonly found breeding divergence, which is that city birds breed earlier and produce smaller clutches, and even more so in more urbanized areas of the city (**Tables 3**, **4**, **Figures 2A,B** and **3A,B**). Such urban effects on the timing of breeding and clutch size have been demonstrated before in great tits (e.g., Dhondt et al., 1984; Wawrzyniak et al., 2015) and many other avian species (see review in Marzluff, 2017). Note however that in a recent comparative analysis of forest and urban passerines at a macro-geographic scale (including 138 great tit populations), Vaugoyeau et al. (2016) found that average

TABLE 4 | Contrasting phenology, reproductive success, behavior, and physiology along urban gradients of habitat naturalness (Naturalness) and pedestrian frequency (Pedestrian) using linear mixed models with female/individual identity and manipulator identity (for handling aggression and breath rate) as random effects.

	Lay date				Clutch size				Hatching success				Fledging success			
Fixed effects	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***
Intercept	42.94	1.05	40.82	<2e-16	9.01	0.58	15.54	<2e-16	−0.58	0.84	−0.69	0.49	3.04	0.82	3.69	3e-04
Naturalness	**1.67**	**0.56**	**2.95**	**4e-03**	**0.30**	**0.10**	**3/00**	**3e-03**	0.04	0.09	0.46	0.64	0.20	0.13	1.49	0.14
Pedestrian	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Year-2014	−9.44	1.38	−6.82	2e-10	−0.05	0.25	−0.21	0.84	−0.51	0.37	−1.37	0.18	−1.02	0.61	61.68	0.10
Year-2015	−4.15	1.46	−2.84	5e-3	−0.61	0.25	−2.46	0.01	0.86	0.40	2.13	0.04	0.10	0.64	0.16	0.87
Year-2016	−6.64	1.44	−4.62	7e-6	−0.39	0.26	−1.52	0.13	1.03	0.39	2.62	0.01	−1.30	0.62	−2.12	0.04
Year*Naturalness-14	**−1.88**	**0.75**	**−2.49**	**0.01**	**−0.27**	**0.12**	**−2.26**	**0.03**	ns	ns	ns	ns	ns	ns	ns	ns
Year*Naturalness -15	**−0.24**	**0.78**	**−0.31**	**0.76**	**−0.35**	**0.13**	**−2.70**	**8e-03**	ns	ns	ns	ns	ns	ns	ns	ns
Year*Naturalness-16	**−0.12**	**0.78**	**−0.15**	**0.88**	**−0.22**	**0.13**	**−1.65**	**0.10**	ns	ns	ns	ns	ns	ns	ns	ns
Lay date	–	–	–	–	−0.03	0.01	−2.02	0.04	–	–	–	–	–	–	–	–
Clutch size	–	–	–	–	–	–	–	–	0.88	0.10	8.59	2e-14	–	–	–	–
Brood size	–	–	–	–	–	–	–	–	–	–	–	–	0.33	0.11	3.06	2e-03

Random effects	**Variance**	**SD**	**Sample size:**	**Variance**	**SD**	**Sample size**	**Variance**	**SD**	**Sample size**	**Variance**	**SD**	**Sample size**
Female ID	21.38	4.62	Nb ♀ = 141	1.20	1.09	Nb ♀ = 141	1.51	1.23	Nb ♀ = 116	2.01	1.44	Nb ♀ = 116
Residual	27.48	5.24	Nb obs. = 183	0.49	0.70	Nb obs. = 183	1.21	1.10	Nb obs. = 133	4.27	2.07	Nb obs. = 133

	Handling aggression				Exploration score				Breath rate			
Fixed effects	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***	***Est.***	***SE***	***t***	***P***
Intercept	2.46	0.17	14.19	7e-12	6.38	0.28	22.81	<2e-16	133.35	3.13	42.54	6e-12
Naturalness	0.03	0.03	0.88	0.38	−0.06	0.15	−0.43	0.67	**−1.97**	**1.06**	**−1.87**	**0.06**
Pedestrian	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	Ns	ns
Sex-Female	−0.44	0.13	−3.50	6e-4	ns	ns	ns	ns	ns	ns	Ns	ns
Age-Juvenile	−0.29	0.12	−2.45	0.01	ns	ns	ns	ns	ns	ns	Ns	ns
Year-2015	−0.34	0.16	−2.16	0.03	ns	ns	ns	ns	−4.88	2.35	−2.08	0.04
Year-2016	−0.36	0.16	−2.28	0.02	ns	ns	ns	ns	−3.47	2.76	−1.26	0.21
Year*Naturalness -15	ns	ns	ns	ns	ns	ns	ns	ns	**3.43**	**1.31**	**2.62**	**0.01**
Year*Naturalness -16	ns	ns	ns	ns	ns	ns	ns	ns	**1.02**	**1.39**	**0.74**	**0.46**

Random effects	**Variance**	**SD**	**Sample size**	**Variance**	**SD**	**Sample size**	**Variance**	**SD**	**Sample size**
Individual ID	0.37	0.61	Nb ind. = 209	9.79	3.13	Nb ind. = 216	101.30	10.06	Nb ind. = 213
Manipulator ID	0.03	0.18	Nb manipul. = 6	–	–		18.79	4.33	Nb manipul. = 4
Residual	0.53	0.73	Nb obs. = 261	7.50	2.74	Nb obs. = 271	164.64	12.83	Nb obs. = 270

Gray areas highlight the focal effect of the naturalness or pedestrian gradients, with significant results in bold (P < 0.05). ns, non-significant (P > 0.05). Sample sizes are provided for the number of females (Nb ♀) or individuals (Nb ind.), the number of observations (Nb obs.) and the number of manipulators (Nb manipul.).

laying date was not correlated with the degree of urbanization, while average clutch size decreased with urbanization in collared (*Ficedula albicollis*) and pied flycatchers (*Ficedula hypoleuca*) but not in great tits. Later laying and smaller clutches in cities have been attributed to the urban heat island effect, as well as the artificial lights and the lack of adequate resources (i.e., arthropods) during the breeding season (Chamberlain et al., 2009; Kempenaers et al., 2010).

Hatching success was lower in urban broods, yet this did not lead to lower fledging success (**Table 3**, **Figure 2D**) nor to lower fledgling numbers (Table S2). Within the city, fledging success varied significantly across years but again was not significantly lower in more urbanized areas. It is possible that the slightly lower fledgling productivity in urban broods (**Table 2**, Table S2) was not significant in our 6 years study because of insufficient power. However, it remains that while the other breeding traits show strong significant differences between forest and city, it did not translate in a similarly strong divergence for fledgling success. This result contrasts with previous findings in great tits (Bailly et al., 2016) and with the common assessment that the urban environment induces strong constraints on avian reproduction (Gil and Brumm, 2014). It raises the very interesting question of whether urban birds might display adaptive breeding strategies that differ from birds in the forest, yet do not lead to lower reproductive success (see further discussion on this possibility, below). Reduced clutch size

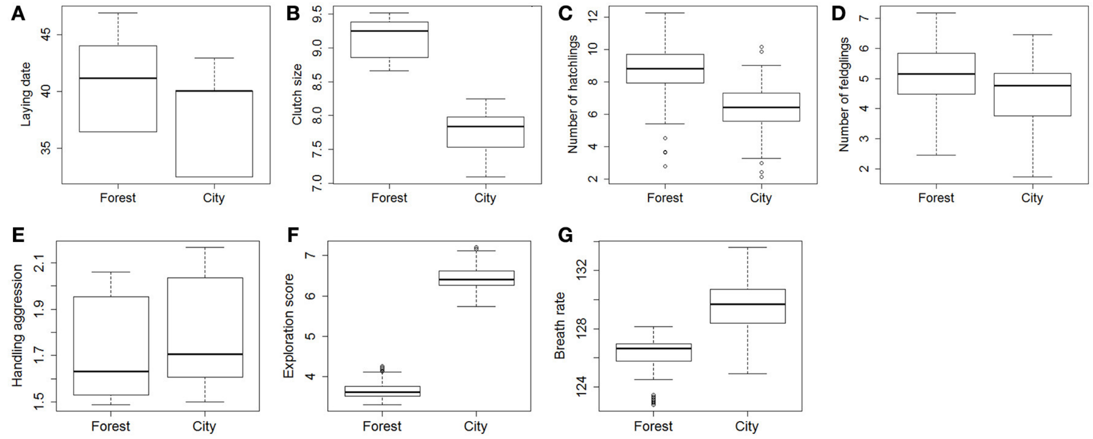

FIGURE 2 | Predicted contrasts in breeding [**A**, lay date; **B**, clutch size; **C**, number of hatchlings (from a model controlling for clutch size), **D**, number of fledglings (controlling for number of hatchlings)], behavior (**E**, handling aggression; **F**, exploration score) and physiological traits (**G**, breath rate) between great tits from the forest of La Rouvière and great tits from the city of Montpellier. The graphs present the boxplot of predicted values from models detailed in **Table 3**. For lay date, 1 = 1st March. Breath rate is in number of breaths/min.

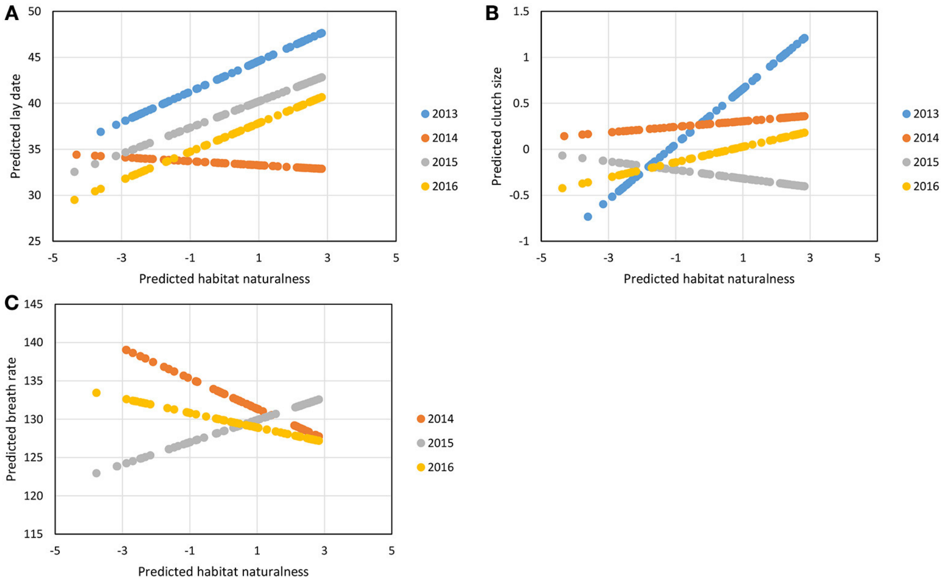

FIGURE 3 | Predicted variation in great tit lay date **(A)**, clutch size **(B)**, and breath rate **(C)** along the naturalness gradient in the city of Montpellier, for each year of data sampling, from the best linear mixed models, presented in **Table 4**. For lay date, 1 = 1st March. Breath rate is in number of breaths/min.

would hence be the result of an adaptive process rather than the expression of energetic constraints. Overall in terms of POLS, the breeding data suggested that city birds displayed one element pointing to slower life histories (smaller clutches) yet did not differ consistently from forest birds in their reproductive output.

Our results on personality traits and breath rate were more consistent with the prediction of faster behavioral syndromes in urban compared to forest habitats. Handling aggression was not significantly influenced by the urban habitat, yet city birds were faster explorers with generally faster breath rate under constraint (**Table 3**). Breath rate also varied across the urban gradient, yet with important annual variation (**Table 4, Figure 3C**). Previous rural vs. urban avian comparisons for these traits found: lower short-term stress response in urban European blackbirds (Partecke et al., 2006), reduced physiological stress and faster exploratory behavior in urban dark-eyed juncos *Junco hyemalis* (Atwell et al., 2012; Abolins-Abols et al., 2016), higher territorial aggression in urban song sparrows *Melospiza melodia* (Evans et al., 2010), urban Australian magpies *Gymnorhina tibicen* (Cilento and Jones, 1999), urban Eurasian coot *Fulica atra* (Minias, 2015), and urban noisy miners *Manorina melanocephala* (Lowry et al., 2011), and finally higher breath rates in urban great tits during handling (Torne-Noguera et al., 2014, but see Senar et al. from this issue).

Mean breath rate during handling for Montpellier great tits was similar to that found in the study by Torne-Noguera et al. (2014, i.e., around 130 breaths/min) yet it was only around 2% higher than breath rate in forest birds, compared to a 14% difference in Barcelona (Torne-Noguera et al., 2014). Our singular analysis along the urban gradient revealed strong annual variation in the breath rate response to urbanization level (**Figure 3C**), which could partly explain the discrepancies between previous studies (Partecke et al., 2006; Torne-Noguera et al., 2014; Senar et al. this special issue). While heart rate and breath rate are highly related (see e.g., Dubuc-Messier et al., 2016), their interpretation is complicated by the fact that they reflect both sympathetic and parasympathetic reactivity (e.g., Koolhaas et al., 1999), and that responses to long term or acute stresses differ (Abolins-Abols et al., 2016). In rodents more aggressive and active individuals have a strong sympathetic response yet a low parasympathetic reactivity to acute stress, which translates into increased heart rate response (Koolhaas et al., 1997). Similarly, domestic hens with active coping styles (high-feather peckers) have faster heart rates during manual restraint (Korte et al., 1999). Hence overall, in the context of POLS, higher breath rate and faster exploration scores in urban great tits are suggestive of a faster pace-of-life.

Plastic vs. Genetic Origin and Adaptive Nature of Divergence

While we have demonstrated in this study that forest and city great tits display strong phenotypic differences for breeding parameters, behavioral traits and physiological reaction to stress, two fundamental questions remain unanswered. The first question is whether these physiological differences emanate from underlying genetic differences, rather than plasticity. This would suggest that urban birds form a genetically distinct ecotype compared to forest birds, possibly because of a process of local adaptation. The second, related question, is whether the phenotypic differences we have described here result from an adaptive process since both plasticity and genetic processes (such as drift) can result in maladaptive responses.

As regards the first issue of a plastic vs. genetic origin in the phenotypic differences, a genetic difference can occur only if the focal traits are heritable, and in the case of restricted gene flow (Lenormand, 2002). An interesting point to make here is that in our study populations, hatching and fledging success were not repeatable, and there is very little evidence for heritability of reproductive success in the literature (review in Postma, 2014). All other focal traits studied here were repeatable and they were previously shown heritable, in great tits or in closely related avian species (for lay date and clutch size see review in Postma, 2014, for aggression and exploration behavior see review in Dingemanse and Dochtermann, 2014, for breath rate see Class et al., 2014). This overview on heritability suggests that a rapid evolution of the focal traits is possible except for hatching and fledging success. Recent results in population genomics also provide useful knowledge on the neutral and adaptive genomic divergence of Montpellier great tits at both scales studied here (forest vs. city and urban gradient). Using a RAD sequencing analysis of nearly 50,000 SNPs in 140 great tits, Perrier and colleagues (Perrier et al. in review) show that the neutral genetic structure is weakly impacted by urbanization, with an average F_{ST} between La Rouvière forest and Montpellier city of 0.009 and an F_{ST} of 0.012 between the most and least urbanized sites within the city of Montpellier. While all F_{ST} were significant, their low value suggests that gene flow is common along the urban gradient and between forest and city. However, it could well be that non-random gene flow, e.g., via individuals matching their habitat choice to their phenotype (Edelaar et al., 2008; Edelaar and Bolnick, 2012) reinforces local adaptation. In fact it has been suggested that urbanization should favor individuals with high behavioral plasticity (Lowry et al., 2013). Our preliminary results on habitat-specific repeatability (Table S3) show lower repeatability in the behavior and physiology of urban compared to forest birds which could be indicative of higher plasticity, although this is not true for breeding traits. An interesting avenue would hence be to focus on plasticity as a trait *per se*, evaluating how/whether it is heritable and selected for in the urban landscape and across the range of traits studied here (e.g., following the approach of Nussey et al., 2005 for plasticity of timing of breeding). We also envisage in the future a common garden study to experimentally test whether birds from more or less urbanized areas yet raised in aviaries display genetically based differences in the traits studied here.

Addressing the second question to find out whether the phenotypic variation described here is adaptive or not will require more long term data (including survival and reproductive data) to compare the direction and force of selection acting on the focal traits across the habitats (Isaksson, 2015). It is commonly assumed that the novelty of the urban environmental features induces new and strong selection pressures providing the potential for rapid trait change (Markó et al., 2013; Alberti, 2015; Hendry et al., 2017). However, direct empirical measures of urban natural selection remain scarce (but see e.g., the emblematic peppered moth case, Cook and Saccheri, 2013). New genomic resources offer a powerful way to bypass the data consuming classical selection analyses by testing for genome-wide signatures of selection associated with urbanization, even in non-model species (Harris et al., 2015). While at first hand, breeding parameters show decreased clutch size and hatching success in city great tits compared to their forest conspecifics (**Table 3**), a very important result from our study is that these city birds do not have lower fledgling rate or success (**Table 3** and Table S2). This stability of reproductive performance in the nest despite strong differences in breeding traits, suggests that city birds might have evolved an adapted breeding strategy in response to urbanization with smaller clutches and broods to adjust to the lack of food for nestlings in the urban habitat (Chamberlain et al., 2009). A comparison of morphological traits in our focal populations also points to possibly adaptive changes since urban great tits are smaller and lighter than forest birds yet they do not differ in body condition (Demeyrier et al. in prep.).

Perspectives on a Multivariate Long-Term Process

Previous studies have shown enhanced annual survival in urban adult birds, in particular because of higher resource availability over winter (Rebolo-Ifran et al., 2015; Marzluff et al., 2016). In the context of the pace-of-life theory, such decreased probability of mortality would be indicative of a slower pace-of-life (Réale et al., 2010). However, new predation pressures in the city are also thought to affect avian survival, especially nestlings', and overall rural/urban survival comparisons are still scarce and show no consistent pattern (Chamberlain et al., 2009). With only 3 years of breeding captures, our study lacked power to conduct capture-mark-recapture analyses, yet it is most definitely our objective to pursue the long term research and compare survival rates across the urbanization gradient in the future. In the same vein, a longer monitoring will enable a comparison of recruitment success across urbanization, providing a complementary perspective to our results on fledgling success. Survival data will also be crucial to understand selection pressures acting on urban great tits, and to compare them with selection in more natural habitats.

While the present analysis focused on first broods following classical approaches in the literature, considering second broods in the study of forest and urban reproductive strategies could reveal further divergences. Between 2011 and 2016, 11.6% of Montpellier urban females laid second broods vs. 4.5% in La Rouvière forest (along with 20.6 and 23.1% of replacement broods). In our study sites in the city of Montpellier, *Pinus* spp. and the evergreen oak *Quercus ilex* are dominant tree species. In these breeding habitats, later breeding could be favored because the food required to rear nestlings peaks later in the season (Blondel et al., 2006). Also, in such habitats, second clutches can be typically larger than first broods, while the reverse is true in deciduous habitats such as found in La Rouvière forest (Lambrechts et al., 2008). Hence a complementary analysis involving both first and second broods might confirm that city birds have adopted a different yet adaptive breeding strategy.

Finally, the colonization of an urban landscape by an avian species is likely to be a long process, possibly leading to a succession of adaptive phenotypes. In line with the general finding that individuals that are superior colonizers are inferior competitors (Yu and Wilson, 2001), it has been suggested that while a proactive fast lifestyle would be favored during the colonization stage of urban habitats (Sol et al., 2013), the higher sensitivity of reactive slow individuals to the environmental conditions might favor them on the long run when facing ecological novelty (Robertson et al., 2013). In the case of such a sequence of colonization, findings regarding the pace-of-life of birds adapted to the urban habitat will depend a. on how advanced the colonization process is, and b. on how much the urban population is isolated from surrounding rural populations. Although Montpellier is a millenary city where great tits have a long history of presence, it is still undergoing a rapid urbanization process, which could explain the high prevalence of bold birds in the most urbanized areas. Also, recent genetic analyses based on genome-wide RAD-sequences have revealed that Montpellier and La Rouvière great tits show significant but low genetic differentiation (Perrier et al. in review). Uncovering the direction of gene flow between city and forest, and testing for a source-sink dynamic would reveal whether the city population could be regularly supplied with new, possibly bold, individuals.

Overall, our study shows strong phenotypic divergence between forest and urban great tits, but also across the urbanization gradient. Behavioral and physiological traits suggested that urban birds were on a faster end of the pace-of-life continuum than their forest conspecifics, yet breeding data did not support this conclusion. Further investigations on the plastic vs. genetic origin of the differences revealed here, along with the selection forces on the focal trait, will improve our understanding of how urban life shapes great tit pace-of-life in the future.

ETHICS STATEMENT

Capture and ringing was performed under individual ringing permits delivered by the CRBPO and all the protocols involved, including the behavioral assays described below, were approved by the Animal Care and Use Committee Languedoc Roussillon (CEEA-LR-12066) as well as by Regional Institutions (bylaw issued by the Prefecture of Montpellier number 2012167-0003).

AUTHOR CONTRIBUTIONS

Conceived study: AC, VD, ML, AG. Collected data: AC, VD, ML, SP, AG. Analyzed videos: VD. Statistical analyses: AC. Wrote the manuscript: AC. Improved the manuscript: VD, ML, AG. Contributed funding and materials: AC, AG.

FUNDING

This research was supported by a recurrent annual funding from the OSU-OREME and by the European Research Council (Starting grant ERC-2013-StG-337365-SHE to AC).

ACKNOWLEDGMENTS

We thank the many participants to the field monitoring, the City of Montpellier (Direction Paysage et Biodiversité) and the Zoo de Lunaret for logistic help. We thank Aude Caizergues, Arnaud Béchet, Gabrielle Dubuc Messier, and Denis Réale for useful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00053/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past collaboration with one of the authors AG and states that the process nevertheless met the standards of a fair and objective review.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 09 August 2017
doi: 10.3389/fevo.2017.00082

Humans and Tits in the City: Quantifying the Effects of Human Presence on Great Tit and Blue Tit Reproductive Trait Variation

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Environmental conditions are key drivers of life-history evolution, and the urban environment is an extreme form of land-use readily inhabited by avian wildlife, whose life-history variation in such altered environment is still poorly understood. Recently, the study of environmental variables associated with urban living—which include shifts in temperature, light, noise or food availability—has attracted increased attention. Another environmental axis that sets the urban space at odds relative to natural habitats is high human abundance, yet very little is known about its effect on avian fitness. We developed a protocol to quantify human presence by performing repeated counts of humans on the ground within a 15 m radius of nestboxes monitored in two centrally-located study areas of a European capital city. In parallel, a GIS-based approach was used to infer nestbox distance to the nearest path and road. Multiple counts of human presence around each nestbox yielded moderate to high repeatabilities ($0.6 \leq r \leq 0.8$) while requiring considerable resources time- and people- wise. In contrast, GIS-based estimates of nestbox distance to paths and roads were time efficient and generated highly repeatable results. The effects of (i) human presence around each nestbox, (ii) nestbox distance to the nearest path and (iii) nestbox distance to the nearest road were tested on reproductive traits of blue tits *Cyanistes caeruleus* and great tits *Parus major* breeding in two urban sites. Human presence did not influence blue tit or great tit life-history traits and reproductive success, suggesting reproductive habituation to humans in an urban landscape. In contrast, nestbox distance to roads shortened incubation time in great tits while nestbox distance to paths increased incubation time in blue tits. Moreover, blue tit offspring 2 weeks after hatching were lighter closer to roads. Our study confirms the reliability of a field protocol capturing human presence around multiple fixed locations that can be easily implemented in either urban or rural landscapes. At the same time, it appears that when applied to two urban sites where habituation to humans might have occurred, it is infrastructural networks rather than human presence *per se* that played a greater role in tit reproductive trait variation.

Keywords: human disturbance, habituation, *Parus major*, *Cyanistes caeruleus*, life-history traits, paths, roads, incubation

Edited by:
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Specialty section:
This article was submitted to Urban Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *10 February 2017*
Accepted: *11 July 2017*
Published: *09 August 2017*

Citation:
Corsini M, Dubiec A, Marrot P and Szulkin M (2017) Humans and Tits in the City: Quantifying the Effects of Human Presence on Great Tit and Blue Tit Reproductive Trait Variation. Front. Ecol. Evol. 5:82. doi: 10.3389/fevo.2017.00082

INTRODUCTION

Humans are a keystone species in an urban habitat, modifying and shaping original ecosystem structures with often drastic consequences for their functionalities (Rees, 1997). Urban space is a habitat that attracts humans: while 54% of the world human population already resides in urban settlements, it is likely to increase to 66% by 2050 (United Nations, 2014). Consequently, 0.5% of the planet's land area has become urban space (Schneider et al., 2009), a value that is expected to grow several-fold in the next decades (Seto et al., 2011).

Urban growth is one of the major causes of natural habitat loss and fragmentation at a global scale (Grimm et al., 2008) as it modifies extensively the original landscapes (Shochat et al., 2006), leading to an overall rearrangement of habitats and animal communities (McDonnell and Pickett, 1990; Chamberlain et al., 2009). Urban areas are characterised by impervious surfaces including buildings and infrastructural networks, but also modified greenspaces; they encapsulate a multitude of ecological niches at a local scale, set in a multidimensional space of modified biotic and abiotic elements and processes (Sprau et al., 2016). Unavoidable interactions between urban wildlife and humans are thus increasing in prevalence due to growing urban sprawl taking over rural areas (Ditchkoff et al., 2006; Grimm et al., 2008). Birds are a convenient model to study the effects of urbanization on wildlife, due to their conspicuousness and ubiquity in both cities and rural sites. Moreover, much knowledge on bird biology is readily available from long-term studies carried out in the wild, thus offering a valuable background to infer change occurring in an urban environment (Savill et al., 2011).

A large number of previous studies have reported changes in bird ecology triggered by the urban environment (reviewed in Chace and Walsh, 2006; Gil and Brumm, 2013); these pointed out how this distinctive habitat may be defined by a multitude of environmental factors which rarely act in isolation. Overall, even though cities and towns are generally characterised by altered climatic profiles (increased minimum temperatures, Marzluff, 2001), predator communities (Churcher and Lawton, 1987) and food resources (i.e., supplementary feeding by humans; Fuller et al., 2008; Robb et al., 2008), the latter is emerging as a paramount in terms of its influence on habitat quality (Solonen, 2001; Robb et al., 2008; Chamberlain et al., 2009).

Urban-driven abiotic factors are also known to impact avian ecology and breeding biology, such as noise, light and chemical pollution (Slabbekoorn and Ripmeester, 2008; Dominoni et al., 2013). Thus, bird species were shown to decline in terms of richness, density and abundance due to noise pollution (Stone, 2000; Bayne et al., 2008; Mockford and Marshall, 2009). At the population level, noise pollution was found to induce an increased pitch in birds or a shift in their singing activity to the night time, avoiding noisy periods (Slabbekoorn and Peet, 2003; Kirschel et al., 2009; Nemeth and Brumm, 2009). Artificial night lighting—light pollution—may attract many birds during their nocturnal migration: harsh consequences for such important detours are an increased number of predation events and/or collisions with artificial structures in cities (van de Laar, 2007, reviewed in Erritzoe et al., 2003), as well as a reduction in energy storage or delayed arrival at wintering or breeding areas (Seress and Liker, 2015). Light pollution can also affect bird behaviour by advancing avian singing time at dawn and dusk (Da Silva et al., 2014). Finally, the increased exposure to environmental toxic chemicals—chemical pollution—can affect birds' physiology and phenotype directly but also indirectly because of habitat alterations or parasites spread (Morrison, 1986; Eeva et al., 1994; Fry, 1995; Isaksson, 2015).

While the effect of a large range of human triggered, biotic and abiotic variables specific to urban landscapes have been tested on avian reproductive traits, knowledge on the "direct" effect of human presence on avian reproduction is much more limited. Humans (often deterministically defined as "human disturbance") are known to trigger various responses in terms of changes in animal distribution, demography and population size (as reviewed in Gill, 2007). Earlier studies often tested the effect of human disturbance on bird species by inducing artificially different levels of noise, but without explicitly linking noise treatment levels to real life distribution of visitors on the ground (Lord et al., 2001; Verhulst et al., 2001; Baines and Richardson, 2007). Moreover, the direct effect of human disturbance at the individual level has largely focused on behavioural responses, where flush distance (Fernández-Juricic and Tellería, 2000; Miller et al., 2001; Tarjuelo et al., 2015), feeding patterns (Fernández-Juricic and Tellería, 2000) or physiological responses (e.g., increased corticosterone levels; Fowler, 1999; Walker et al., 2006; Ellenberg et al., 2007; Almasi et al., 2015) were measured. However, knowledge on the direct effect of human presence on individual fitness in an urban habitat is limited, as variation in life-history traits and reproductive success caused by human presence has been largely investigated in non-urban habitats and largely in non-cavity nesting bird species (**Table 1**). While we may expect that birds breeding in urbanised habitats are continuously exposed to humans, thus habituated to their often not-threatening activities, no evidence in terms of life-history trait variations for such specific stressor was reported in cities. Even though many studies underlined how avian populations breeding in habitat characterised by frequent human disturbance, might often reduce their flee distance as a result of habituation (Metcalf et al., 2000; Rodriguez-Prieto et al., 2009; Clucas and Marzluff, 2012), it is also known that the presence of humans near the nest might ordinarily trigger strong behavioural responses in parental behaviour while feeding their young—for example, by alarm calling and avoiding entering nests for a certain time (Müller et al., 2006).

Human presence at the site and at the nest level is not often easy to monitor due to its inherent property of being variable in space and time. It can depend on season and weather conditions (sunny vs. rainy days; Glądalski et al., 2016), days of the week (weekends vs. working days; Remacha et al., 2016) or hours of the day (morning peak hour vs. afternoon). It is thus important that such variability is taken into account when quantifying human presence and its possible disturbance to wildlife. Moreover, and in order to detect the potential biological effect of human presence on avian reproduction, protocols for capturing human disturbance need to be repeatable—an information that is only

TABLE 1 | Summary of studies reporting the impact of human presence on avian life-history traits.

Bird species	**Life-history traits tested[a]**	**Habitat structure**	**Method to quantify human presence[b]**	**Repeatability test for human presence quantification[c]**	**Trend[d]**	**References**
Blue tit (*Cyanistes caeruleus*)	Number of fledglings	Urban parkland *vs.* deciduous forest	Number of tickets sold in the urban parkland, not quantified in the deciduous forest	Not tested	(–)	Glądalski et al., 2016
	Nestling growth rate and development	Oak forest: recreational areas *vs.* quiet areas in the woods	Not quantified: nests located in proximity of paths considered as disturbed	Not tested	(–)	Remacha et al., 2016
Woodlark (*Lullula arborea*)	Nest survival, number of fledglings	Heathlands	Estimation performed on transects for every given site	Yes, paired t-test: no difference in the number of people measured (in space and time)	(ns), (+)	Mallord et al., 2007
Black grouse (*Lyrurus tetrix*)	Clutch size, hatching success	Managed grouse moors	At nest level, disturbance induced	Not tested	(ns), (ns)	Baines and Richardson, 2007
Dottereles (*Charadrius obscurus aquilonius*)	Incubation duration	Beaches	At nest level, disturbance induced	Not tested	(–)	Lord et al., 2001
European oystercatcher (*Haematopus ostralegus*)	Incubation duration, foraging behaviour	Salt marshes (breeding sites) and mudflats (foraging sites)	At nest level and in feeding territories, disturbance induced	Not tested	(–), (–)	Verhulst et al., 2001
Blackbird (*Turdus merula*), Woodpigeon (*Columba palumbus*)	Occupancy rate	Urban tree-lined streets	In two random points along tree-lined streets: pedestrians and cars passing by were counted for 3 minutes in the morning and at midday	Not tested	(–)	
Black skimmer (*Rynchops niger*)	Laying date, hatching success	Coastal	At nest level, three different levels of disturbance	Not tested	(–), (–)	Safina and Burger, 1983
Herring gull (*Larus argentatus*)	Hatching success, nestling survival	Coastal	At site level, disturbance detected	Not tested	(–), (ns)	Hunt et al., 1972

[a] *Life-history traits tested in response to human presence.*

[b] *Disturbance quantified as observational data or experimentally induced.*

[c] *Specifies whether a repeatability test for human presence detection was performed.*

[d] *Reports whether human presence affected the inferred life-history traits positively (+), negatively (–) or had no effect (ns). The symbols' order refers to life-history traits listed in "a."*

rarely reported in studies of human disturbance on wildlife (**Table 1**).

This study quantifies the biological effect of humans on great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) life-history traits and reproductive success, characterised at the nestbox level and estimated in two contrasted yet centrally located urban study sites. Great tits and blue tits are two well-studied songbird species that breed in nestboxes in a large range of environments, from undisturbed rural areas to the chaos of urban settlements. The two study sites presented in this study—an urban park and a cemetery—offer the opportunity to compare two different urban habitat structures whose distributions of visitors vary gradually from a regularly visited site during specific opening hours (cemetery) to frequently visited without specific rules (urban park). Here, the effects of human presence—quantified with an easily implemented and repeatable protocol capturing human presence on the ground and at the nestbox level—is inferred and contrasted with the effects of nestbox distance to the nearest path and road, which are infrastructural networks acting as key sources of human presence in urban areas.

METHODS

Study Sites

This study was conducted in 2016 as part of a new long-term research project whose main aim is to study the effect of cities on the genotype and phenotype of two wild passerine species—great tits *P. major* and blue tits *C. caeruleus*. 196 Schwegler woodcrete nestboxes (type 1b with an entrance hole of 32 mm and suitable for both great tits and blue tits) were erected in two study sites—91 in a cemetery and 105 in an urban park (Pole Mokotowskie), located in the city centre of Warsaw, Poland. Warsaw (52°14′N, 21°1′E) is the largest city of Poland, and ranks as the 9th most populous capital city in the European Union with a population of over 1.7 million people (Eurostat, 2017). While both study sites are centrally located, the two areas are defined by different habitat structures, whose spatial location and actual surface use leads wildlife to face contrasted environmental pressures, especially in terms of human presence. Nestboxes were laid out in a grid, with an inter-nestbox distance of *c.* 50 m, an average south-east orientation ($N = 196$, mean = 106.7 degrees, $SE = 5.6$), and fixed at a height ranging from 2.5 to 3.0 m above ground level.

The urban park Pole Mokotowskie (52°12′N, 20°59′E) covers *c.* 65 hectares. Despite its current design, the park lays on a surface initially used as an airport until 1945. Pole Mokotowskie is now a typical managed green space framed by four large roads, where grass, flower beds and trees alternate to generate covered and open areas. Due to both size and central location, this urban public park plays an important role for city dwellers both as recreational area and a bike-friendly commuting space.

The Jewish Cemetery (52°14′N, 20°58′E), with its area of 34 hectares, is one of the largest Jewish necropolis in the world and still serves as burial place today. Established in 1806, the cemetery was largely neglected and abandoned during the German invasion. Due to a strongly reduced number of visitors after the II World War, only a small portion of the site was regularly visited, leading to a general overgrowth of trees and shrubs in the rest of the area. Because of selective forces mainly driven by important historical events, the Jewish cemetery is now formed by a naturally regenerating habitat. It is distinguished by a particular landscape of moss-covered tombstones in a large wild urban forest mainly composed by a mixture of both native and exotic tree species; oaks (*Quercus* spp.), silver birch (*Betula pendula*), Norway maples (*Acer platanoides*), elms (*Ulmus* spp.) and black locust (*Robinia* spp.) are the most common tree species in the site.

Human abundance and space use is highly contrasted in the two study sites: human presence in the cemetery is heterogeneous (many areas have few or hardly no visitors), and it is limited in terms of opening hours to daytime and outside of religious holidays. Cemetery visitors often arrive in groups and remain on marked paths. In contrast, the urban park is an open site where one can roam freely, and is visited by numerous commuting urban dwellers (pedestrians or cyclists) or those interested in recreational activities.

Life-History Data

Starting from mid-March, each nestbox was visited at weekly intervals to record the date of the first egg laid. The following breeding variables were compiled: nest occupancy (a nestbox was considered occupied if at least one egg was laid by a blue tit or great tit; empty nestboxes and those occupied by other species were considered as not occupied in our analyses), egg laying date (lay date recorded as day 1 = 1st of April), standardised lay date (standardised for each site and species), clutch size, incubation duration (defined as the number of days between the last egg laid to the first egg hatched), hatching success (number of hatchlings/clutch size), fledging success (number of fledglings/number of hatchlings) and chick mass 15 days after hatching (hatching day = day 1). Birds were ringed at day 15 using standard-numbered metal rings supplied by the Polish Bird Ringing Centre (Museum and Institute of Zoology, Polish Academy of Sciences). Nestboxes were checked *c.* 25 days after hatching to establish fledging success. Key summary statistics of reproductive success for each study site are presented in Supplementary Material Table 1.

Ethics Statement

Research was carried out under permit from the Regional Directorate for Environmental Protection in Warsaw, Poland.

Quantifying Human Presence

The possible disturbance to great tit and blue tit reproductive biology caused by human presence was quantified in both sites and for each nestbox by measuring: (i) the number of humans and dogs present around each nestbox in a 15 m radius, (ii) nestbox distance from the nearest path and (iii) nestbox distance from the nearest road.

Human Presence Protocol

The ground-based data collection for human presence was performed from March until July 2016 on specific days throughout the season and aimed to capture human and dog presence at each nestbox. Each site was split in two paths grouping a similar number of nestboxes to minimize the time between the start and the end of each count. During each trial, two fieldworkers per site were counting for 30 s all humans (i.e., bikers and pedestrians) and dogs within a 15 m radius of each nestbox in the two study sites, following two assigned paths (i.e., track 1 and track 2, see maps in Supplementary Material Figure 1). In order to guarantee that all counts at each nestbox were performed at similar timeframes during the respective counts, the tracks' directions followed by the two fieldworkers during the human presence protocol were switched at each trial. The time needed to perform counts of human presence around each nestbox in the field did not vary significantly between sites: fieldworkers spent on average 83.2 min in the cemetery ($SE = 1.75$, $N = 36$) and 80.15 min in the urban park ($SE = 1.45$, $N = 96$) on their assigned path. To capture variation in human presence that can be influenced by human activities, and which might depend on time of day and working *vs.* weekend day, counts in the urban park were replicated across 2 working days (Mondays and Fridays) and 2 days during the weekend (Saturdays and Sundays). In each of these days, counts of human presence for each nestbox were performed 4 times throughout the day (at 8:00 a.m., 12:00 p.m., 3:00 p.m., and 6:00 p.m. for the urban park). Due to restricted opening hours, the cemetery counts were performed twice daily, and were restricted to Mondays, Fridays, and Sundays (**Table 3**). Dogs represented only 6% of the total number of humans and dogs ($N = 7,586$) recorded in a 15-m radius to each nestbox, we therefore use for simplicity the term "human presence" to refer to any human (pedestrian or biker) or dog presence around any given nestbox.

Distances from Paths and Roads

We recorded the spatial location of nestboxes using a GPS Garmin Map 64s and all the nestboxes coordinates were downloaded using the open source Software DNRGPS Minnesota. All nestbox distances to the closest path and road were calculated using the free and Open Source Geographic Information System Quantum GIS 2.8.2 "Wien," (version released on the 9th of May 2015), with a default projection of WGS84-Geographic Coordinate System. Built on the Plugin OpenLayers, the OpenStreetMap has been used as base map to measure all distances with the Distance Matrix tool of the software. Specifically, the distances were measured in meters from the nestbox point to the middle of both the closest path and road. Since the measurements recorded with the Distance Matrix tool may change in precision due to the zoom function manually selected by the observer at each record, the repeatability of the process was tested comparing the measurements taken in two different days by the same person (M.C.).

Statistical Analyses

Repeatability

The repeatability of human presence around each nestbox measured on the ground as well as the repeatability of distance measurement of every nestbox to the closest path and road computed in qGIS was calculated using the "rptR-package" in R following Stoffel et al. (2017). This package allowed for repeatability estimation using a generalised linear model. Specifically, the repeatability test to infer human presence on the ground was performed for four different temporal combinations (early *vs.* late in the day, between working days, weekend *vs.* working days, early *vs.* late in the season) within which the total number of visitors around each nestbox was inferred and the nestbox ID was fitted as random effect in the model. The same method was extended to verify the reliability of the GIS-based approach, where the same observer (M.C.) measured nestbox distances to roads and paths in two separate days. Finally, correlation tests were performed to quantify the strength of association between human presence and distance from the closest path and from the closest road within sites.

Statistical Analyses

Statistical analyses were implemented using the computing environment R (version 3.3.1, released on the 21st of June 2016). To test the effects of human presence and the effects of distance to paths and roads on blue tit and great tit life-history traits and reproductive success, we used the *glm* function of Generalised Linear Models (GLM) in R. All interactions between human presence index, distance from paths and roads and site were tested; however, these were found non-significant and consequently removed from the final models. The following traits were inferred, tested for blue tits and great tits in separate analyses: egg laying date, clutch size and incubation time were analysed with a general linear model with normal error distribution. Occupancy rate, hatching success (i.e., the number of hatchlings on clutch size) and fledging success (i.e., the number of fledglings on the number of hatchlings in nests with at least one hatched offspring) were tested with a quasi-binomial distribution to control for over-dispersion. Human presence and nestbox distance to paths and roads were fitted as a continuous variable in all models while the site was fitted as a fixed effect with two levels corresponding to the two study areas in blue tits: the cemetary was excluded as predictor in great tits due to the low sample size recorded in the site ($N = 2$). Egg laying date (standardised for site and species) was fitted in all models with the exception of two models where occupancy rate and lay date were fitted as response variables. Finally, we used a linear mixed model (*lme* function in R) to test the effects of human presence and the effects of nestbox distance to paths and roads on individual nestling mass at day 15 (response variable), where nestbox ID was fitted as random effect to control for non-independence of nestlings.

RESULTS

Repeatability of Human Presence on the Ground and With a GIS-Based Approach

Human presence recorded in a 15 meters radius around each nestbox during a 30 second long scan varied from 0 to 33 detected humans, and was on average 7.6 times higher in the urban park than in the cemetery (**Figures 1A,B**, **Table 2**). Interpolated maps illustrating average human presence for each nestbox (**Figure 1**) show that the flux of people varied in terms of intensity and scatter between and within sites: while people were heterogeneously distributed and most abundant in the southern areas of the urban park (**Figure 1C**), visitors in the cemetery were usually detected while walking on the main path near the entrance gate (**Figure 1D**). Due to renovation works in the cemetery, workers were systematically detected in the North-Eastern part of the site during the trials.

The repeatability of human presence, measured on the ground for each site separately, was high in the urban park, reaching values between 0.808 and 0.840 and overlapping confidence intervals across the four temporal combinations tested (**Table 3**). The repeatability of human presence in the cemetery was lower, with values ranging between 0.205 and 0.563, which is likely to be the consequence of both reduced human presence and fewer counts (**Table 3**).

Distances from paths and roads at each nestbox, estimated using a computer-based GIS approach, showed near-perfect measurement repeatability, which ranged between 0.991 and 1 (**Table 3**). Additional tests were carried out to determine the strength and direction of any possible linear relationship between human presence (human abundance detected at each nestbox) and distances from paths and roads (obtained *via* GIS-based approaches). Correlations were tested at a within site levels and were all found non-significant but for the exception of the urban park where there was a weak yet significant negative correlation between the number of people around any given nestbox and its distance to the nearest path (**Table 4**).

Effects of Human Presence, Distance to Paths and Roads on Life-History Traits and Reproductive Success

Human presence estimated in a 15 m radius around each nestbox did not influence any of the investigated blue tit or great tit life-history and reproductive traits (**Table 5**). In contrast, in blue tits incubation duration was significantly longer closer to paths while chick mass 15 days after hatching was significantly lower closer to roads. In great tits, incubation duration was significantly shorter closer to roads (**Table 5**). No effect of human presence, distance to paths and roads was detected on hatching and fledging success (Supplementary Material Table 2).

TABLE 2 | Human presence in a 15 m radius from each nestbox in two urban sites.

Site	N nestboxes	N counts	Human presence at the nestbox during a 30 s scan
Urban Park	105	Working days: 24 Weekends: 24 Total: 48	Mean ± SD: 1.354 ± 1.08 Median: 1.02 Min: 0 Max: 32
Cemetery	91	Working days: 12 Weekends: 6 Total: 18	Mean ± SD: 0.178 ± 0.47 Median: 0 Min: 0 Max: 22

"N counts" refers to the number of times human presence was assessed around each nestbox on any given site.

DISCUSSION

This study is one of the very few testing the biological effect of human presence on great tit and blue tit life-history and reproductive success in an urban environment (see also **Table 1**). To the extent of our knowledge, this is the first study that takes into consideration both the intensity of human presence at the nestbox level and its repeatability. The nestbox-based protocol of human presence quantification, as well as the heterogeneous human distribution in space reported in this study that occurred both at a within-site and between-site levels (**Figure 1**), allows for the testing of human disturbance at the finest biological level—the breeding event itself.

Available reports testing the effects of human presence were usually carried out in natural and semi-natural areas, and the methods applied in quantifying such dynamics varied among studies. Human presence was often induced experimentally (Lord et al., 2001; Verhulst et al., 2001; Baines and Richardson, 2007), and the relationship to a natural distribution of these variables is not always explicit. In addition, while most of the previous studies were conducted in colonial waterbirds far from an urban context (see review by Carney and Sydeman, 1999) only a few studies testing the effect of human presence were carried out in urban settings (Fernández-Juricic, 2000; Fernández-Juricic and Tellería, 2000).

The protocol for capturing human presence on the ground required considerable effort in terms of time and number of observers relative to a computer-based GIS approach. Repeatability tests were performed for several temporal combinations that could detect whether human presence is consistent (or not) between times of the day (mornings vs. afternoons), between working days (Mondays vs. Fridays), between working days and weekends, and finally testing whether human presence is stable at the nestbox level throughout the season. The 4 types of combinations tested generated highly equivalent repeatabilities ($0.81 < r < 0.84$) with overlapping confidence intervals, overall revealing that human presence in the urban park is temporally stable. In the cemetery site in contrast, we detected lower intra-day and intra-week repeatabilities (**Table 3**), yet a satisfactory repeatability of $r = 0.56$ when human presence was compared early vs. late in the breeding season. The lower repeatability of human presence recorded in the cemetery is likely to be the result of fewer counts combined with a low and irregular distribution of humans within the study site (**Figure 1**). Thus, our study reveals that the repeatability of human presence was considerably larger in the site with higher human presence (the urban park) and with a greater heterogeneity in human presence between nestboxes. In the urban park where human presence was high, the high repeatability of ground-based counts ($r > 0.8$) confirmed the reliability of our protocol to accurately capture human presence, thereby revealing a stable yet heterogeneous human distribution in both urban sites.

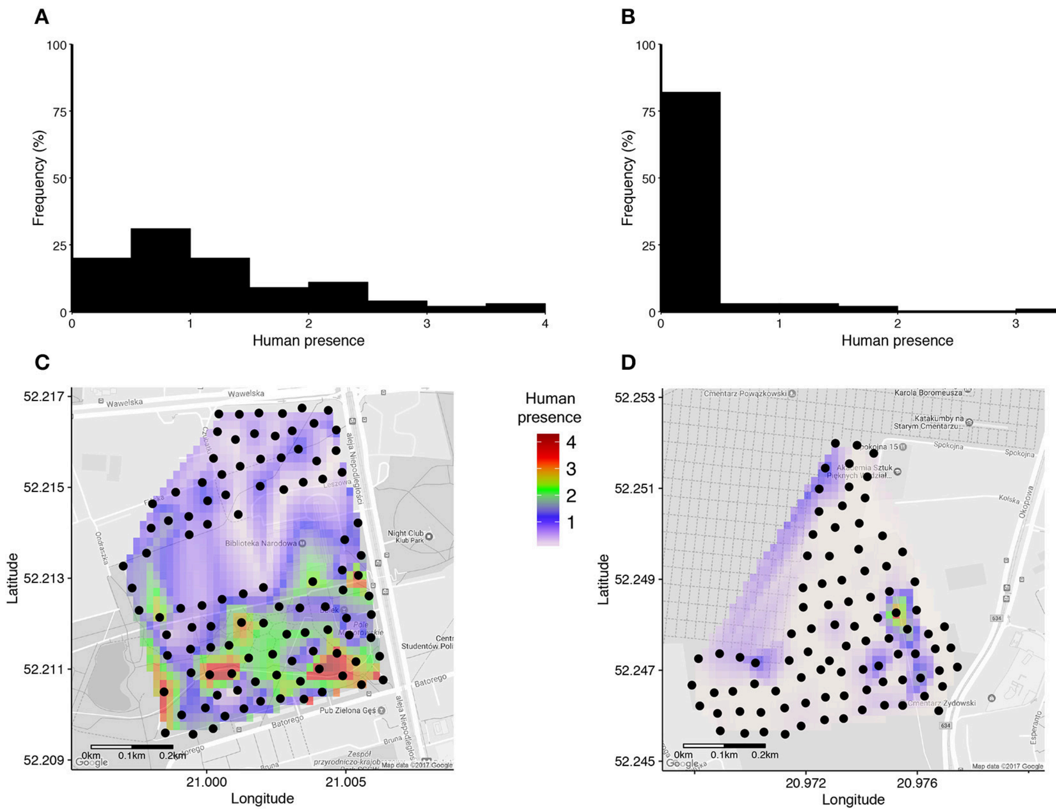

FIGURE 1 | (A,B) Histograms of average human presence around each nestbox in the urban park **(A)** and in the cemetery **(B)**. Average human presence was calculated as the total number of humans and dogs in a 15 m radius to each nestbox standardised by the number of counts performed within each site (i.e., $N = 48$ in **A**, $N = 18$ in **B**). **(C,D)** Interpolated maps of average human presence around each nestbox (black dots) are indicated for the urban park **(C)** and the cemetery **(D)**, respectively.

TABLE 3 | Repeatability of the human presence protocol and GIS-based approaches, with standard errors (SE) and 95% confidence intervals (CI) reported on their original scale).

	Urban park				Cemetery			
	N	**R**	**SE**	**CI**	**N**	**R**	**SE**	**CI**
GROUND-INFERRED HUMAN PRESENCE								
Mornings vs. Afternoons[a]	105	0.821	0.037	0.74–0.881	91	0.281	0.105	0.091–0.501
Seasonal variation[b]	105	0.812	0.035	0.75–0.875	91	0.563	0.062	0.426–0.655
Weekend vs. week days[c]	105	0.840	0.033	0.771–0.894	91	0.438	0.091	0.232–0.588
Mondays vs. Fridays[d]	105	0.808	0.044	0.725–0.898	91	0.205	0.098	0.03–0.375
GIS-BASED INFERENCE								
Paths[e]	105	0.999	0	0.999–0.999	91	0.999	0	0.998–0.999
Roads[e]	105	0.991	0.002	0.988–0.994	91	1	0	1–1

[a] *Intra-day variation: all counts of human presence performed in the morning were tested against counts of human presence performed in the afternoon.*

[b] *Seasonal variation: the first half of the dataset was tested against the second half of the dataset.*

[c] *Weekday variation: counts of human presence made on Saturdays and Sundays were compared with counts performed on working days, e.g., Mondays or Fridays.*

[d] *Working day variation: counts performed on Mondays were compared with counts performed on Fridays. For GIS-based repeatabilities.*

[e] *Computer-based distance measurements collected on the first day were tested against measurements collected on the second day.*

Further work is required to test (i) the extent to which human presence can be captured with high repeatability in a gradient of urbanization with contrasted human presence at the nestbox and site level (indeed, repeatability values for human presence in the cemetery, characterised by considerably lower human presence, were also lower) and (ii) to define a minimum number of counts that would reduce ground effort while securing satisfactory repeatability ($r \geq 0.6$) of human presence at the level

TABLE 4 | Pearson's correlations and significance tests investigating collinearity between human presence and distances to infrastructural facilities.

Site	N	*r*	*p*-value	Type of interaction
Urban Park	105	0.08	0.42	Human presence–roads
		−0.29	0.002*	Human presence–paths
		−0.07	0.44	Roads–paths
Cemetery	91	−0.12	0.26	Human presence–roads
		−0.16	0.13	Human presence–paths
		−0.37	0.0002***	Roads–paths

*Significance levels: *p < 0.05, ***p < 0.001.*

of individual breeding events (Corsini et al., in preparation). Human preferences in terms of space use were particularly marked in the urban park, which was also the site with the highest number of visitors (**Figures 1A,C**). Indeed, the pattern of space use within this area was associated with the presence of recreational facilities, mostly located in the southern part of the study site. The GIS-based approach, in contrast, was a considerably less-time consuming method compared to the protocol for detecting human presence on the ground (involving only one observer and 2 days of measurements), and proved to be highly repeatable ($r \geq 0.991$). Importantly, the easily-computed GIS-based approach for measuring nestbox-level distances to paths and roads provided an alternative approach to capture human presence on the ground, and was found to have a much greater influence on tit life-history trait variation than human presence *per se*: while no direct effect of human presence was detected in great and blue tit life-history, incubation time in blue tits and great tits, as well as blue tit offspring mass 2 weeks after hatching were associated with distance to infrastructural facilities such as paths and roads.

Policy regulations of a given area are likely to play an important role in shaping the dynamics of people flow within sites. While in both sites visitors can roam freely, paths remain the major communication channels throughout both study sites. However, human presence correlated with distance to paths only in the urban park, which may be caused by a larger detection power of such associations due to a larger number of visitors overall. Our results are thus in line with the findings of Remacha et al. (2016), who reported that distance to paths is a reliable index of human disturbance. In the case of roads, there is no evidence that this infrastructure is correlated with distribution of human presence detected on the ground, thereby suggesting that the presence of such infrastructural facilities at the site level is not always an important source of human presence, although it can impact reproduction through independent means, as explained below.

In this study, variation in blue tit or great tit life-history traits and reproductive success was not explained by human presence estimated using the ground-based protocol, suggesting that human presence does not affect birds' survival or life-history traits in the long-term. In contrast with our findings, Remacha et al. (2016) showed that blue tit nestlings from forested areas used as a recreational site that hatched on days with increased activity of visitors (weekends and other non-working days) were found to grow slower and fledge with lower body mass; this difference may be due to differences in birds' environmental backgrounds: in a more semi-natural context, where human presence is higher during the breeding season, it is possible that birds are more sensitive to human disturbance. Birds inhabiting rural realities already showed a higher physiological and behavioural stress response than their urban counterparts, often revealed through a higher flight-initiation distance (McGiffin et al., 2013; Abolins-Abols et al., 2016). On the other hand, birds inhabiting urban parks are almost continuously exposed to humans—so despite the fact that each nestbox can be consistently exposed to high or low human presence, birds may become habituated through their daily experience of humans while foraging. Studies of the effect of human presence in other bird species reveal mixed results. Similarly to our findings, in a wild population of black grouse located in a recreational area, the presence of visitors did not affect productivity in terms of clutch size, hatching success and breeding success (Baines and Richardson, 2007). In the woodlark, on the other hand, productivity was related to the level of human disturbance, although in the opposite direction than expected, with birds breeding in more disturbed areas raising more fledglings (Mallord et al., 2007). Human presence was also found to extend the duration of incubation period in the dotterel and the European oystercatcher (Lord et al., 2001; Verhulst et al., 2001), while recreational activities had a negative-marginal effect on incubation behaviour in American oystercatchers (McGowan and Simons, 2006).

These results suggest that behavioural responses are usually context-dependent and related to the trade-offs experienced by individuals: indeed, it is easier to expect habituation in birds that are used to human presence, which is a common scenario in an urban park, while birds in rural environments may react to human presence adversely (Remacha et al., 2016). Furthermore, the impact of human disturbance is likely to change among avian species or species with variable ecological niches: ground and shrub nesting birds, for example, are likely to be more sensitive to human disturbance. The same pattern was reported for ground-foraging species (Rodgers and Smith, 1995; Gill, 2007). In addition, while there is an often-held assumption that human presence triggers changes in behaviour [like flush responses, reduction in incubation duration or changes in foraging behaviour (Lord et al., 2001; Verhulst et al., 2001; Thiel et al., 2007)], which in turn may be translated into reduced fitness, those fitness consequences are in fact rarely tested (Gill et al., 2001). Here, we did not find any evidence that human presence affects the life-history or reproductive success of birds in an urban context, and suggest that structural properties of the environment, such as paths and roads, overrides the weak or non-existent effect of human presence in cities.

In contrast to a lack of effect caused by human presence, nestbox distance to paths and roads influenced incubation duration and chick mass on day 15 after hatching. A larger dataset (in terms of study sites and years of study) is likely to be required to detect finer scale effects of the effect of human

TABLE 5 | Effects of human presence, paths and roads on blue tit and great tit reproductive traits.

	Blue tits				**Great tits**			
Occupancy	**Estimate**	***SE***	***Z*-value**	***p***	**Estimate**	***SE***	***Z*-value**	***p***
Intercept	−2.859	0.735	−3.89	< 0.001	−1.111	0.673	−1.644	0.103
Human presence	0.084	0.189	0.443	0.658	−0.199	0.290	−0.687	0.494
Distance from path	0.003	0.011	0.244	0.807	−0.008	0.016	−0.490	0.625
Distance from road	0.002	0.002	0.810	0.418	−0.002	0.004	−0.563	0.575
Site	1.913	0.617	3.101	0.002**	–	–	–	–
	$n = 196$				$n = 105$			
Egg laying date	**Estimate**	***SE***	***t*-value**	***p***	**Estimate**	***SE***	***t*-value**	***p***
Intercept	14.778	2.432	6.077	< 0.001	15.466	5.405	2.861	0.0187
Human presence	−0.295	0.657	−0.449	0.656	3.064	2.324	1.318	0.220
Distance from path	−0.012	0.034	−0.351	0.727	0.224	0.150	1.493	0.170
Distance from road	0.011	0.007	1.607	0.116	−0.041	0.027	−1.540	0.158
Site	−0.457	2.207	−0.207	0.837	–	–	–	–
	$n = 44$				$n = 13$			
Clutch size	**Estimate**	***SE***	***t*-value**	***p***	**Estimate**	***SE***	***t*-value**	***p***
Intercept	10.145	0.991	10.237	< 0.001	9.514	2.465	3.862	<0.001
Egg laying date	0.094	0.259	0.361	0.720	0.086	0.869	0.099	0.923
Human presence	0.023	0.265	0.088	0.931	−1.010	1.137	−0.887	0.401
Distance from path	0.009	0.014	0.672	0.505	−0.093	0.075	−1.239	0.250
Distance from road	−0.001	0.003	−0.223	0.825	0.005	0.014	0.361	0.728
Site	−0.726	0.898	−0.809	0.424	–	–	–	–
	$n = 44$				$n = 13$			
Incubation duration	**Estimate**	***SE***	***t*-value**	***p***	**Estimate**	***SE***	***t*-value**	***p***
Intercept	17.180	1.339	12.834	< 0.001	16.905	1.591	10.623	< 0.001
Egg laying date	−1.796	0.365	−4.913	< 0.001***	−0.062	0.550	−0.113	0.913
Human presence	−0.190	0.411	−0.463	0.646	−1.596	0.729	−2.191	0.065
Distance from path	−0.048	0.018	−2.615	0.013*	−0.061	0.049	−1.234	0.257
Distance from road	0.002	0.004	0.462	0.647	0.031	0.009	3.519	0.010**
Site	1.580	1.235	1.279	0.209	–	–	–	–
	$n = 42$				$n = 12$			
Chick mass Day 15	**Estimate**	***SE***	***F*-value**	***p***	**Estimate**	***SE***	***F*-value**	***p***
Intercept	7.363	0.878	1, 356.932	< 0.001	6.503	4.361	158.664	<0.001
Egg laying date	−0.066	0.266	0.062	0.806	−1.602	2.043	0.615	0.490
Human presence	0.250	0.325	0.771	0.388	2.365	1.693	0.879	0.418
Distance from path	−0.001	0.012	0.006	0.940	0.029	0.108	0.023	0.890
Distance from road	0.006	0.003	5.406	0.029*	0.035	0.026	1.342	0.331
Site	0.398	0.815	0.062	0.806	–	–	–	–
	$n = 171$ (31 groups)				$n = 39$ (8 groups)			

Nest occupancy was fitted as a quasi-binomial response variable, while egg laying date, clutch size and incubation duration were modelled with a Gaussian error distribution. Chick mass was modelled with a linear mixed model and nest ID as random effect. In blue tits, the cemetery was used as reference level to test differences between sites. Significance levels: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

presence or distance to paths and roads on tit reproductive trait variation. Roads have already been shown to have a strong impact on fitness in birds (e.g., Halfwerk et al., 2011; Dietz et al., 2013; but see review by Kociolek et al., 2011). Their negative effect may arise through noise pollution leading to disruption in communication, for example in the context of mate attraction, predator avoidance or parent-offspring communication (Slabbekoorn and Ripmeester, 2008; Francis, 2015). Thus, communication breakdown caused by noise may at least partly be responsible for lower body mass in nestlings located closer to roads. A more direct mechanism associating proximity to roads with lower offspring body mass is the fact that a greater proportion of impervious surface close to the nestbox automatically results in lower levels of biomass and resulting food availability for our focal species (but see the opposite effect in Florida scrub jays Morgan et al., 2010). Moreover, roads are associated with many biotic and abiotic changes, for example: increased mortality in birds due to direct collisions with vehicles, but also the general alteration of physical and chemical properties in these novel environments (Reijnen et al., 1995; Erritzoe et al., 2003; but see review by Trombulak and Frissell, 2000).

The fact that blue tits significantly increased their incubation time closer to paths (while great tits show an equivalent yet non-significant trend in the same direction) independently of human presence is surprising and suggests that paths act as a disruptor to incubation that may be independent of human presence. Indeed, the correlation between human presence and distance from paths was significant but weak ($r = -0.29$, $p = 0.002$, **Table 4**), suggesting that due to the frequency of off-trail events, the presence of visitors in the urban park is not strictly connected with infrastructural networks. However, previous studies reported that habituation to walkers is common in several species, especially when people remain on marked paths: however, this level of habituation may change during the breeding season, whenever off-trail events occur (Nisbet, 2000). In addition, it is possible that the mere presence of humans in proximity of a nest can induce increased alertness in both adults affecting parental care (while feeding the nestlings or, as in this case, during incubation). In contrast, incubation duration was significantly shorter in great tits breeding in proximity to paved roads, which also coincides with *c.* 0.5°C increase every 100 m closer to the largest road neighbouring the urban park (daily averages over 4 months starting on the 25th of March; Corsini, unpublished data).

In conclusion, we confirm that the protocol for quantifying human presence developed in this study proved to be highly repeatable in a site with high intra-site variance of human presence, thereby confirming its usefulness to other studies testing the effects of human presence around fixed point locations in avian and non-avian species alike. At the same time, our results suggest that urban great tits and blue tits are habituated to human presence, a keystone species in the urban environment, and generally perceive humans as harmless stimuli (Lowry et al., 2013). While the process of habituation is known to reduce stress responses in avian species (Walker et al., 2006), fitness consequences due to a long-term exposure to human generated stressors may be complex, especially in an urban context where several environmental variables other than human presence interact. Finally, in the context of urban study sites, it is mostly infrastructural networks rather than human presence *per se* that played a greater role in tit reproductive trait variation.

AUTHOR CONTRIBUTIONS

MS designed the study; MC, AD, and MS collected the data; MC, PM, and MS performed the analyses, MC prepared a first draft of the manuscript; MC, AD, PM, and MS edited and approved the final version of the manuscript.

FUNDING

This study was financed with a Sonata Bis grant 2014/14/E/NZ8/00386 from the National Science Centre, Poland.

ACKNOWLEDGMENTS

We thank Tomasz Mazgajski, Jerzy Bańbura and Anne Charmantier for constructive discussions on urban ecology. We also thank Karol Kobiałka, Marta Celej, Justyna Kubacka and Fatima Hayatli for help in the field. We are grateful to ground managers for enabling research on their sites.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00082/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 16 May 2017
doi: 10.3389/fevo.2017.00047

Does Seasonal Decline in Breeding Performance Differ for an African Raptor across an Urbanization Gradient?

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Specialty section:
This article was submitted to Urban Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *31 January 2017*
Accepted: *28 April 2017*
Published: *16 May 2017*

Citation:
Rose S, Sumasgutner P, Koeslag A and Amar A (2017) Does Seasonal Decline in Breeding Performance Differ for an African Raptor across an Urbanization Gradient?. Front. Ecol. Evol. 5:47. doi: 10.3389/fevo.2017.00047

As human populations have increased globally, so too has the transformation of natural landscapes into more urban areas. Within Africa, population growth rates and urbanization rates are amongst the highest in the world, but the impacts of these processes on Africa's wildlife are largely un-explored. In this study, we focus on a recently established population of black sparrowhawks *Accipiter melanoleucus* in Cape Town, South Africa. Using long-term data (16 years), we investigate the relationship between the timing of breeding and breeding performance along an urban-rural habitat gradient. We found no evidence for a direct effect of urbanization on either timing of breeding or breeding performance. However, we did find some evidence for a differential seasonal decline in breeding performance dependent on the levels of urbanization. Thus, higher productivity was found in more urbanized habitats earlier in the season, but towards the end of the breeding season, birds in less urbanized habitats performed better. Our study represents one of the first to examine avian productivity in relation to urbanization in Africa, and also represents one of the first studies to suggest that seasonal declines in productivity may differ according to levels of urbanization. These results have considerable implications for potential changes in phenology or productivity for the regions' avifauna as African urbanization continues.

Keywords: urban ecology, timing of breeding, reproduction, South Africa, *Accipiter melanoleucus,* raptors

INTRODUCTION

Urbanization is a rapid and global process in which natural environments are transformed into new urban systems (Berry et al., 1998) which are characterized by an increase of unproductive sealed surfaces in highly disturbed fragmented landscapes (McDonnell and Pickett, 1990). By 2050, 66% of the global population is predicted to live in urban areas (United Nations, 2015). Urbanization significantly alters the physical, chemical, and biotic environment (McDonnell and Pickett, 1990). The resulting decrease of natural habitat forces ecosystems and species to adapt, avoid or tolerate these new conditions (Blair, 1996; Isaksson, 2015).

Key processes that contribute to the impact of urbanization on birds include vegetation changes, habitat fragmentation, introduction of exotic species, changes in food abundance and quality, and novel predator assemblages (Chace and Walsh, 2006). An urban gradient characterizes different levels of urban stressors, such as pollution (light, noise, and air), the abundance of novel predators like cats and corvids (e.g., Stracey, 2011), and of anthropogenic food resources that may be of lower quality ("urban junk food," Pierotti and Annett, 2001; Schoech and Bowman, 2001). All of these factors can potentially affect demographic parameters of city-dwelling species (Isaksson, 2015). In order to anticipate the costs and benefits for birds living in urban areas, it is useful to quantify the relative breeding performance within urbanized areas compared to more natural habitats.

There is a growing body of literature comparing breeding performance of birds living in urban and rural habitats, with some general patterns emerging. The onset of breeding tends to be earlier in urban areas as a potential result of increased food availability (Chamberlain et al., 2009), the urban heat island effect (i.e., higher temperatures in the city center, Shochat et al., 2006; since the beginning of incubation can be temperature dependent, Visser et al., 2009) and light pollution (Spoelstra and Visser, 2014). Clutch sizes tend to be smaller and nestling conditions tend to be poorer in urban vs. natural habitats (reviewed by Chamberlain et al., 2009). However, these studies have mostly been conducted in the Northern Hemisphere with very different climatic conditions. Despite an increased focus on urban avian ecology, as far as we know no studies have yet focused on these questions for any bird species in Africa.

Many bird species show seasonal declines in breeding performance during the course of a breeding season (e.g., Both et al., 2004; Grüebler and Naef-Daenzer, 2010), and this has also been found for some urban nesting species (Chamberlain et al., 2009), including urban raptors (e.g., Sumasgutner et al., 2014a). The explanation for these patterns often focuses on declines in food availability that might also influence sibling competition (Gil et al., 2008), which may in theory vary across levels of urbanization. If so, it might be expected that the patterns of such seasonal declines might vary across an urban gradient. In the only study to explore this issue so far, Wawrzyniak et al. (2015) found seasonal declines in clutch size of blue tits (*Parus major*) nesting in urban habitats but not for those nesting in forests.

Because raptors belong to the upper trophic level, they can be particularly susceptible to changes in the environment (Newton, 1998). Despite this, some raptors are increasingly associated with urbanized areas (e.g., *Falco peregrinus*, Cade and Bird, 1990; *F. columbarius*, Sodhi et al., 1992; *F. tinnunculus,* Village, 1990; Charter et al., 2007; *Accipiter cooperii,* Boal and Mannan, 1999, 2000; *A. gentilis*, Rutz, 2008). This is potentially due to their relatively high mobility that could allow them to escape from some of the limitations of urban areas (Chace and Walsh, 2006), while still benefitting from the advantages, such as lower direct persecution (Rutz, 2008) and novel resources like higher abundance of avian prey or nesting sites (e.g., Palomino and Carrascal, 2007; Cavalli et al., 2014; Rullman and Marzluff, 2014; Sumasgutner et al., 2014b). However, some rodent specialists such as Barn owls *Tyto alba* or Eurasian kestrels *F. tinnunculus* show decreased fledgling success with increasing urbanization, probably due to decreased prey availability connected to sensory interference due to noise pollution for owls (Senzaki et al., 2016), or nocturnal rodents being harder to locate for a diurnal raptor in an urban environment (Hindmarch et al., 2014; Sumasgutner et al., 2014a). Dietary requirements may be an important factor in determining the success of an urban raptor, specifically the degree of specialization in their diet choice. Highly specialized raptors may have to spend more energy and time seeking preferred prey, whereas generalists may have more flexibility in coping with fluctuating food availability (see Terraube et al., 2011 for a natural setting).

In this study we focus on the black sparrowhawk *Accipiter melanoleucus* in South Africa, a raptor that colonized the city of Cape Town in the last two decades after expanding its distribution range from the North-East into the South-West of Southern Africa (Amar et al., 2014). Black sparrowhawks appear to have adapted well to urban areas, with more than 50 breeding pairs (Martin et al., 2014b) within our study area. Together with the colonization of the urban habitat, the species extended its breeding season toward earlier egg-laying, resulting in a breeding season of 9 months (Mar-Nov; Martin et al., 2014b). Potentially contributing to the success of the species in this area, the health of black sparrowhawks' chicks does not appear to be negatively influenced by levels of urbanization (Suri et al., 2017, but see van Velden et al., 2017 for a specific *Knemidokoptes* mite infection in adults). This might be linked to the high abundance of suitable prey species across all habitat types that may buffer any potential negative health effects of urban stressors (Suri et al., 2017). Building on these results, our study aims to explore how the breeding performance of urban black sparrowhawks varies with urbanization in Cape Town. Using a long-term study (16 years), we investigated the timing of breeding and breeding performance along an urban-rural habitat gradient. Additionally, we specifically explore whether the established relationship between timing of breeding and breeding performance (Martin et al., 2014b) differed depending on levels of urbanization. This question is particularly relevant to our study area, since it is unique within a South African context in having an extended 9 months long breeding season (Martin et al., 2014b). Given the results of several other studies exploring timing of breeding, we predicted the onset of breeding to be earlier in more urbanized areas. The expected relationship between urbanization and breeding performance is harder to predict, however, given that Suri et al. (2017) tended to find higher abundance of doves and pigeons in more urbanized habitats, we might expect higher productivity in more urban areas. Likewise the precise relationship between timing of breeding and breeding performance in relation to urbanization was difficult to predict, but if urbanized areas do have higher or more regular prey availability, the established seasonal declines in breeding performance may be somewhat buffered within more urbanized areas.

METHODS

Study Site and Species

This study focused on the resident population of black sparrowhawks in Cape Town, Western Cape, South Africa (**Figure 1**). The study area covers various habitats over 595 km^2 (Martin et al., 2014b), including heavily urbanized areas, plantations of exotic tree stands, patches of Afromontane forest and indigenous fynbos vegetation. Cape Town experiences a

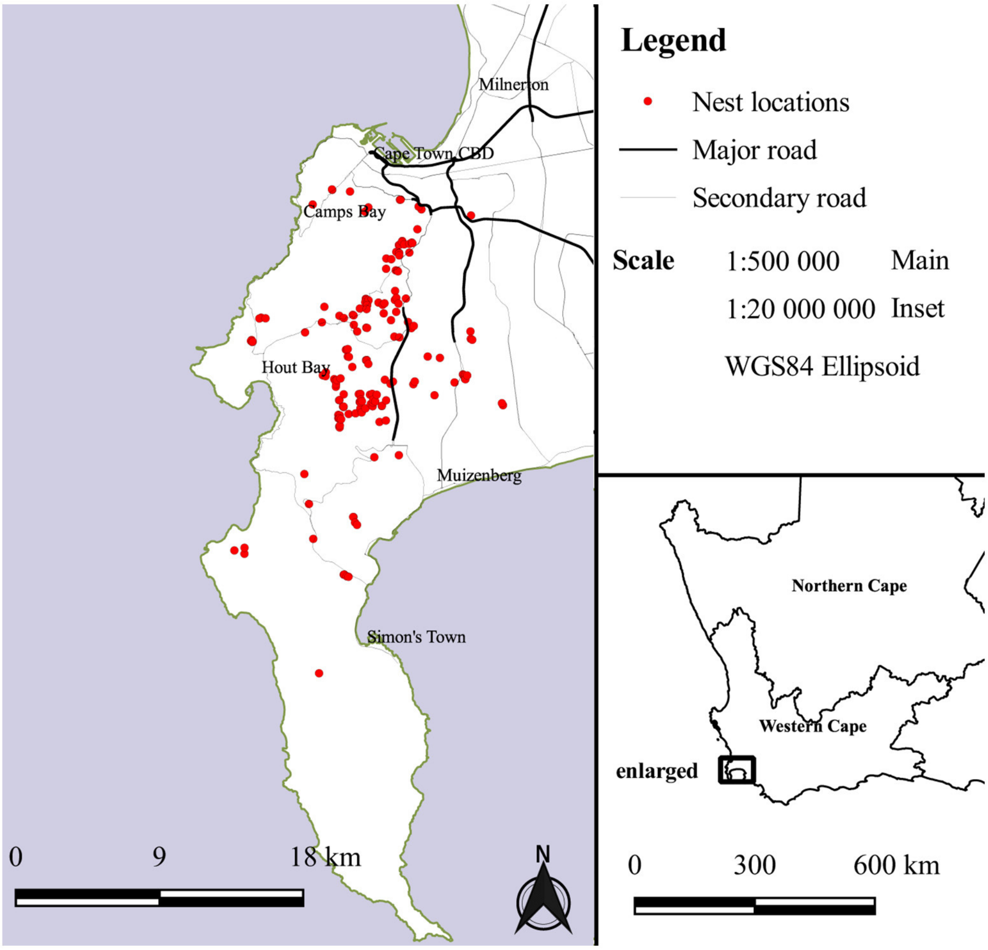

FIGURE 1 | Distribution of black sparrowhawk nest localities on the Cape Peninsula over a 16-year monitoring period. Insert situates the Cape Peninsula in the South African context.

Mediterranean climate, with an average annual rainfall of 788 mm and average monthly temperatures ranging from 15 to 30°C. This population breeds mainly during the winter months, which coincides with the rainy season (Amar et al., 2014; Martin et al., 2014b). The population has been actively monitored since 2000 (Amar et al., 2014).

Systematic monitoring by a team of experienced researchers occurred during the breeding season from March to November. Territories were located by surveying suitable stands of trees. Occupancy was confirmed by activity indicators such as calling, whitewash, prey remains or nest structures. Following confirmation of breeding attempts, pairs are then monitored approximately weekly. About 90% of the population is individually color ringed, allowing individual identification of many breeding pairs (see Martin et al., 2014a for further details). Adults were trapped on territories using a bal-chatri trap baited with a live white "racing" pigeon, *Columba livia* (Berger and Mueller, 1959; see Ethical Note in Martin et al., 2014a for details). Chicks were ringed when they were 3.5–4.5 weeks old. Age of chicks was estimated visually by the extent of wing and tail feather growth (using reference photographs from nest cameras). This study uses data collected between 2000 and 2015.

Defining the Urban Gradient

Quantification of the urban gradient was based on a circular buffer with a radius of 2,000 meters (m) assigned to each nest; the chosen scale was based on previous work in which adult males with global positioning system (GPS) tags were tracked and their home range sizes during the breeding season where determined (Sumasgutner et al., 2016b). Urban cover was calculated based on land cover classes from the 2013–2014 South African National land-cover dataset produced by GEOTERRA (Department of Environmental Affairs, 2015). This land cover dataset compiled 72 land types at a 30 m resolution, from which sealed, unproductive areas of land were classified as "urban." Percentage urban cover was derived for each nest buffer using the packages *raster* (Hijmans, 2015), *sp* (Pebesma and Bivand, 2005; Bivand et al., 2013), and *rgdal* (Bivand et al., 2016). **Figure 2** illustrates the binary urban/non-urban land covers as compared with aerial photographs.

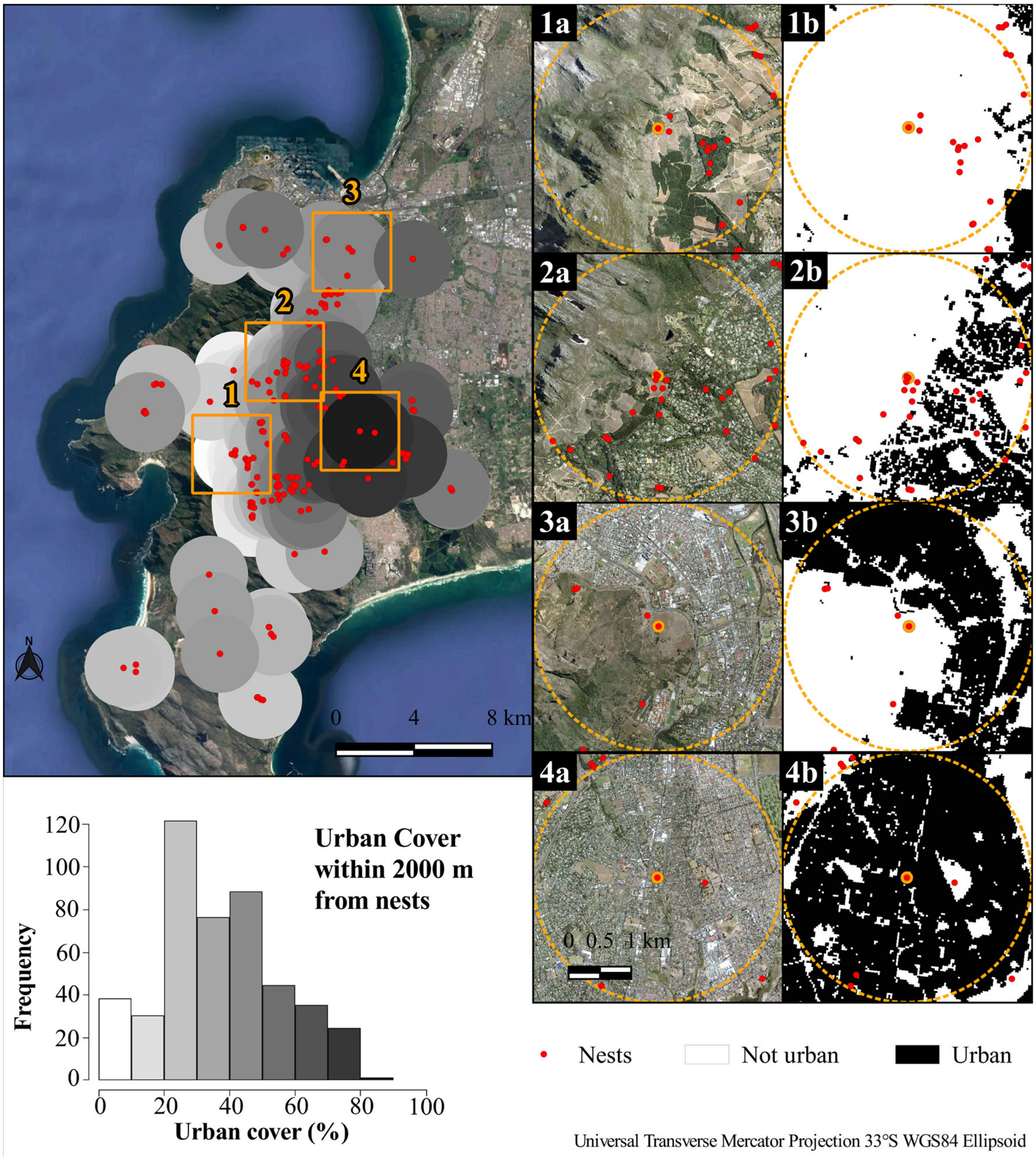

FIGURE 2 | Percentage urbanization for each black sparrowhawk nest on the Cape Peninsula over a 16-year monitoring period (top left), frequency plot of the distribution of nests along an urban gradient (bottom left), illustrations of representative individual nest locations with aerial imagery (center column) and land cover reduced to urban/non-urban binary (right column). Urban cover is 1.4, 12.7, 47.1, and 87.0%, respectively, for nests 1, 2, 3, and 4. Data from Department of Department of Environmental Affairs (2015); Google Earth Pro (2016), and City of Cape Town (2016).

Statistical Analysis

The "lay month" (the only accurate measurement of the timing of breeding possible) for successful nests is backdated from the age of the chicks; we used the incubation behavior (female sitting low on nest) to time the onset of breeding for unsuccessful nests. "Breeding success" is a binomial variable distinguishing between successful and unsuccessful nests, "brood size" only includes successful nests (1-3 chicks that fledged) and "productivity" all active nests where 0–3 chicks fledged. All analyses were performed in relation to urbanization using Linear Mixed Models (LMMs) for the timing of breeding and Generalized Linear Mixed Models (GLMMs) for breeding performance, in R studio (R Core Team, 2016) with the packages *nlme* (Pinheiro et al., 2014), *lme4* (Bates et al., 2015), *MASS* (Venables and Ripley, 2002), *lattice* (Deepayan, 2008), *ggplots2* (Wickham, 2009) *effects* (Fox, 2003), and *car* (Fox and Weisberg, 2011). All quantitative variables were scaled in order to bring the variables to comparable dimensions to interpret effect sizes for interaction terms. We tested the dispersion of our response variables using the "qcc.overdispersion.test" in the *qcc* package (Scrucca, 2004). Residual distributions of the models were inspected visually to assess model fit.

Unless otherwise stated (when using successful nests only), the sample size in all following analyses was 456 broods surveyed over 16 years starting in 2000. Year, territory ID, and nest ID were included as random terms to account for pseudoreplication arising from same nests sampled repeatedly within and between years (nest ID), and breeding pairs occupying territories over several years (territory ID), and multiple measures from different nests within the same year (year). All analyses were conducted at the nest level since some pairs build multiple nests in different locations within and between seasons, resulting in varying urban scores for some individuals. The lay month (Gaussian distribution) was modeled as the response variable with the urban gradient as the explanatory variable. For the other analyses examining breeding performance, we accounted for the timing of breeding (using lay month) as a quadratic term and explored the interaction of timing of breeding with the urban gradient. We included the lay month as linear and quadratic term to investigate a potential polynomial relationship. Error structures for the GLMMs were: negative binomial (productivity), Poisson (brood size) and binomial (breeding success). Least squares means (*post-hoc* contrasts for mixed-models, Lenth and Hervé, 2015) are not yet implemented for interaction terms between continuous variables. Thus, to understand the nature of the relationship between the timing of breeding and levels of urbanization better, we performed additional tests whereby we selected the earlier 25% of nests (egg-laying Mar-May, $n = 123$) to see if there is a significant difference in the breeding performance in relation to the urban gradient. Then we repeated this analysis for the latest 25% of nests (egg-laying Sep-Nov, $n = 120$); see Tate et al. (2016) for a similar approach. Throughout, reported χ^2 and p-values are based on an ANOVA Table of Deviance using Type II Wald χ^2 tests for mixed models.

Ethical Note

The University of Cape Town's Science Faculty Animal Ethics Committee has approved all the procedures required for the long-term study of the black sparrowhawk population in Cape Town (permit number: 2012/V37/AA). Furthermore, all procedures were carried out under a Cape Nature Hunting Permit (No. 0035-AAA004-00428), a South African National Parks Permit and a SAFRING ringing permit (no. 1439).

RESULTS

Urban Gradient

The urban gradient around each nest ranged from 87% to 1.4%. See **Figure 2** for representations of (1) 1.4%, (2) 12.7%, (3) 47.1%, and (4) 87.0% urban cover, and the frequency of different urbanization scores for black sparrowhawk nests over the 16-year monitoring period.

Timing of Breeding and Breeding Performance along the Urban Gradient

Timing of breeding did not differ in relation to the urban gradient (**Table 1**, **Figure 3**), nor was there a direct relationship between breeding performance and the urbanization gradient term. However, an interesting interaction was noted between timing of breeding and urbanization in relation to breeding performance (**Table 1**, **Figures 4A,C**). Firstly, for pairs with nests in the most urbanized areas (**Figure 4A**, blue dotted line), we found that productivity declined strongly as the season progressed, whereas this decline was less steep for pairs in less urban areas (**Figure 4A**, black solid line), indicating that the timing of breeding had greatest influence on productivity in more urbanized areas. This interaction term shifts after approximately one third of the breeding season (June/July). A very similar relationship was also seen for breeding success (**Figure 4C**). However, the interaction term was statistically not significant, but suggested a tendency for differential seasonal declines in relation to urbanization (productivity GLMM: $\chi^2 = 3.45$, $P = 0.063$; breeding success GLMM: $\chi^2 = 3.20$, $P = 0.074$). This apparent relationship was not observed for brood size (**Figure 4B**, successful nests only, 1–3 chicks) where we found slightly more chicks fledging in urban areas throughout the season and less chicks fledging in more rural areas. Thus, our results suggest that the interaction between timing of breeding and urban gradient was due to nest failures, rather than the number of fledglings produced in successful nests. When splitting the data for early nests vs. late nests these observations were confirmed: we found slightly higher productivity in early nests in more urbanized areas ($\chi^2 = 3.41$, $P = 0.065$), but no such difference in late nests ($\chi^2 = 2.20$, $P = 0.138$). Additionally, we found significantly higher probability of nest failure in more urbanized areas later in the season ($\chi^2 = 5.28$, $P = 0.022$; vs. breeding success in early nests: $\chi^2 = 2.74$, $P = 0.098$).

DISCUSSION

The most fundamental result from our analysis was that the extent of urbanization surrounding nest sites did not seem to have a strong influence on breeding performance. Thus, despite many additional stressors that may come from breeding within a more urbanized environment (Isaksson, 2015), black sparrowhawks appear able to cope with these challenges and produced similar number of chicks irrespective of the levels of urbanization around their nest sites. This result therefore adds to the findings of Suri et al. (2017), which explored influences of urbanization on chick health for this same black sparrowhawk population, and found no negative effect on a range of health parameters. Suri et al. (2017) suggested that the main prey species (doves and pigeons, *Streptopelia semitorquata*, and *Columba livia*) were plentiful across all habitats, and this abundance of prey may explain why there were no negative impacts of urbanization either on chick health and ultimately on the population's productivity. Thus, from these two studies it does seem that the species is able to cope well with the urban environment within the Cape Town region.

TABLE 1 | LMMs (for lay month) and GLMMs investigating the relationship between breeding parameters (productivity, brood size, and breeding success) and the urban gradient.

Model	Error structure	*N*	*Df*	Estimate	*SE*	χ^2	*P*	Sign
Lay month	**Gaussian**	**456**						
Urban gradient				−0.097	0.070	1.89	0.168	
Intercept				0.003	0.077		< 0.001	***
Productivity	**Negative binomial**	**456**	**446**					
Urban gradient				−0.024	0.064	0.123	0.726	
Lay month				−0.211	0.050	16.024	< 0.001	***
Lay monthˆ2				−0.095	0.048	3.986	0.046	*
Urban gradient:lay month				−0.088	0.047	3.449	0.063	•
Urban gradient:lay monthˆ2				−0.003	0.046	0.005	0.945	
Intercept				0.300	0.068		< 0.001	***
Brood size	**Poisson**	**323**	**314**					
Urban gradient				0.014	0.062	0.339	0.560	
Lay month				−0.022	0.044	0.240	0.625	
Lay monthˆ2				−0.002	0.056	0.002	0.968	
Urban gradient:lay month				−0.007	0.041	0.031	0.860	
Urban gradient:lay monthˆ2				0.009	0.041	0.052	0.820	
Intercept				0.609	0.061		< 0.001	***
Breeding success	**Binomial**	**456**	**447**					
Urban gradient				−0.223	0.214	1.367	0.242	
Lay month				−0.671	0.149	18.354	< 0.001	***
Lay monthˆ2				−0.210	0.142	2.290	0.130	
Urban gradient:lay month				−0.251	0.140	3.200	0.074	•
Urban gradient:lay monthˆ2				0.081	0.140	0.335	0.563	
Intercept				1.344	0.232		< 0.001	***

*Note that the interaction term between the urban gradient and the lay month accounted for a linear and quadratic relationship and that all quantitative variables were scaled. ":" indicating an interaction term, "ˆ2" indicating a quadratic term, significance codes: "***" < 0.001, "*" < 0.05, "•" < 0.1.*

Contrary to our expectations, there was no direct relationship between the percentage of urban cover and timing of breeding. Many urban species are known to start breeding earlier compared to their rural counterparts (see reviews by Chamberlain et al., 2009), but these studies were located in the Northern hemisphere where the urban heat island effect might be beneficial (but see advanced incubation in Australian magpies *Gymnorhina tibicen,* Rollinson and Jones, 2002; and earlier first nest attempts for White-winged chough *Corcorax melanorhamhos,* Beck and Heinsohn, 2006). However, for black sparrowhawks in South Africa breeding coincides with the colder periods of the year, and within Cape Town with the rainy season (Martin et al., 2014a).

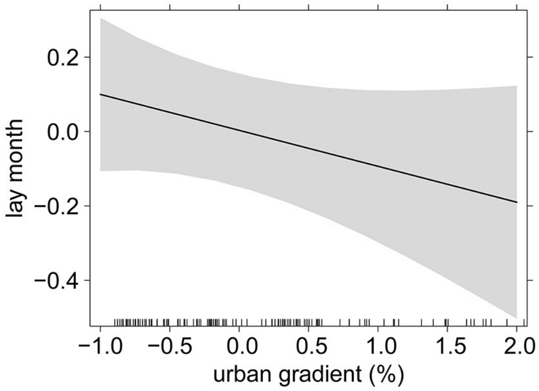

FIGURE 3 | The relationship between the timing of breeding (lay month, scaled) and the urban gradient (% of urban cover, scaled), based on predicted values of LMM, 95% CIs in shaded gray; black bars on the x-axis represent sample size. Model details in **Table 1** ($\chi^2 = 1.89$, $P = 0.17$).

One of the most interesting findings from this study was the apparent difference in the degree of seasonal decline in breeding performance depending on the surrounding levels of urbanization. Declining productivity as breeding seasons progress is a well-known phenomenon, that has been documented in many bird species mostly in the Northern hemisphere (e.g., Both et al., 2004; Grüebler and Naef-Daenzer, 2010), but increasingly also in the Southern hemisphere (e.g., Garcia-Heras et al., 2016; Murgatroyd et al., 2016), including previously in our study system (Martin et al., 2014b). In addition to this pattern, however, we also found a convincing indication that the level of this decline differed depending on the extent of urbanization surrounding the nests. Thus, it

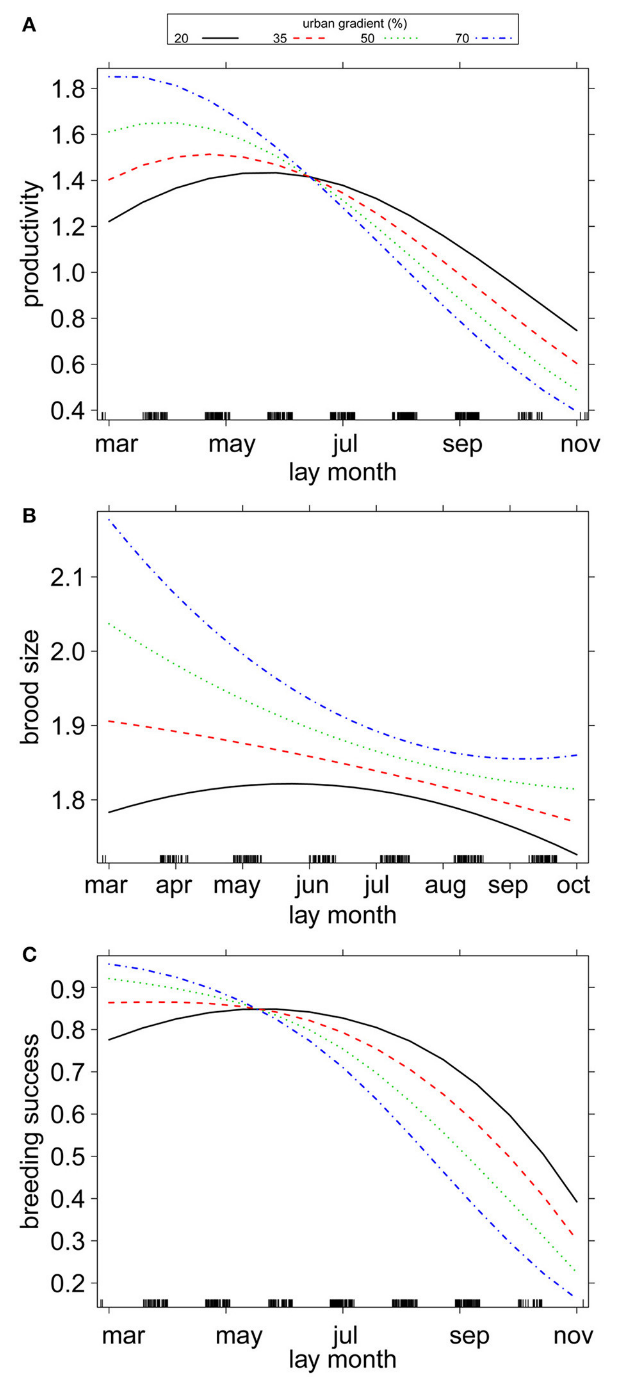

FIGURE 4 | Plots showing the relationship between the urban gradient (% urban cover) and (A) productivity ($\chi^2 = 3.45$, $P = 0.06$); **(B)** brood size ($\chi^2 = 0.03$, $P = 0.86$); and **(C)** breeding success ($\chi^2 = 3.20$, $P = 0.07$), based on predicted values of GLMMs, black bars on the x-axis represent sample size. Note that all quantitative variables were scaled. Model details in **Table 1**.

appears that earlier on in the season productivity (the number of chicks produced) is highest for pairs breeding in more urban habitats, but later on this advantage disappears. In fact, when focussing on breeding success, we found significantly higher probability of nest failure in more urbanized areas later in the season.

Several other recent studies have documented differences in seasonal declines in breeding parameters between habitats, but usually on a larger geographical scale. For example, a similar result was noted for another South African raptor, the black harrier *Circus maurus*. Although the nests in that study were located in the different habitats, they were also often separated by large distances. The differences in seasonal declines in clutch size and to a lesser degree productivity that were found in that study were thought to be driven by differences in weather conditions between the areas (Garcia-Heras et al., 2016). Likewise Zárybnická et al. (2015) found different levels of seasonal declines between Tengmalm's owl *Aegolius funereus* breeding in either boreal or temperate forests. Here the differences were attributed to varying prey availability (small mammals) over the season influencing nestling mortality and failure rates. The only other study, as far as we know, to explore this issue in relation to urbanization, is the study by Wawrzyniak et al. (2015) on blue tits (*Parus major*). This study found that clutch size tended to decline during the breeding season in an urban setting but not within forest habitat. They also attributed this result largely to better prey availability within the forest habitat. Thus, in these other studies changes in either weather or prey were thought to be the drivers for differences in seasonal declines.

What might then be responsible for the relationship witnessed in our current study? It could be that food is more abundant in urban habitat earlier in the season. This could be the case if timing of breeding of avian prey species in urban areas follows the more usually trend seen for birds (Chamberlain et al., 2009). Thus prey may be more available, especially inexperienced juvenile prey, earlier on in the season, which may account for the higher productivity of earlier breeding black sparrowhawks within urban habitats compared to their rural conspecifics. Later on in the season, the situation may be reversed due to the later breeding of prey species in the more rural habitats. Unfortunately we have no information on the timing of breeding on any of the key prey species in the area in relation to urbanization nor are we aware of a seasonal variation in prey abundance (Suri et al., 2017). Additionally, we found that differences in productivity were principally driven by nest failures rather than differences in brood sizes of successful nests. Thus, the mechanism for the differential seasonal decline may not be food related, since prey abundance would be expected to impact brood sizes rather than to induce complete nest failures. Alternatively, we know from other studies that one of the main causes of nest failure in this black sparrowhawks is nest usurpation by Egyptian geese (*Alopochen aegyptiaca*, Curtis et al., 2007) and that this increases later in the breeding season (Sumasgutner et al., 2016a). Thus it may be that usurpation in more prevalent in more urbanized areas, since both species have recently expanded their South African range into the urban and suburban habitats of Cape Town (see Mangnall and Crowe, 2002 on Egyptian geese). This might further explain the significantly lower breeding success in more urbanized habitats later in the season, since the breeding season of Egyptian geese peaks later in the year (MacLean, 1997; Carboneras and Kirwan, 2016); when also the threat of nest usurpation increases (Sumasgutner et al., 2016a).

CONCLUSION

In this study we found no evidence that black sparrowhawks suffer from a decrease in breeding performance as a result of living in an urban environment, but timing of breeding was important, specifically in more urbanized areas. To summarize, black sparrowhawks appear to have successfully navigated various steps outlined by Møller (2014) of becoming an urban raptor species, which was likely facilitated by a reliable food supply (Suri et al., 2017) and abundant nesting opportunities in the form of alien tree species (Malan and Robinson, 1999).

AUTHOR CONTRIBUTIONS

SR and PS contributed equally to this manuscript (shared first authorship). The original idea and study design came from PS and AA. AK performed most of the field work; help by others is accordingly acknowledged. SR and PS analyzed the data. The manuscript was prepared by SR, PS, and AA.

FUNDING

The Black Sparrowhawk project is funded by the DST-NRF Centre of Excellence. SR was supported by the NRF and PS by the Claude Leon Foundation.

ACKNOWLEDGMENTS

We are grateful to the Black Sparrowhawk team—Johan Koeslag, Mark Cowen, Odette Curtis, Gareth Tate, Margaret MacIver and Sharon Pryce—for the long-term population monitoring, data collection and extensive support in the field.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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in Ecology and Evolution

ORIGINAL RESEARCH
published: 02 August 2017
doi: 10.3389/fevo.2017.00085

Mechanisms Associated with an Advance in the Timing of Seasonal Reproduction in an Urban Songbird

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OPEN ACCESS

Edited by:
Diego Gil,
Museo Nacional de Ciencias Naturales (CSIC), Spain

Reviewed by:
Pierre J. Deviche,
Arizona State University, United States
Oscar Gordo,
Estación Biológica de Doñana (CSIC), Spain

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: 31 January 2017
Accepted: 12 July 2017
Published: 02 August 2017

Citation:
Fudickar AM, Greives TJ, Abolins-Abols M, Atwell JW, Meddle SL, Friis G, Stricker CA and Ketterson ED (2017) Mechanisms Associated with an Advance in the Timing of Seasonal Reproduction in an Urban Songbird. Front. Ecol. Evol. 5:85. doi: 10.3389/fevo.2017.00085

The colonization of urban environments by animals is often accompanied by earlier breeding and associated changes in seasonal schedules. Accelerated timing of seasonal reproduction in derived urban populations is a potential cause of evolutionary divergence from ancestral populations if differences in physiological processes that regulate reproductive timing become fixed over time. We compared reproductive development in free-living and captive male dark-eyed juncos deriving from a population that recently colonized a city (∼35 years) and ceased migrating to that of conspecifics that live in sympatry with the urban population during winter and spring but migrate elsewhere to breed. We predicted that the earlier breeding sedentary urban birds would exhibit accelerated reproductive development in the spring along the hypothalamic-pituitary-gonadal (HPG) axis as compared to migrants. We found that free-living sedentary urban and migrant juncos differed at the level of the pituitary when measured as baseline luteinizing hormone (LH) levels, but not in increased LH when challenged with Gonadotropin-Releasing Hormone (GnRH). Among captives held in a common garden, and at the level of the gonad, we found that sedentary urban birds produced more testosterone in response to GnRH than migrants living in the same common environment, suggesting greater gonadal sensitivity in the derived urban population. Greater gonadal sensitivity could arise from greater upstream activation by LH or FSH or from reduced suppression of gonadal development by the adrenal axis. We compared abundance of gonadal transcripts for LH receptor (LHR), follicle stimulating hormone receptor (FSHR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) in the common-garden, predicting either more abundant transcripts for LHR and FSHR or fewer transcripts for GR and MR in the earlier breeding sedentary urban breeders, as compared to the migrants. We found no difference in the expression of these genes. Together these data suggest that advanced timing of reproduction in a recently derived urban population is facilitated by earlier increase in upstream baseline activity of the HPG and earlier release from gonadal suppression by yet-to-be-discovered mechanisms. Evolutionarily, our results suggest that potential for gene flow between seasonally sympatric populations may be limited due to urban-induced advances in the timing of reproduction and resulting allochrony with ancestral forms.

Keywords: seasonal breeding, phenology, supplemental cues, photoperiod, migration, songbird, gonads, urbanization

INTRODUCTION

Over the past several centuries, urban landscapes have replaced native habitat in many parts of the world (Marzluff, 2016; Alberti et al., 2017). Urban environments can differ from the environments they replace in many ways, and while human activity in urban areas can pose novel challenges to animals (Shanahan et al., 2014), relatively mild urban microclimates and enhanced resource availability can also lead to increased opportunities to reproduce (Deviche and Davies, 2013).

A common observation is that urban bird populations begin their breeding season earlier compared to rural populations (Deviche and Davies, 2013). Seasonal fluctuations in climate and resource availability are often reduced in urban environments, which can lead to longer breeding seasons and year-round occupancy by formerly migratory lineages (Adriaensen and Dhondt, 1990; Partecke and Gwinner, 2007; Chamberlain et al., 2009; Atwell et al., 2014). If the physiological processes that lead to differences between urban and rural populations in reproductive timing and other seasonal traits become fixed over time, then gene flow and interbreeding could be limited (Evans et al., 2010; Fudickar et al., 2016a).

Though it is clear that urbanization can advance timing of reproduction, relatively little is known about the sources of variation in reproductive physiology that modulate the advancement or extension of breeding. The hypothalamic-pituitary-gonadal (HPG) axis plays a central role in the seasonal regulation of reproduction in birds (Dawson, 2015; **Figure 1**). The timing and rate of the seasonal activation of the HPG is modulated by a combination of predictive (day length) and supplementary cues (e.g., food and temperature; Hahn et al., 2005; Schaper et al., 2012; Wingfield et al., 2012; Dawson, 2015; Watts et al., 2015). Advancement in breeding phenology in urban habitats, therefore, may result from a plastic response to the novel habitat by phenotypically flexible individuals and/or a genetic response to selection for earlier seasonal activation of the HPG. Whether or not differences in timing are fixed or flexible may have important evolutionary implications. For example, if advanced reproduction in urban populations is the result of selection for greater sensitivity of the HPG at shorter day lengths—either by an earlier seasonal response or responding at a faster rate—then the resulting reproductive allochrony (asynchronous reproduction) may limit gene flow between urban and rural populations, ultimately leading to selection against hybrids. Alternatively, if advanced timing in urban populations is modulated by plastic responses, then the scope for divergence may be limited, as immigrants would be capable of adopting the timing of established urban populations. These alternatives also have important implications for the adaptability of both urban and rural populations to cope with future environmental change.

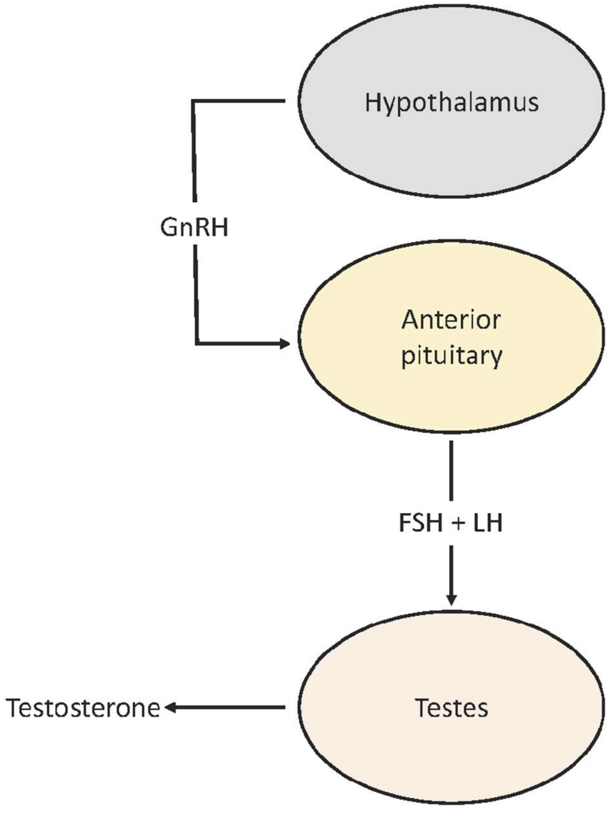

FIGURE 1 | Simplified HPG axis. GnRH released by the hypothalamus reaches the anterior pituitary where it stimulates production and release of FSH and LH which enter the general circulation. When LH and FSH bind to receptors on the testes (FSHR and LHR) in early spring, testes grow and increase testosterone production.

To date, there is limited evidence from studies that have compared the endocrine axes of urban vs. nearby rural populations in both field and common garden settings to support the importance of both plasticity and genetics in modulating earlier reproduction and HPG activation in urban habitats (Partecke et al., 2004, 2006; Schoech, 2009; Atwell et al., 2014; Davies et al., 2015). While these studies highlight the importance of differential HPG activity in facilitating earlier and extended breeding in urban habitats, much remains to be learned about the generality of those results, as well as the specific mechanistic sources of variation across the many "levels" of the HPG axis. Additionally, one interpretive challenge with such prior studies is that they typically sample putatively related populations that inhabit nearby yet radically different urban vs. rural habitats during the sampling period. In our study, we utilized both field and common garden settings to probe multiple levels of HPG activity in both urban-breeding and migratory non-resident populations during a critical life-history stage in which they co-occur in urban habitats, yet pursue divergent seasonal strategies.

Heteropatry—where closely related populations coexist during the non-breeding season (e.g., fall, winter, and /or early spring) yet pursue independent phenological and geographical trajectories during breeding—provides a unique opportunity to address the physiological mechanisms that underlie reproductive timing differences between populations (Winker, 2010; Ketterson et al., 2015; Bauer et al., 2016; Fudickar et al., 2016a,b; Greives et al., 2016; Ramenofsky et al., 2017). Broadly distributed avian species often have heteropatric distributions, in which some populations migrate while others do not (Winker, 2010). Despite experiencing the same photoperiodic and supplementary cues for much of the annual cycle (e.g., from the time that migrants arrive in the fall until they depart in the spring), there is often distinct phenological divergence in spring, when sedentary individuals transition into breeding, while migrants delay reproduction and prepare for and complete a migration before breeding. As a result, closely related sedentary and migratory birds are effectively sympatric during the non-breeding season as well as the transitional periods between non-breeding and breeding, yet are entirely allopatric during the core breeding season. In the current study, we compared specific neuroendocrine mechanisms predicted to underlie reproductive timing differences between a recently established (∼35 years) urban population of juncos vs. heteropatric ancestral migrant juncos, under both free-living (field) and common-garden (captive) conditions.

This study focused on a population of dark-eyed juncos (*J. hyemalis thurberi*) that became established in urban San Diego CA USA in the early 1980s, likely as a result of overwintering migratory birds remaining to breed (Rasner et al., 2004; Yeh, 2004; Atwell et al., 2012, 2014). Since then, an isolated sedentary urban breeding population has persisted with limited subsequent immigration despite a sympatric distribution with migratory juncos between October and April (Yeh and Price, 2004; Unitt, 2005). The colonization event and current heteropatric distribution thus provides a unique opportunity to examine mechanisms associated with contemporary adaptation to an urban environment. Although the precise breeding location of the migrants is not known, previous studies have shown that first egg laying dates in the San Diego population are ∼2.5 months earlier than the closest non-urban populations (Yeh and Price, 2004; Atwell et al., 2014). A recent study, in which juncos from San Diego and the closest non-urban breeding population (∼50 km) were captured on their respective breeding grounds as juveniles and observed in a common-garden experiment, found that population differences in the seasonal timing of mean peak HPG activity (plasma testosterone and post Gonadotropin-Releasing Hormone (GnRH) challenge testosterone) observed in the wild did not persist in the common garden (Atwell et al., 2014), which suggests a degree of flexibility in reproductive timing in both populations.

Here, we asked whether differences in the timing of the seasonal onset of reproduction between heteropatric sedentary urban and migratory juncos result from differential activation of the HPG in early spring and whether any difference is linked to differences in sensitivity to day length. While Atwell et al. (2014) compared the duration and peak timing of HPG activity of sedentary urban juncos from San Diego with that of the closest non-urban population, in the current study we focused on a comparison of sedentary urban breeders with migrants that are sympatric during winter and early spring. Based on previous reports indicating that winter flocks of juncos consist of migrants from a range of breeding populations (Fudickar et al., 2016a; J. Atwell personal observations), we expected that migrant juncos in San Diego would originate from multiple breeding populations.

First, we compared free-living sedentary urban and migratory juncos on their shared overwintering grounds in February for responsiveness of the HPG endocrine axis—specifically the response of the pituitary gland to repeated GnRH stimulation. By repeatedly injecting birds with GnRH, our aim was to more accurately replicate the pulsatile nature of GnRH that occurs naturally in spring. By measuring luteinizing hormone (LH) following repeated injections of GnRH, we assessed the potential for variation in pituitary sensitivity to modulate differential HPG axis activity in resident vs. migrant cohorts during the period when their observed seasonal strategies diverge, despite exposure to identical environmental conditions. We have previously demonstrated in longer diverged (∼15,000 years) overwintering juncos that pituitary sensitivity to repeated GnRH injection may serve to functionally limit HPG axis activation in female, but not male migrants (Greives et al., 2016). The hypothesis to be tested here was whether migrant birds have a weaker response to GnRH as would be predicted if reduced pituitary sensitivity to repeated GnRH acts as a mechanism to delay reproduction in birds that will forego breeding in the urban environment and migrate prior to reproduction.

Next, we measured morphological and physiological correlates of reproductive development across 4 weeks in early spring in sedentary urban and migrant junco males held in a captive common-garden experiment that simulated natural photoperiod (during the same early spring period), and provided *ad libitum* food, mild temperatures, and reduced social interactions. If the seasonal timing of reproduction in sedentary urban juncos is advanced due to increased sensitivity to photoperiodic cues in early spring, we predicted that timing of gonadal development and endocrine profiles of urban-breeding and migrant juncos should remain divergent. In contrast, if the seasonal timing of reproduction differs in sedentary urban and migrant juncos due to flexible responses to the environment, we predicted convergence of seasonal timing of gonadal development and endocrine profiles in captivity. We limited our study to male juncos because, in captivity, females do not advance into reproduction in the spring as readily as males (E.K., personal observation).

Finally, we compared gonadal transcript abundance of LH receptor (LHR), follicle stimulating hormone receptor (FSHR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) of sedentary urban and migrant juncos in early spring. LHR and FSHR are important for seasonal gonadal growth in birds, and GR and MR can suppress gonadal growth and activation in birds (McGuire et al., 2013; Blas, 2015; Vizcarra et al., 2015). Therefore, we predicted higher LHR and FSHR transcript abundance in sedentary urban juncos in early spring and higher GR and/or MR transcript abundance in migrant juncos in early spring.

We interpret our collective results in the context of both the proximate mechanisms underlying phenotypic divergence in reproductive timing, as well as the ultimate factors and evolutionary implications associated with the observed physiological divergences and convergences.

METHODS

Field Study

Capture and Morphological Measures

Thirty-seven male juncos were captured in the urban habitat of the University of California-San Diego (UCSD) campus in La Jolla, California, USA, using continuously monitored walk-in traps baited with seed. Birds in the field study were classified *post hoc* as sedentary urban breeders or migrants using a combination of morphological measures and stable isotopes in feather tissue (hydrogen; details below). We collected the outermost secondary feather from each bird's right wing during handling to examine stable hydrogen (δ^2H) signatures. Using calipers, we measured the length of the right tarsus (± 0.1 mm). Using a ruler, we measured the length of the right wing (flattened) and the tail (± 0.5 mm). Previous studies have reported that juncos that breed in San Diego have reduced white on their tail feathers and black on their heads compared to rural migratory populations (Yeh, 2004; Atwell et al., 2014). We visually estimated the percentage of white in the four outermost right tail feathers of each bird at capture. Using calipers, we measured (a) the length of the entire hood from the bill to the ventral terminus and (b) the length from the bill to the ventral terminus of homogenous black feathers. Age class was determined based on wing plumage as juncos have a limited first prebasic molt, making it possible to distinguish between first-year and after-first-year birds (Nolan, 2002). Capture dates were from 13 February to 23 February 2014, ∼4 weeks prior to the historical first egg date in the resident population, and roughly 2–3 weeks prior to the apparent peak of migrant passage through southern California (J.W. Atwell personal observation). All individuals received a unique numbered metal band to avoid repeat sampling. All procedures were approved by the Indiana University, North Dakota State University, and University of California San Diego Institutional Animal Care and Use Committees and conducted under appropriate federal banding permits (permit #20261) and the California Department of Fish and Wildlife (permit #007852).

Blood Sample Collection—Field

Immediately after capture, a 50 μl blood sample was obtained from the alar wing vein for baseline measures of LH. The birds then received an intramuscular injection of 1.25 μg chicken GnRH (American Peptide, Sunnyvale, CA) in 50 μl of phosphate-buffered saline into the pectoralis muscle. This dose fully activates the HPG axis in this species (Jawor et al., 2006). Five minutes following the first GnRH injection, a second 50 μl blood sample was taken from the wing vein for LH. Previous work in this species and white-crowned sparrows (*Zonotrichia leucophrys gambelii*) indicated LH levels reach a peak 5 min following injection and then significantly decline by 20 min and return to baseline at 30 min (Wingfield et al., 1979; Bergeon Burns et al., 2014). The birds then received a second injection of GnRH (same dose as above) 30 min after the first injection, followed by a third injection of GnRH (same dose as above) 30 min following the second injection. A final 50 μl blood sample was collected 5 min following the third injection of GnRH (65 min after the first GnRH injection). All birds were held in an opaque paper bag between injections. Blood samples were stored on ice until later processing in the laboratory. Samples were centrifuged to separate red blood cells from plasma. Plasma was collected with a Hamilton syringe and stored at −20°C until it was shipped on dry ice to The Roslin Institute, The University of Edinburgh for LH quantification.

Luteinizing Hormone Assay

To determine plasma LH, we used a micromodification of the radioimmunoassay described previously (Sharp et al., 1987). Briefly, the assay reaction volume was 60 μl, comprised of 20 μl of plasma sample or standard, 20 μl of primary rabbit LH antibody, and 20 μl of I^{125}-labeled LH. The primary antibody was precipitated to separate free and bound I^{125} label using 20 μl of donkey *anti*-rabbit precipitating serum and 20 μl of non-immune rabbit serum. All samples were measured in duplicate in a single assay. The intra-assay coefficient of variation was 2.9% and the minimum detectable dose was 0.2 ng/ml. This LH radioimmunoassay has been used extensively in many avian species including dark-eyed juncos (Deviche et al., 2000; Meddle et al., 2002; Wingfield et al., 2012; Bergeon Burns et al., 2014; Greives et al., 2016).

Common Garden Study

Bird Capture and Housing

From December 13 to 18, 2014 we captured 30 male juncos (population of origin classification details described above) from the UCSD campus using baited walk-in traps. After capture, we collected the outermost secondary feather from each bird's right wing and measured plumage and morphological traits as we did for the field study for *post hoc* classification of sedentary urban and migrant individuals. We housed all birds for 1–6 days (depending on capture date) in cages in an indoor facility with natural sunlight, where they were provided food (2:1 mixture of white millet and cracked corn) and water *ad libitum*. On December 19, 2014 the birds were transported overnight by air to Indiana University. At Indiana University, all birds were held together in a climate controlled free flight aviary (6.4 × 3.2

× 2.4 m) and were provided with water containing Nekton-S Multi-Vitamin for Birds (Arcata Pet, Arcata, CA) and food *ad libitum*. Birds were fed a seed mix containing white millet and sunflower chips (2:1), live mealworms, orange slices, and a soft diet containing ground puppy chow, hard-boiled eggs, and carrots. We adjusted lights in the aviary every 3 ± 1 days to simulate the natural seasonal changes in day length in San Diego. Throughout the experiment, the temperature in the aviary was maintained at 16 ± 2°C.

On 29 January 2015, we individually housed the birds in 61 × 46 × 46-cm cages and separated them into 5 identical indoor aviary rooms (2.5 × 2.1 × 2.4 m). In each room, we arranged cages so that birds were visually isolated from each other. All procedures were approved by the Indiana University and University of California San Diego Institutional Animal Care and Use Committees and conducted under appropriate scientific collecting permits issued by the USFWS (permit #MB093279) and the California Department of Fish and Wildlife (permit #007852).

Blood Sample Collection—Captivity

We collected blood samples (∼150 μl) from each bird every 6 or 7 days from February 26 to March 20 2015 to measure baseline plasma corticosterone (CORT), initial plasma testosterone, and plasma testosterone in response to a standardized GnRH challenge protocol (testosterone post-GnRH). The GnRH challenge protocol involves collecting a blood sample 30 min after an intramuscular injection of GnRH, which is administered following the initial blood sample. This assay allows for a robust and repeatable measure of an individual male's ability to produce testosterone in response to a standardized physiological stimulus and is known to vary seasonally in relation to reproductive development in juncos (Jawor et al., 2006). Blood samples for CORT were always collected within 3 min of entering a room (range = 1 min 30 s to 3 min; average = 2 min 41 s), and rooms were entered only once per sampling day. Blood samples for plasma testosterone were collected immediately following sampling for CORT and were always taken within 5 min of entering a room. Exactly 5 min after entering a room, we injected birds with 1.25 μg chicken GnRH (American Peptide, Sunnyvale, CA) in 50 μl of phosphate-buffered saline. Thirty minutes after each injection, we collected a second blood sample (∼75 μl) to assess an individual male's potential maximum testosterone. Samples were kept at 4°C until they were centrifuged (within 4 h). After centrifugation, we collected plasma using a Hamilton syringe, and plasma and red blood cells were frozen at −20°C until they were assayed.

Morphological Measurements

Each sampling day, after the last blood sample was collected from an individual, we measured subcutaneous fat and cloacal protuberance volume. Fat varies seasonally in relation to transitions between both (1) wintering and breeding condition (reduced fat) and (2) wintering and pre-migratory condition (increased fat; Clark, 1979). Migration is a metabolically taxing behavior, and migrants use fat as the primary source of fuel during the migratory stage (Jenni and Jenni-Eiermann, 1998). We visually estimated fat score (0–4) for subcutaneous fat deposition in furcular and abdominal regions separately as follows: 0 = no fat present, 1 = trace amounts, 2 = half full, 3 = full, and 4 = bulging. We summed furcular and abdominal fat scores to obtain a total fat score (range 0–8; Fudickar et al., 2016a).

The cloacal protuberance (CP), the terminal part of the passerine ductus deferens, is the primary location of sperm storage, is testosterone sensitive and its seasonally variable size is a reliable measure of breeding condition in male passerines (Wolfson, 1952). Cloacal protuberance volume (V) was estimated by using the formula to calculate the volume of a cylinder [$V = (\text{radius})^2(\text{height})$]. Cloacal width (diameter, w, ±0.1 mm) was measured with dial calipers, and height (h, ±0.5 mm) was measured with a ruler, which allowed us to ensure that our measurement started flush against the body.

Gonadal Development

To investigate differences in the seasonal timing of gonadal development, at the end of the study (24–25 March 2015), we euthanized birds with an isoflurane overdose and dissected both testes, which were immediately flash frozen on dry ice and transferred to −80°C, where they were stored until they were weighed and we extracted mRNA. To compare testis size among individuals, we measured frozen testis mass of one testis from each bird in the lab using a digital microbalance accurate to ±0.001 g.

Corticosterone and Testosterone Assays

Following established methods for juncos (Jawor et al., 2006), we determined plasma CORT and testosterone concentrations from 10 to 20 μl aliquots, respectively, after a diethyl-ether extraction of steroids using enzyme immunoassays (CORT: 500655, Cayman Chemical; testosterone: ADI-900-065, Enzo Life Sciences). Coefficients of variation of the assays were as follows: CORT: $n = 4$ plates, intraplate = 5.55 ± 1.8% (mean ± SE), range = 0.22–7.71%; testosterone: $n = 8$ plates, intraplate = 8.59 ± 0.8% (mean ± SE), range = 5.45–10.85%. Sampling dates were assigned equally across plates.

mRNA Extraction and qPCR

Total RNA was extracted from one testis for each bird using the Trizol method (Invitrogen, Carlsbad, CA). Following spectrophotometry to quantify total RNA, 1 μg RNA was treated with DNAse (Promega, Madison, WI) and underwent reverse-transcription PCR using oligo dT primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting cDNA was used as a template for quantitative real-time PCR (qPCR) to measure gene expression of LH receptor (LHR), FSH receptor (FSHR), glucocorticoid receptor (GR), and mineralcorticoid receptor (MR). qPCR reactions were run on a Stratagene MX3000P using PerfeCTa SYBR Green SuperMix (low ROX, Quanta Biosciences, Gaithersburg, MD, USA). We used MxPro software (v.4.10, Agilent, Santa Clara, CA, USA) to analyze gene expression with the comparative Ct method, where abundance of transcripts from individual samples is expressed against a calibrator sample, and normalized against expression of a housekeeping gene (RPL4). RPL4 is one of the most stable housekeeping genes in testes of passerines (Zinzow-Kramer et al., 2014), and its expression did not differ between the sedentary urban and migrant juncos in our study (Mann-Whitney $U = 32.50$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.234$ two-tailed). Primer sequences are reported in the Supplementary Material.

DNA Extraction

Genomic DNA (gDNA) was extracted from red blood cells in the Center for the Integrative Study of Animal Behavior (CISAB) at Indiana University, using IBI Scientific MINI Genomic DNA kits (IB46701). Red blood cells collected during the captive study were used for the DNA extractions. DNA was measured with a Nanodrop spectrophotometer (Take 3).

Feather isotopes

The outermost (distal) secondary feather from each bird's right wing was collected during handling to examine stable hydrogen isotope (δ^2H) signatures. Juncos complete their annual molt cycle before leaving breeding habitats, or retain juvenile wing feathers throughout their first annual cycle (Nolan, 2002). δ^2H of these feathers therefore can, in some cases, permit assignment of breeding latitude based on known patterns of isotopic variation in precipitation (Bowen et al., 2005). Feathers were cleaned of oils using a 2:1 chloroform: methanol solution and allowed to air-dry under a fume hood at Indiana University. Approximately 0.50 mg of vane material was clipped from the proximal end of each feather and loaded into 3 × 5 mm pressed silver capsules. The non-exchangeable hydrogen isotope composition was measured by continuous flow isotope ratio mass spectrometry using a Finnigan TC/EA interfaced to a Finnigan DeltaPlus XL mass spectrometer (Thermo Scientific, Bremen, Germany), using established methods at the U.S. Geological Survey Stable Isotope Laboratory in Denver, Colorado (Wunder et al., 2012). δ^2H values are reported in per mil notation (‰) relative to V-SMOW, using internal standards (−78 and −172‰, respectively) calibrated to CFS-CHS-BWB (Wassenaar and Hobson, 2003). Benzoic acid ($\delta^2\text{H} = -61$‰) and IAEA-CH-7 ($\delta^2\text{H} = -100$‰) were also analyzed within analytical sequences with a precision of $< \pm 4$‰.

Genotyping-by-sequencing

We took advantage of the genomic data generated in a study running in parallel to the study reported here to confirm the genetic identity of the UCSD resident population as a differentiated group from migrant populations of *Junco hyemalis thurberi* of California. To do so, we used genotyping-by-sequencing (GBS, Elshire et al., 2011) to obtain the genotypes of 43 *J. hyemalis thurberi* sampled in the UCSD campus. 32 of these 43 samples were collected at the time of the captive study (30 birds held in the captive study and 2 additional birds caught at the same time) and 11 were collected during previous studies from known breeders at UCSD. Fifty additional samples were collected from individuals from surrounding migratory populations of *Junco hyemalis thurberi* from California. Specific sampled populations were (sample sizes in parenthesis): UCSD campus (43), Mount Laguna (12), Palomares (7), San Jacinto (8), Santa Barbara (7), Eldorado (8) and Mendocino (8). GBS libraries were prepared and sequenced at Cornell University's Institute for Genomic Diversity, using the restriction enzyme PstI for digestion. Sequencing was carried out in an Illumina HiSeq 2000 platform and resulting reads were trimmed and quality filtered with PRINSEQ (Schmieder and Edwards, 2011). We used the Genome Analysis Toolkit (GATK, McKenna et al., 2010) version 3.6-0 to perform the variant calling after mapping the reads against a *Junco* draft genome assembled for this purpose with the Burrows-Wheeler Aligner (BWA, Li and Durbin, 2009). We then used VCFTOOLS (Danecek et al., 2011) to retain only highly confident biallelic single nucleotide polymorphisms (SNPs) with a genotyping quality phred score over 70, a minimum allele frequency of 1.5% and per site missing rate lower than 10%. We also implemented a threshold for SNPs showing highly significant deviations from Hardy-Weinberg equilibrium (HWE) with a p-value of 10^{-4} to filter out false variants arising from the alignment of paralogous loci. The resulting SNP matrix was further filtered to exclude variants potentially under selection with BayeScan (Foll and Gaggiotti, 2008) and for linkage disequilibrium using the package SNPrelate (Zheng, 2012) in R (R_Core_Team, 2015) version 3.2.2 for a final dataset of 23,051 SNPs.

Captive study classification

Using the 23,051 SNP matrix, we ran a STRUCTURE (Pritchard et al., 2000) analysis for two populations ($K = 2$) to test for differentiation between UCSD residents and the remaining populations of Californian juncos. We ran the analysis five times after running a preliminary analysis to infer the lambda value. The burning was set to 50 K iterations and the analysis ran for an additional 100 K iterations. Two main clusters were identified in all five runs, clearly consistent with a resident group of UCSD breeders and a second cluster encompassing the remaining populations. From the 43 individuals sampled on the UCSD campus, we could confidently assign 37 individuals (those with a posterior assignation probability to either group over 0.9): 8 were clear migrants, 6 of which were collected for the captive study (posterior assignment probability to the UCSD breeders group = 0), while the other 29 were clear residents, 19 of which were collected for the captive study (posterior assignment to the UCSD breeders group ranging from 0.93 to 1). The remaining 6 individuals presented different degrees of admixture, 3 of which were collected for the captive study (posterior assignment to the UCSD breeders group ranging from 0.14 to 0.81). Due to uncertainty in their classification, the 3 individuals were excluded in our comparisons. We were not able to classify 2 additional birds captured for the captive study due to insufficient DNA quality. Final sample size for the captive comparison was 19 sedentary urban birds and 6 migrants. A plot of the STRUCTURE analysis is included in the Supplementary Material.

Field study classification

We did not have genomic data to classify birds used in the field study. To classify birds from the field study, we first ran a discriminant function analysis (DFA) using the morphologic and isotopic data from the birds held in the captive study that were confidently classified individuals based on genomic data (described above). Next, we used the DFA model generated from genotyped birds to classify 35 UCSD campus individuals for which we had morphometric and isotope data but not genomic data, including them as *blind* cases in the model construction. To inform the DFA model, we used isotope and morphometric data from genotyped birds with sufficient isotope and morphometric data ($N = 25$, 6 migrants and 19 residents from the captive study) to run a DFA in SPSS 22 (SPSS Inc. 2008, Chicago, IL, USA) for the two groups based on (i) the isotopic scores of hydrogen; (ii) the length of the tarsus, the wing and the tail; (iii) the percentage of white in the right tail feather 1, 2, 3, and 4; and (iv), the distance from the front of the crown to where the black ends, the distance from the front of the crown to the very back and the percent of the crown that is black (discriminant function coefficients are reported in the Supplementary Material). Because of the relatively low overall and per group sample size, we included in the analysis a "leave-one-out" cross validation test to assess the power of our model to correctly classify individuals. The validation test correctly classified five of the six migrant individuals (an 83.3% of migrants correctly classified) and 17 of 19 residents (an 89.5% of residents correctly classified), yielding a total of 88% of cases correctly classified.

Finally, we used the DFA model to classify the 35 UCSD campus individuals for which we had morphometric and isotope scores but not genomic data, including them as *blind* cases in the model construction. The predicted group membership resulted in 9 migrants (probability of group membership ranging from 0.96 to 1) and 26 residents (probability of group membership ranging from 0.98 to 1). In light of the cross validation test, these outcomes should be taken with caution. Two additional birds in the field study were captured the following winter for the captive study, and therefore were genotyped (both migrants). The final sample size of our comparison of free-living birds was 11 migrants and 26 residents.

Statistical Analyses of Hormone and Physiology Data

We used non-parametric statistical tests for all physiological measures, due to unequal sample sizes and unequal variances in both the field and captive studies.

Field study

Age distribution was equal in sedentary urban and migrant juncos sampled for LH in the field (Fisher's exact test, $P = 0.443$; first-year sedentary urban = 7, after-first-year sedentary urban = 19, first-year migrant = 5, after-first-year migrant = 6). To test for within-subject variance in plasma LH levels across the three sample times (baseline LH, LH 5 min after a single GnRH injection, and 5 min after the final GnRH injection), we used a Friedman test with sedentary urban and migrant juncos combined. We used Mann-Whitney U test to test for between-group differences in LH at each sample time.

Captive study

Age distribution was equal in sedentary urban and migrant juncos held in the captive study (Fisher's exact test, $P = 0.630$; first-year sedentary urban = 6, after-first-year sedentary urban = 13, first-year migrant = 3, after-first-year migrant = 3). To test for within-subject variance in physiological measures (cloacal protuberance volume, plasma testosterone, testosterone post-GnRH challenge, subcutaneous fat score, and CORT) across the four sampling weeks, we used a Friedman test for each week with sedentary urban and migrant juncos combined. We used a Mann-Whitney *U* test to test for between-group differences in physiological measures each week. We were not able to sample enough plasma from all birds at each time point, therefore, sample numbers vary across sampling times. To test for differences between sedentary urban and migrant juncos in gonadal FSHR, GR, LHR, and MR transcript abundance, we used independent Mann-Whitney U tests. We performed all statistical analyses using SPSS, version 24 (IBM). All reported *p*-values are two-tailed.

RESULTS

Field Study

Luteinizing Hormone

Sedentary urban juncos had higher baseline LH than migrant juncos ($U = 48.50$, $n_{\text{urban}} = 26$, $n_{\text{migrant}} = 11$, $p = 0.002$). LH increased after injection ($n = 32$, Chi-Square $\chi^2 = 39.44$, df = 2, $p < 0.001$), but there was no difference between the groups following GnRH challenge (LH 5 min: $U = 106.00$, $n_{\text{urban}} = 25$, $n_{\text{migrant}} = 10$, $p = 0.488$; LH 65 min: $U = 107.50$, $n_{\text{urban}} = 24$, $n_{\text{migrant}} = 10$, $p = 0.637$) **(Figure 2)**.

Common Garden Study

Gonads

At the end of the 4-week sampling period, sedentary urban juncos had larger gonads than migrant juncos ($U = 18.00$, $n_{\text{urban}} =$

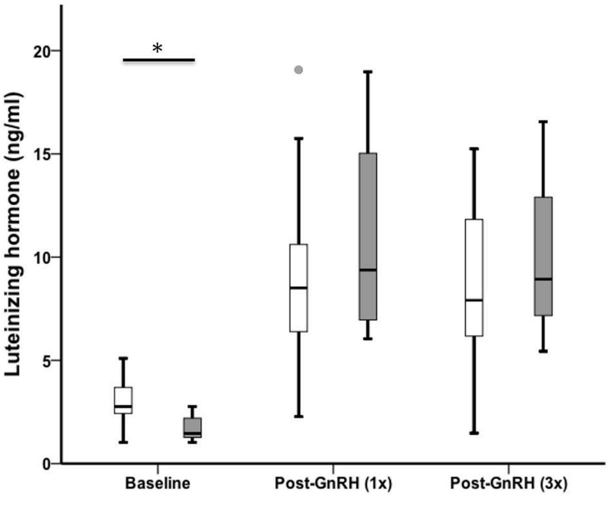

FIGURE 2 | Male sedentary urban juncos (white boxes) had higher baseline levels of LH than male migrant juncos (gray boxes). LH increased in both groups 5 min after a GnRH-challenge and remained high after two additional injections timed 30 min apart (*$p \le 0.05$). The groups did not differ in LH from one another post-GnRH injections. Boxes represent upper quartile, median, and lower quartile. Whiskers include the range of values except for outliers. Outliers include values > 1.5 box lengths (circles).

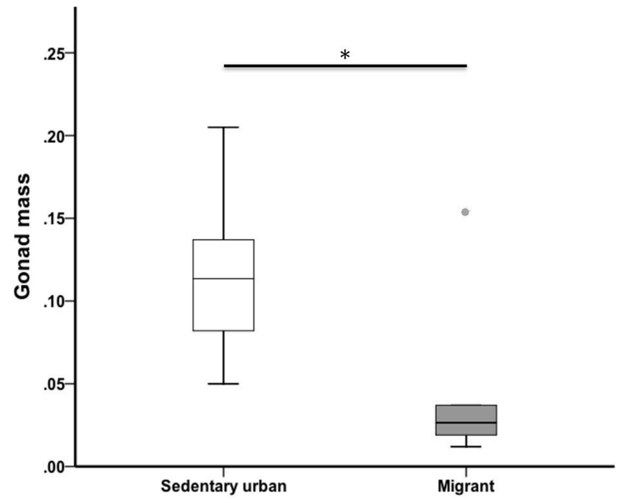

FIGURE 3 | Testis mass of male sedentary urban juncos was higher than that of migrant juncos in early spring. Boxes represent upper quartile, median, and lower quartile (*$p \leq 0.05$). Whiskers include the range of values except for outliers. Outliers include values >1.5 box lengths (circles).

19, $n_{\text{migrant}} = 6$, $p = 0.013$) **(Figure 3)**. Median gonad mass was 0.113 g for sedentary urban juncos and 0.024 g for migrants.

Cloacal Protuberance Volume

Cloacal protuberance (CP) volume increased across the 4-week sampling period in all birds ($n = 25$, Chi-Square $\chi^2 = 35.10$, df $= 3$, $p < 0.001$) however there was no difference when comparing sedentary urban and migrant juncos (CP week one: $U = 56.50$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.975$; CP week two: $U = 41.50$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.324$; CP week three: $U = 55.50$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.924$; CP week 4: $U = 46.50$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.50$).

Testosterone

Baseline testosterone increased across the 4-week sampling period in all birds ($n = 23$, Chi-Square $\chi^2 = 7.85$, df $= 3$, $p = 0.049$; **Figure 4A**). Sedentary urban and migrant juncos had similar baseline testosterone during the first 2 weeks of the study (baseline T week one: $U = 40.00$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.279$; baseline T week two: $U = 32.50$, $n_{\text{urban}} = 18$, $n_{\text{migrant}} = 6$, $p = 0.152$) but sedentary urban juncos had higher levels in weeks 3 and 4 (baseline T week three: $U = 21.50$, $n_{\text{urban}} = 18$, $n_{\text{migrant}} = 6$, $p = 0.030$; baseline T week 4: $U = 14.00$, $n_{\text{urban}} = 18$, $n_{\text{migrant}} = 6$, $p = 0.008$). Sedentary urban juncos had higher GnRH-induced testosterone than migrants throughout the study (GnRH T week one: $U = 24.00$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.036$; GnRH T week two: $U = 19.00$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.016$; GnRH T week three: $U = 19.00$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.016$; GnRH T week 4: $U = 19.00$, $n_{\text{urban}} = 18$, $n_{\text{migrant}} = 6$, $p = 0.020$; **Figure 4B**).

CORT

CORT increased across the 4-week sampling period in all birds ($n = 21$, Chi-Square $\chi^2 = 9.49$, df $= 3$, $p = 0.023$; **Figure 5A**). Sedentary urban and migrant juncos had similar baseline CORT in all 4 weeks of the study (CORT week one: $U = 38.00$, $n_{\text{urban}} = 17$, $n_{\text{migrant}} = 6$, $p = 0.363$; CORT week two: $U = 44.50$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.426$; CORT week three: $U = 35.00$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.162$; CORT week 4: $U = 32.00$, $n_{\text{urban}} = 16$, $n_{\text{migrant}} = 6$, $p = 0.238$).

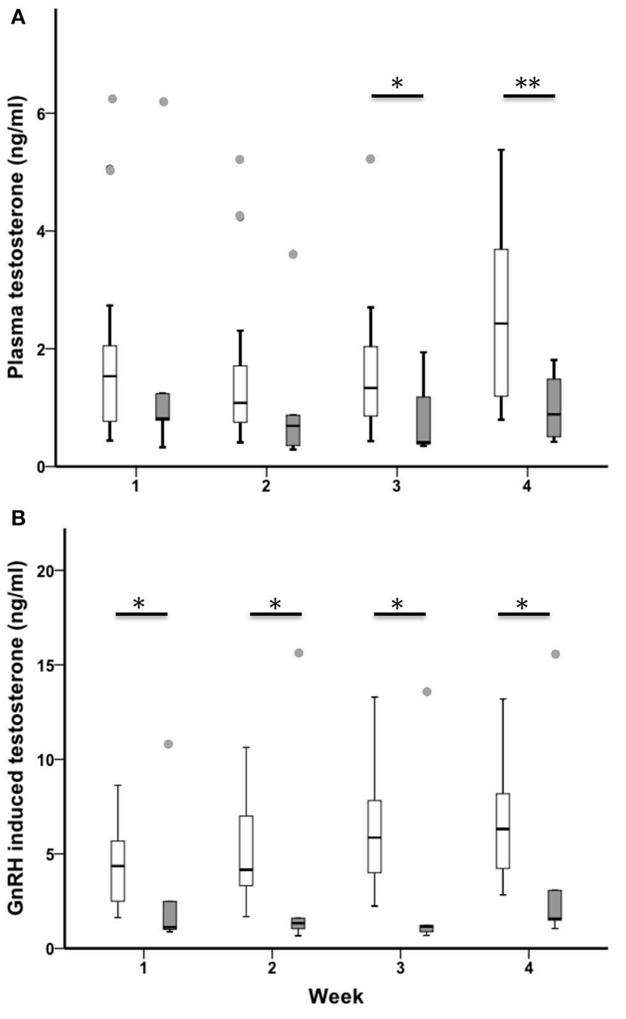

FIGURE 4 | Male sedentary urban juncos (white boxes) and migrant juncos (gray boxes) did not differ in plasma testosterone until the third week of sampling (12 & 13 March) **(A)**. Sedentary urban juncos had higher levels of testosterone in response to GnRH-challenge **(B)** than migrant male juncos throughout the study (*$p \leq 0.05$; **$p \leq 0.01$). The samples were collected on the following dates: 1 = 26 & 27 February, 2 = 5 & 6 March, 3 = 12 & 13 March, 4 = 19 & 20 March. Boxes represent upper quartile, median, and lower quartile. Whiskers include the range of values except for outliers. Outliers include values >1.5 box lengths (circles).

Fat

Migrants exhibited more subcutaneous fat than sedentary urban juncos throughout the study (Fat week one: $U = 13.00$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.002$; Fat week two: $U = 13.500$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.003$; Fat week three: $U = 20.50$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.018$; Fat week 4: $U = 19.00$, $n_{\text{urban}} = 19$, $n_{\text{migrant}} = 6$, $p = 0.004$; **Figure 5B**).

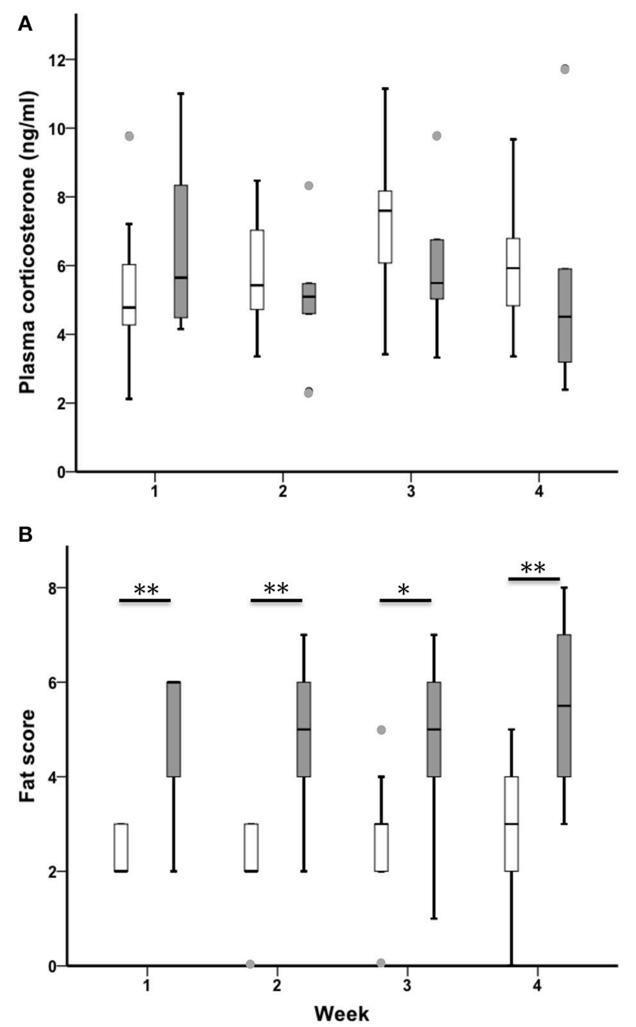

FIGURE 5 | Male sedentary urban juncos (white boxes) and migrant juncos (gray boxes) did not differ in plasma corticosterone at any point during the study **(A)**. Male migrant juncos exhibited larger subcutaneous fat scores than urban male juncos **(B)** ($^*p \leq 0.05$; $^{**}p \leq 0.01$). The samples were collected on the following dates: 1 = 26 & 27 February, 2 = 5 & 6 March, 3 = 12 & 13 March, 4 = 19 & 20 March. Boxes represent upper quartile, median, and lower quartile. Whiskers include the range of values except for outliers. Outliers include values >1.5 box lengths (circles).

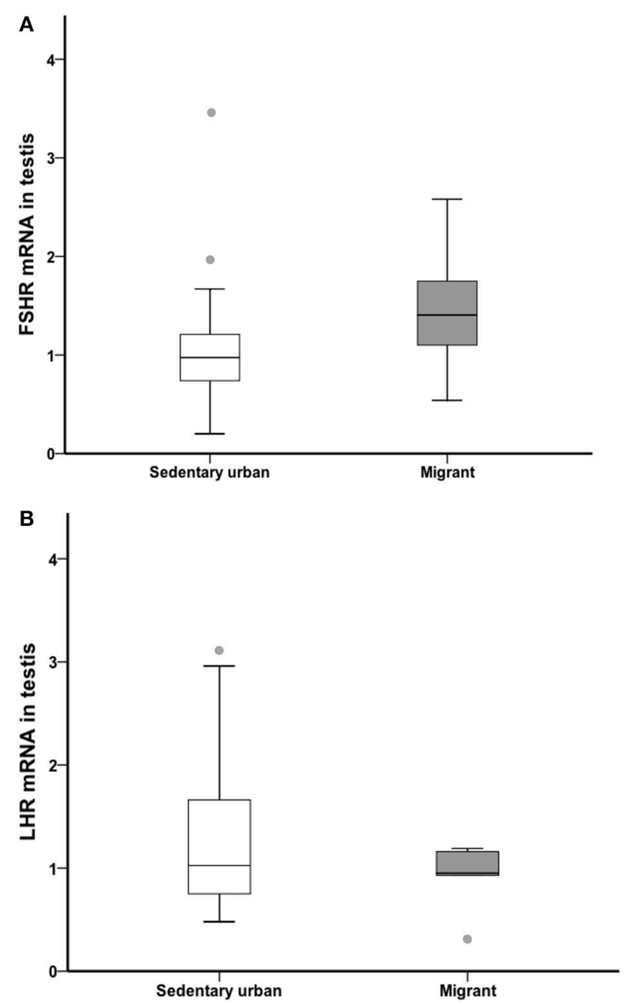

FIGURE 6 | Male sedentary urban juncos (white boxes) and male migrant juncos (gray boxes) did not differ in FSHR **(A)** or LHR **(B)** transcript abundance in the gonad. Measures of transcript abundance are $\log_2$-fold change relative to a calibrator. Boxes represent upper quartile, median, and lower quartile. Whiskers include the range of values except for outliers. Outliers include values >1.5 box lengths (circles).

There was no difference in fat across the four sampling periods when sedentary and migratory juncos were combined ($n = 25$, Chi-Square $\chi^2 = 2.97$, df = 3, $p = 0.396$).

LHR and FSHR

At the end of the 4-week sampling period, there was no difference in gonad LH receptor or FSH receptor transcript abundance between sedentary urban and migrant juncos (LHR: $U = 46.00$, $n_{\text{urban}} = 18$, $n_{\text{migrant}} = 6$, $p = 0.594$; FSHR: $U = 31.50$, $n_{\text{urban}} = 18$, $n_{\text{migrant}} = 6$, $p = 0.133$; **Figure 6**).

GR and MR

At the end of the 4-week sampling period, there was no difference in gonad glucocorticoid receptor or mineralocorticoid receptor transcript abundance between sedentary urban and migrant juncos (GR: $U = 34.00$, $n_{\text{urban}} = 18$, $n_{\text{migrant}} = 6$, $p = 0.182$; MR: $U = 37.00$, $n_{\text{urban}} = 18$, $n_{\text{migrant}} = 6$, $p = 0.257$; **Figure 7**).

DISCUSSION

Findings from this study resemble those from other studies that have shown rapid advancement in the seasonal onset of reproduction in urban avian populations (Deviche and Davies, 2013). However, this study is unusual in the degree to which it addresses the physiological mechanisms that allow earlier reproduction in urban populations and the implications for genetic divergence over time because population differences were exhibited under identical environmental cues.

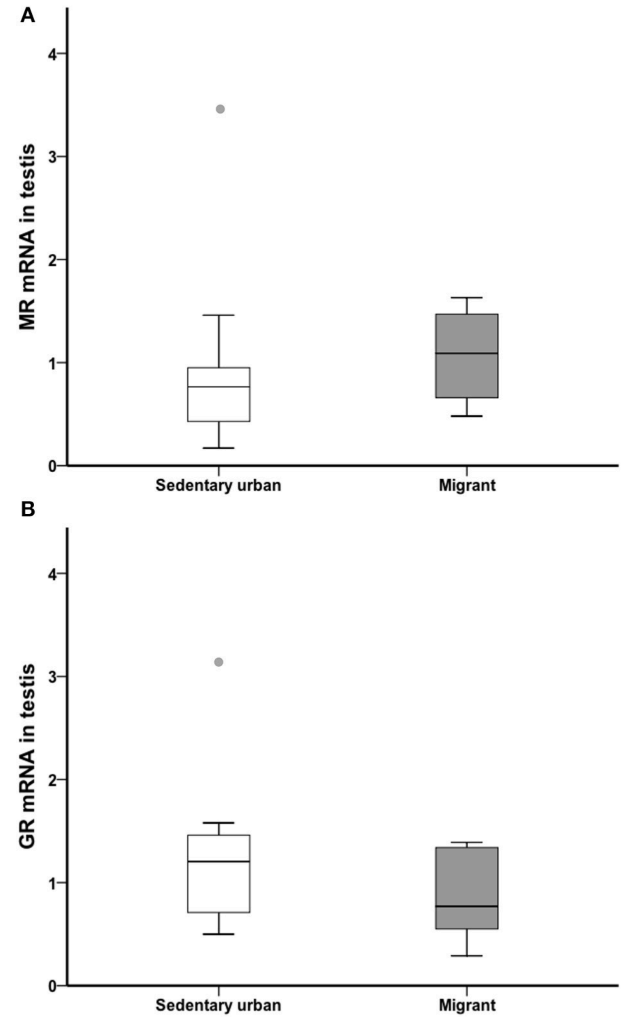

FIGURE 7 | Male sedentary urban juncos (white boxes) and male migrant juncos (gray boxes) did not differ in MR **(A)** or GR **(B)** transcript abundance in the gonad. Measures of transcript abundance are $\log_2$-fold change relative to a calibrator. Boxes represent upper quartile, median, and lower quartile. Whiskers include the range of values except for outliers. Outliers include values >1.5 box lengths (circles).

Timing of reproduction is critical to determining levels of gene flow between closely related populations and is subject to change as avian populations colonize rapidly expanding urban habitats. In this study, we compared indices of reproductive timing in two populations living in the same environment, a derived population that has recently become sedentary and begun to breed in San Diego CA and its ancestral relatives that co-exist with these urban colonists during winter but depart to breed elsewhere. By some but not all measures, reproductive development occurred earlier in the urban colonists. In the wild, baseline LH was greater in urban breeders but LH in response to GnRH did not differ between urban breeders and migrants (**Figure 2**). When birds from both populations were held in a common garden and exposed to the same simulated natural increases in day length with ample food, they nevertheless diverged in gonadal mass (**Figure 3**) and gonadal development as determined from testosterone release after stimulation by GnRH. Sedentary urban and migrant juncos were similar in baseline T until the third week of our sampling. However, throughout the sampling period, sedentary urban juncos elevated T more than migrant juncos, suggesting increased sensitivity at the level of the pituitary or the gonad (**Figure 4**). The populations did not differ in gonadal sensitivity to stimulation by LH or FSH when sensitivity was measured as abundance of transcripts for receptors for LH and FSH (**Figure 6**). The delay in reproductive timing in migrants is not likely due to suppression by the HPA axis because migrants did not differ from colonists in circulating levels of corticosterone (**Figure 5A**) or in the abundance of transcripts for gonadal glucocorticoid receptors (**Figure 7**). Whatever the mechanisms underlying differences in timing of reproductive development, they may help to explain divergence in recently separated and seasonally sympatric populations.

In combination, field LH and captive T results from our study suggest that differences in the timing of the seasonal increase in GnRH secretion could help explain differences in reproductive timing between sedentary urban and migrant juncos. While we found no difference between sedentary urban and migrant LH in response to GnRH (either 1x or 3x), baseline LH and captive baseline T (weeks 3 and 4) were both higher in sedentary urban juncos, which suggests that GnRH secretion could increase seasonally earlier in sedentary urban juncos. In captivity, sedentary urban juncos also increased T more than migrants in response to GnRH. In combination, these results suggest similar activity of the pituitary (no difference in LH after GnRH challenge) but earlier activation of hypothalamic and gonadal activity (higher baseline LH and T post-GnRH challenge). However, the underlying causes for these differences in seasonal activation are still unknown. The differences that we observed in the captive common-garden suggest a differential response between urban sedentary and migrant juncos to similar environmental cues. Urban sedentary juncos could be more sensitive than migrants to seasonal cues, such as photoperiod and food availability at the shared overwintering site and in the common-garden. As a result, the reproductive system of urban sedentary juncos could either respond earlier or develop at a faster rate in early spring.

Our finding of advanced seasonal elevation of baseline LH in sedentary urban juncos is similar to the results of a recent study that compared the seasonal timing of reproductive development in an urban population of Abert's Towhees (*Melosone aberti*) in Phoenix Arizona USA with that of a nearby rural population. Urban Abert's Towhees increased plasma LH, but not plasma testosterone, earlier than non-urban birds (Davies et al., 2015). In our captive common-garden, sedentary urban and migrant juncos also had similar plasma testosterone in the first 2 weeks of comparison, but diverged in the final 2 weeks of our study, with sedentary juncos displaying elevated baseline testosterone levels. Davies and colleagues suggest there has been a seasonal advancement in the endocrine activity of the anterior pituitary gland and/or hypothalamus in urban Abert's Towhees. Identifying and comparing the mechanistic basis for advances in the seasonal timing of reproduction in different species could be useful for identifying common mechanisms by which populations adapt to urban habitat.

At the conclusion of our captive study we also compared gonadal transcript abundance of LHR and FSHR in urban sedentary and migrant juncos. FSHR and LHR are important for seasonal gonadal growth, and LHR is critical to avian seasonal testosterone production (Vizcarra et al., 2015). Accordingly, we predicted higher FSHR and LHR transcript abundance in sedentary urban juncos in early spring. However, we did not find differences in transcript abundance of either FSHR or LHR (**Figure 6**). The absence of differences in gonadal LHR and FSHR transcripts, combined with elevated baseline LH observed from urban sedentary birds and elevated baseline testosterone during the final 2 weeks of our captive study, suggests that the earlier gonadal growth and responsiveness was not the result of a change in gonadal LH sensitivity to LH or FSH. Recent work has identified an inhibitory role of Gonadotropin Inhibitory Hormone (GnIH) in the gonads of male songbirds (McGuire et al., 2013; Ernst et al., 2016). Future work is needed to identify whether decreased gonadal GnIH production and/or gonadal GnIH receptor abundance in sedentary urban juncos in early spring could be acting as a mechanism for earlier onset of reproduction. Another potential explanation for the variation that we observed in T production is that the populations could differ in expression of genes in the steroid biosynthetic pathway. A recent comparison of junco subspecies that differ in testosterone-mediated traits found variation in gonadal mRNA for key regulatory genes for testosterone production including StAR, CYP17, and p450scc (Rosvall et al., 2016).

Previous work has identified a relationship between plasma CORT and the activity of the reproductive system in birds (Schoech, 2009). Recent work on Rufous-winged Sparrows (*Peucaea carpalis*), a sedentary sparrow that breeds in arid habitat, reported reduced plasma T following stressors during pre-breeding and breeding (Deviche et al., 2014, 2016). However, plasma CORT and T were not found to be correlated at the individual level in Rufous-winged Sparrows, and there was no evidence for a functional relationship between elevated CORT and T levels.

In the current study, we evaluated whether either decreased plasma corticosterone and/or decreased gonadal receptors for corticosterone (GR and MR) might play a role in greater testosterone production in sedentary urban juncos in early spring. We compared circulating corticosterone and gonadal GR and MR transcript abundance in sedentary urban and migrant juncos in our common garden study. Both in the field and a common garden, sedentary urban juncos, and migrant juncos that breed close-by had similar baseline levels of corticosterone during the breeding season (Atwell et al., 2012). Similar to these observations, our data collected during the period preceding the breeding season found no differences in baseline levels of corticosterone; sedentary urban and migrant juncos had similar levels of corticosterone over our 4-week captive study in early spring (**Figure 5A**). Further, we also did not identify any differences in gonadal GR or MR transcript abundance in sedentary urban and migrant juncos (**Figure 7**). Thus, observations from the current study do not support a role for baseline glucocorticoids during the pre-breeding period in inhibiting reproductive development in migrant juncos overwintering with urban sedentary juncos.

Another possibility is that stress-induced levels of corticosterone might act to delay reproductive development in migrants. A previous study (Atwell et al., 2012) conducted during the breeding season, found higher stress-induced corticosterone in a nearby rural breeding population compared to breeders from the urban San Diego population. We did not measure stress-induced corticosterone in this study, so we cannot say whether differences in stress-induced corticosterone levels might have delayed reproductive development in migrant juncos in San Diego. However, we think that is unlikely because in a similar comparison in Virginia USA, *migrant* juncos captured during spring had lower, not higher, levels of stress-induced corticosterone as compared with seasonally sympatric residents (Bauer et al., 2016).

Fat varies seasonally in passerines in relation to transitions between wintering and migratory stages (Clark, 1979). Despite having access to the same amount of food in the common-garden environment, migrant juncos had more subcutaneous fat than sedentary urban juncos throughout early spring (**Figure 5B**). Beginning in late March, migrant juncos that overwinter in San Diego migrate to breeding sites prior to reproduction (Yeh and Price, 2004). Elevated fat reserves in migrant juncos in early spring likely serve as fuel for migration. Results from our captive comparison suggest this is a seasonal change that is not expressed in the sedentary urban colonists. Perhaps the absence of migration-related traits in the sedentary urban population allows them to respond to the favorable environmental conditions encountered in their newly urbanized habitat, facilitating earlier onset of reproduction.

CONCLUSIONS

Avian populations face novel challenges with ongoing global urbanization. By identifying the mechanistic underpinnings of adaptive traits associated with successful colonizations of urban areas, we will be better able to predict which populations will persist and adapt to rapidly changing environments. We have demonstrated differences between heteropatric sedentary urban and migrant juncos in the seasonal timing of gonadal responsiveness and upstream endocrine activity of the pituitary gland and hypothalamus. However, we found no differences between these recently diverged groups in expression of key genes associated with reproduction and sex steroid production in the gonads. Urbanization is an ongoing process and future studies should aim to identify the mechanisms responsible for advanced reproductive physiology in individuals colonizing urban habitats.

AUTHOR CONTRIBUTIONS

AF, TG, MA, JA, and EK, All contributed to the design of the experiments, collection of data, performance of lab work, and writing of the manuscript. SM and CS, Contributed to the design of the experiments, lab work, and writing of the manuscript. GF

Contributed to design of the experiments, ran the GBS and DFA analyses, and contributed to writing the manuscript.

ACKNOWLEDGMENTS

We would like to thank Alexander Kauffman at UCSD for use of lab space and equipment and Tim Gentner at UCSD for serving as our representative to the IACUC. John McCormack and Brant Faircloth assisted with sequencing of the junco reference genome and provided support to GF. Jessica Graham, Emily Stewart, Katie Needham, Rachel Hanauer, Alex Hughes, Abby Kimmitt, Sam Slowinski, and Charli Taylor helped to collect and process samples. This work was funded by the National Science Foundation (IOS-1257474 to EK and IOS-1257527 to TG). SM acknowledges Roslin Institute strategic grant funding from the Biotechnology and Biological Sciences Research Council (BB/J004316/1 and BB/J004332/1). Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00085/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 05 May 2017
doi: 10.3389/fevo.2017.00040

Urbanization Alters the Influence of Weather and an Index of Forest Productivity on Avian Community Richness and Guild Abundance in the Seattle Metropolitan Area

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Fluctuations in weather and forest productivity influence the abundance and richness of bird populations, however in a rapidly urbanizing landscape the relative importance of each factor may vary. We assessed this possibility in the Seattle, WA, USA region by correlating 10 years of bird richness and relative abundance of nine guilds indicative of their tolerance of human development, migration, diet and use of human food subsidies with an annual index of forest productivity (vegetation greenness derived from a 250 m resolution Normalized Difference Vegetation Index (NDVI) product from the MODIS satellite) and weather (variation in the Oceanic Niño Index, which estimates the strength of the El Niño-Southern Oscillation, a major driver of local temperature and precipitation). We found that variation in NDVI exerted a strong influence on the richness of the avian community and the abundance of guilds in landscapes undergoing active development, but was less influential in areas of established housing development or forested reserves. Relative to NDVI, weather was much less influential on the abundance of guilds at actively changing sites, and slightly more influential in forest reserves and established developments. Following the warm winter and during the dry summer associated with a strong El Niño, migrants and herbivores declined in changing landscapes, insectivores declined in established developments, and herbivores declined while synanthropic species increased in reserves. These changes may presage the effects of climate change in the Pacific Northwest, which are expected to be similar to El Niño conditions. To buffer these changes in native bird communities, planners, developers, regulators, and home owners should minimize the loss of vegetation during development and attempt to quickly achieve mature landscaping that preferably provides food and shelter for birds.

Keywords: primary productivity, richness, avian community, El Niño-Southern Oscillation, NDVI, climate change

Edited by:
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Specialty section:
This article was submitted to Urban Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *28 January 2017*
Accepted: *18 April 2017*
Published: *05 May 2017*

Citation:
Shryock B, Marzluff JM and Moskal LM (2017) Urbanization Alters the Influence of Weather and an Index of Forest Productivity on Avian Community Richness and Guild Abundance in the Seattle Metropolitan Area. Front. Ecol. Evol. 5:40. doi: 10.3389/fevo.2017.00040

INTRODUCTION

Animals increasingly confront new challenges, many resulting from human activities that are extensive and enduring. While many challenges may be difficult to eliminate in a human-dominated world, by understanding their effects it may be possible to affect minor changes that significantly improve the chances for animals to thrive. Two such novel challenges are urbanization

(Miller and Hobbs, 2002) and climate change (IPCC, 2007; Møller, 2013). Both dynamic, abiotic factors affect primary productivity and the biodiversity dependent upon it. In most ecosystems, urbanization influences primary productivity in a predictable way, with a decrease in productivity in proportion to the level of urbanization (Bino et al., 2008). Urbanization also interacts with climate, most obviously in forming the urban heat island, a pattern that has been intensifying in recent years (Hoffmann and Schlünzen, 2013). Concurrent changes in precipitation are less consistent than increased temperatures associated with the urban heat island, but increased downwind precipitation is a common effect of urbanization (Blake et al., 2011). Climate change has observable effects on plant and animal species as well as the communities and ecosystems they compose; changes include phenological mismatches, shifts in species ranges, and response to disturbance (Walther, 2010). Urbanization is exerting an equally pervasive force on ecological communities (Chace and Walsh, 2006; Marzluff et al., 2008), particularly as it removes, fragments, and degrades key habitat elements (Alberti, 2005; Marzluff, 2016) and changes biogeochemical cycles and trophic structure (Goldman et al., 1995; Kaye et al., 2006; Coleman et al., 2011). Animals that find themselves in these newly fragmented landscapes must cope with the changes in vegetation cover and the changes in energy provided by the remaining plants. Despite the severity of these novel changes, the interactive effects of urbanization and climate on plant and animal populations are rarely studied (Liu et al., 2007; Sehgal, 2010; Zuckerberg et al., 2011; Møller, 2013). Investigating the effects of current climatic variability, such as the El Niño-Southern Oscillation (ENSO), in urban ecosystems can help fill this important void.

Birds living in suburban temperate forests experience both seasonal and yearly climatic variability and changes in vegetation associated with urbanization. The severity of the extremes and local habitat conditions dictate which species will thrive and which will decline. Numerous recent studies have found primary productivity or available energy, as measured by satellite based remote sensing, to be a good predictor of species richness at various spatial scales (Laurent et al., 2005; Luck, 2006; Marshal et al., 2006; Goetz et al., 2007; Bino et al., 2008). However, there are relatively few published reports investigating the use of productivity as a predictor of avian species richness in urban landscapes (Gottschalk et al., 2005) and fewer yet that relate this to the effects of climate. In a large-scale study, Luck (2006) found that species richness tracks human population density, despite the effects of urbanization, because humans routinely select the most productive areas for settlement.

Given the inherent variability associated with suburban landscapes, methods that can quickly and accurately assess important variables are imperative. The patchiness of the suburban landscape makes ground based measurement of primary productivity difficult; fortunately, advances in remote sensing techniques allow for rapid and extensive assessment. Normalized Difference Vegetation Index (NDVI), which synthesizes both vegetation quantity and health, has been shown to be an accurate predictor of productivity (Tucker et al., 1985; Piñeiro et al., 2006; Goetz et al., 2007; Bino et al., 2008; Buyantuyev and Wu, 2009). MODIS (MODerate resolution Imaging Spectroradiometer) allows a researcher to derive frequent NDVI observations over a wide scale of time and space without a debilitating sacrifice in spatial resolution (Buyantuyev and Wu, 2009). The improved continuous temporal resolution may provide a more accurate predictor of avian species richness and abundance than simply maximum NDVI of one or two images, particularly in an area undergoing rapid land clearing and subsequent construction. Similarly to using a synthetic variable such as NDVI to represent vegetative cover and primary productivity, the Oceanic Niño Index (ONI), a measure of ENSO that utilizes ocean buoys to measure sea temperature, provides a synthetic climate variable that may represent future patterns if climate change models are accurate (Praskievicz and Chang, 2009; Chen et al., 2011; Todd et al., 2011). Although it is clear that no single weather variable, such as temperature or precipitation, is adequate to predict changes in productivity or bird populations, a composite climate variable that summarizes changes in temperature as well as precipitation, such as the strength of the ENSO, may have stronger predictive power.

The Seattle metropolitan area is an excellent location to study the convergence of a changing landscape and climate. The significant climatic effects of ENSO are relatively well understood and predictable (Kogan, 2000). Due to a change in normal storm track El Niño winters in the PNW are warmer than normal which may benefit resident bird survival; however, the decrease in soil moisture content for the subsequent summer (Baker et al., 2008) can reduce primary productivity (Li and Kafatos, 2000), which could negatively effect bird reproduction. La Niña winters in the PNW are cooler and wetter than normal, which may increase the energetic demands on birds breeding in a cool, rainy spring, and lower reproductive success (Bloxton, 2002; Nott et al., 2002), but will provide increased soil moisture during the plant growing season. In addition, the urbanizing fringes of Seattle are undergoing rapid and extreme changes in land cover, land use and wildlife habitat (Donnely and Marzluff, 2006), which often results in a sharp decline and subsequent, although moderate, rebound in plant primary productivity. Distinct changes in the patterns of avian species richness, which are, at least partially, explained by urbanization, have occurred near Seattle (Marzluff, 2005). However, the degree to which interactions between climate, urbanization and forest extent and productivity (as summarized by NDVI) are shaping this shift is not well understood.

The abnormal weather that results from ENSO in the Pacific Northwest, warm El Niño winters and drier summers, resembles the predicted effects of sustained climate change and provides an opportunity to examine the interaction of a rapidly changing landscape and climate. By comparing long-established residential developments (developed sites) and forested reserves (reserve sites) with sites that were cleared and developed between 2001 and 2010 (changing sites), we examine how weather variability associated with ENSO and changes in NDVI during and following development influence bird populations over time.

We expect NDVI to be strongly correlated with bird populations at changing sites (Marzluff et al., 2016), but less so at developed or reserve sites. The over-riding effects of forest loss at changing sites suggest that weather patterns will have less influence on birds and forest productivity at these sites. However, at reserves and developments we hypothesize that weather will explain more variation in bird populations than NDVI because productivity is less variable than weather in these sites.

METHODS

Study Region and Site Selection

Within the urbanizing fringe of the Seattle Metropolitan area, we selected 21 one-kilometer study sites spanning a gradient of forest land cover, impervious surface and forest patch connectivity (**Figure 1**; details of site selection and composition are in Marzluff et al., 2016). These sites were all below 500 m elevation in dense urban to sparsely populated exurban and second-growth forest (Donnely and Marzluff, 2004, 2006; Blewett and Marzluff, 2005). They represent 3 treatments (5 reserve, 7 developed, and 9 changing sites) in a quasi-experimental, temporal study (Marzluff et al., 2016). Reserve sites are dominated by mid-successional vegetation with at least 70-year-old Douglas-fir trees interspersed with deciduous trees and an understory of ferns and fruiting shrubs closely surrounded by suburban matrix. Developed sites are long established housing subdivisions that have been human dominated for at least 20 years. Changing sites were forested either when the study began or cleared in the few years prior but have since become suburban developments.

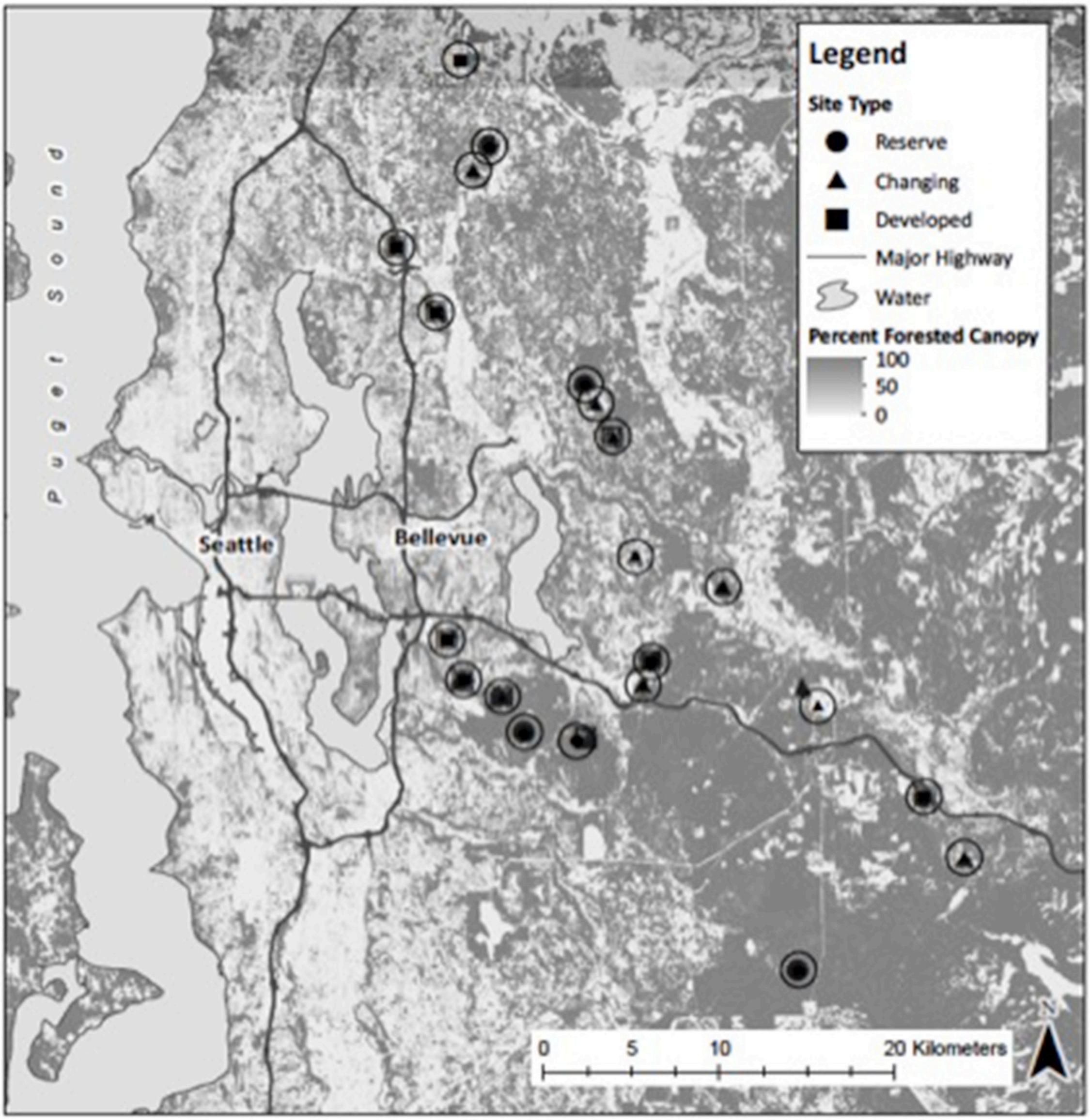

FIGURE 1 | Study sites are spread throughout the Seattle Metropolitan area and across a range of canopy coverage. Canopy cover was obtained from the National Land Cover Database 2001 (Homer et al., 2007).

Although minor development may have continued through 2010 at some changing sites, forest clearing, road building and intense home building typically occurred over 3 years at any one site, with construction starting at the first site in ∼2000.

Bird Surveys

We surveyed bird abundance during the breeding seasons (April to August) of 2001 to 2010 by conducting four 50 m fixed radius point count surveys at four to eight points in each study site each year (Ralph et al., 1993). We conducted the surveys between the hours of 05:00 and 12:00 on days when conditions allowed adequate visibility and audibility of birds (time of surveys were not biased by site type and did not affect detection; Marzluff et al., 2016). Rarely (9% of site years), we only completed three surveys per year due to weather or denial of land access. Additionally, three sites had fewer than eight points; however, as each site was either homogenously forested, small in size or both, fewer points were adequately representative. At all developed and changing sites, the majority, typically six of eight, of survey points were located in the far more heterogeneous urban matrix and the remaining were located in the remnant forest (Donnely and Marzluff, 2006).

Our study goals were to investigate the relative influence of ecological and climatological factors on species abundance and richness within a large avian community. We were not interested in estimating absolute richness or abundance, therefore we chose to design our study to minimize bias in detection, rather than adjust for incomplete detection after data collection. We minimized detection bias by: (1) limiting our survey area to small (50 m-radius), easily defined plots; (2) surveying across the entire breeding season to remove seasonal variation in bird activity; (3) using only 16, expert and seasonally trained observers; (4) curtailing surveys during periods of inclement weather; and (5) limiting surveys to the morning hours and randomizing start times across study sites (Marzluff et al., 2016). Due to inadequate sampling resulting from fixed-radius point counts we did not include nonbreeding birds, raptors, waterfowl, and other species with very specific habitat requirements or large home ranges in this analysis (Ralph et al., 1993).

Our design is appropriate, especially for community studies, where many birds are naturally rare (Banks-Leite et al., 2014). Moreover, while distance sampling is often employed to determine and correct for imperfect detection in bird studies, the basic assumptions of this approach: (1) that birds do not move between the start of the survey and the time at which they are observed, (2) that measurements from the observer to the bird are exact, and (3) that sampled areas are homogeneous are violated in most field settings, including ours (Johnson, 2008; Hutto, 2016; Marzluff et al., 2016). Although failure to correct abundance estimates for imperfect detection may create a bias toward underestimation of relative abundance, rarely does correction affect a study's findings (Banks-Leite et al., 2014). Indeed, correction did not affect conclusions concerning the abundance of birds across changing, developed, or reserve landscapes in our study (Marzluff et al., 2016). In the last year of our study we compared distance sampling and fixed-radius sampling. Our detection probability was high and variation in the density of vegetation among site types did not systematically affect abundance estimates (Marzluff et al., 2016). However, detection was 20% higher at points in open developed sites relative to heavily forested reserves. Despite this potential bias, estimated bird abundance was not uniformly greatest in sites with high detection. Rather, we found that species known to associate with open landscapes were most abundant in developments, while those known to associate with closed forest were most abundant in reserves, regardless of whether we corrected for imperfect detection or did not (Marzluff et al., 2016).

Estimation of occupancy, rather than relative abundance, is a metric applicable to field studies of avian communities such as ours (Hutto, 2016). Small, fixed-radius plots are appropriate for the estimation of occupancy. Several, repeated visits to plots are needed to estimate occupancy, and these repeats should be within a biologically homogeneous sampling period. While we visited each plot three to four times, these visits spanned a period of variable breeding activity (including early sampling of settlement and late sampling after the fledging of nestlings). An increase in sampling effort during early, mid, and late breeding season would be needed to accurately estimate occupancy. Increased sampling was inconsistent with our study goals of wide spatial replication (Banks-Leite et al., 2014); therefore we felt estimation of occupancy was inappropriate for our study.

Avian Richness and Relative Abundance

We classified birds into guilds based upon the following life history characteristics: tolerance of human development, migration, diet, and use of human food subsidies (**Table 1**; assignments for all 58 species in Table S1).

To account for differential survey effort between sites and to normalize our data we calculated annual mean relative abundance as the average number of detections per 10 min, fixed-radius survey per year for each of the adequately sampled bird species and guilds known to breed in the study region (Tables S2, S3, S4). Annual species richness was calculated at the total number of species observed at each site during the four annual surveys (Table S5).

Vegetation Greenness Index

We used images from NASA's MODIS satellite to obtain NDVI, an index of forest greenness in our region, for each research site over the course of the 10-year study. We downloaded the Version 5 MOD13Q1 250 m resolution 16-day composites

TABLE 1 | Bird species were separated in to guilds based on the following life history traits.

Development	Migration	Diet	Food Subsidies
Forest (21)	Neoptropical (24)	Herbivore (18)	Consumer (28)
Early Succession (39)	Short Distance (7)	Insectivore (35)	Avoider (30)
Synanthropic (8)	Resident (27)	Generalist (5)	

Yearly abundances of each guild were calculated for each site taking in to account survey effort. The number of species in each guild is in parentheses. Fifty−eight species total were included in this study. A table that details which species fall into each guild is provided in the supplemental material.

of a gridded level-3 product from the NASA Warehouse Inventory Search Tool (WIST) for January 2001 to December 2010. This results in a maximum of 23 measurements of NDVI for each year, or 230 for each site over the course of this study. In this product NDVI is calculated using ratios of reflectance in the red (645 nm) and near-infrared (858 nm) bands from the best daily images of each 16-day period. Both bands are atmospherically corrected using bi-directional surface reflectances with appropriate feature masking. MODIS13Q1 is a Validated Stage 2 product with extensive accuracy assessments over numerous locations and time points. The MODIS13Q1 product is distributed in Hierachical Data Format–Earth Observation System (HDF-EOS) and visualized in an integerized sinusoidal projection (ISIN) mapping grid (Land Processes Distributed Active Archive Center, 2013).

We obtained research grade GPS (Trimble GeoXT) coordinates for each point count location and projected them in UTM NAD83 using ARCGIS 9.3.3 (ESRI, 2008). The many steps we used to extract usable NDVI data from the MODIS13Q1 product are summarized in **Figure S1**. Briefly, we calculated the geometric mean of the survey point locations within a study site and buffered these with a 1 km^2 circle. Due to the inherent challenges of collecting NDVI data from a satellite, Pixel Reliability classes are provided as a subdataset of the original MODIS13Q1 files. We used the Set Null tool from the Conditional toolset in the ARCGIS Spatial Analyst toolbox to remove any unreliable data by converting pixels with any Pixel Reliability class other than zero (most reliable) to No Data in the NDVI layer. This essentially removed any unreliable data from subsequent calculations. Finally, we calculated the mean NDVI for each composite image at each site using ARCGIS Zonal Statistics with the 1 km^2 buffer around each site center as the zone. At each time point there was a maximum of 21 NDVI pixels included in the each zone; after removing unreliable pixels the actual average was 18.4 with a standard deviation of 2.82. We dropped sites that had <10 reliable pixels from the final calculation of annual mean NDVI. Due to unreliable NDVI data each site had an average of 16.5 (*S.D.* = 1.23) out of a total possible 23 time points, with most of the missing composites coming in the winter months. We calculated the yearly mean NDVI for each site using the values from each composite image. As a result we had annual mean NDVI for each 1 km^2 site drawn from multiple time-points throughout each year (**Figure S1**).

We utilized annual mean NDVI because we were interested in the effects of forest greenness throughout the year. Additionally, we used annual mean NDVI rather than mean summer or annual peak NDVI, because it was calculated from approximately 16 time points, rather than approximately six or one, respectively. Mean annual NDVI was strongly correlated with annual peak NDVI at Changing ($r = 0.93$, $p < 0.01$, $N = 90$), Developed ($r = 0.93$, $p < 0.01$, $N = 70$) and Reserve sites ($r = 0.88$, $p < 0.01$, $N = 50$) throughout the study period, indicating that either measure would correlate similarly with our bird population metrics.

Annual Variation in Weather

We estimated the annual strengths of the El Niño Southern Oscillation using the National Oceanic and Atmospheric Association's Oceanic Niño Index (ONI). This index is calculated as the difference between a 3-month running mean of sea surface temperatures from the historical mean from 1971 to 2000. Numbers >0.5 indicate a strong El Niño and numbers < -0.5 indicate a strong La Nina. Because the effects of the ENSO are felt more strongly during the winter in the study areas, the yearly metrics are the means from the winter preceding the breeding season. For example the metric for 2010 is the mean of September 2009-February 2010.

Rainfall and temperature data was obtained from the NOAA weather station at the Seattle Tacoma International Airport (**Table 2**). The station is 113 m above sea level with Latitude 47°27′N, Longitude 122°19′W. Since specific weather data is not available for each of our study sites we selected one representative station that provided the most complete and accurate data over our study period.

Statistical Analyses

We viewed the experimental unit as the site-year when we describe patterns of variability in abiotic factors and as the correlation between birds and abiotic factors within a site when we assess the relative importance of abiotic factors to birds within each of our three landscapes. Although some sites were monitored for fewer than 10 years, each was monitored over a set of years that represented variation in weather and productivity (**Table 3**). Changing sites, with more variable bird populations were monitored more intensively than reserve and developed sites, where bird populations were relatively stable. We did not sample developed sites from 2004 to 2007 because sufficient field teams were not available.

We used two-way ANOVAs followed by Tukey HSD, *post-hoc* analysis, to describe how bird richness and the abundance of each guild varied with year, site type, and the interaction of year and site.

We quantified the relationship between richness or guild abundance and NDVI or ENSO with univariate and partial correlations that included every adequately sampled year at a given site (**Table 3**). Correlation coefficients (e.g., between richness and NDVI) for each site became the raw data for subsequent description of effect size (average coefficient) and hypothesis testing (whether 95% CI of coefficient includes 0). Coefficients were Fisher-transformed, a variance-stabilizing transformation that makes correlation coefficients suitable for hypothesis testing, before being averaged for comparison among site types and guilds.

All statistical tests were done using SPSS (v.19; IBM, 2010).

RESULTS

Variation in NDVI and Weather among Years and Sites

NDVI varied between reserve, developed and changing sites ($F = 115.51$, $p < 0.01$, $df = 2, 207$), but not across years at each site type (**Figure 2**). Reserve sites had the highest and most consistent annual, mean NDVI (0.83, *S.E.* = 0.004; $n = 50$). This exceeded NDVI at developed sites (mean = 0.68; *S.E.* = 0.006; $p < 0.01$; $n = 70$) and changing sites (mean = 0.72; *S.E.* = 0.007; $p < 0.01$; $n = 90$). Variance in mean annual NDVI was significantly less at reserve sites relative to developed sites ($F = 4.98$, $p < 0.01$,

TABLE 2 | Weather and climate data utilized in this study.

Year	ONI winter	ONI breeding	Mean high (C°)	Mean low (C°)	Yearly mean (C°)	Precipitation (cm)
2001	−0.55	0.0	11.67	4.42	8.06	37.26
2002	−0.05	0.65	11.44	5.09	8.29	74.32
2003	1.25	0.25	12.57	5.46	9.04	52.65
2004	0.45	0.45	12.10	5.32	8.73	74.42
2005	0.75	0.4	11.86	5.20	8.55	47.68
2006	−0.4	0.1	12.14	5.72	8.94	77.06
2007	0.85	−0.1	11.73	4.74	8.24	90.20
2008	−1.2	−0.45	10.99	4.81	7.91	63.50
2009	−0.4	0.3	11.44	4.75	8.11	52.02
2010	1.45	0.1	12.81	5.88	9.35	72.87

Weather data was collected from Seattle Tacoma International Airport. The station is 370 ft. above sea level with Latitude 47°27′N, Longitude 122°19′W. The Oceanic Niño Index (ONI), a measure of El Niño strength was obtained from NOAA sea temperature monitoring buoys in the Pacific Ocean. ONI below −0.5 indicates La Nina conditions, −0.5 to 0.5 is neutral and above 0.5 indicates El Niño conditions; below −1.0 or above 1.0 is considered a strong La Nina or El Niño, respectively.

TABLE 3 | Total number of years of data for each site type and site.

Site type and name	Years of data
Changing sites	87
Beaver Cleaver	10
Cougar Neighborhood	9
Issaquah Highlands	10
Montare	10
Redmond Ridge	10
Snoqualmie Ridge	10
Treemont Estates	10
Union Hill	9
Uplands	9
Developed sites	35
Beardslee	5
Forster Woods	4
Hawthorne	5
Highlands	3
Old Black Nugget	6
Somerset	4
Totem Falls	4
Westwood	4
Reserve sites	40
Cedar Watershed Preserve	7
Cougar Mountain Park	9
Lee Forest	10
Redmond Watershed Preserve	9
Squak Mountain	5

Every site was not monitored over the 10-year study period, but each has data from representative years.

$df = 4, 6$) and changing sites ($F = 4.02$, $p < 0.01$, $df = 4, 8$). Changing and developed sites had similar variance ($F = 1.25$, $p = 0.15$, $df = 8, 6$).

The large-scale weather pattern of ENSO (ONI) was a uniform variable across all study sites, but was variable from year to year (**Figure 2**; $F = 20.4$, $p < 0.01$, $df = 9, 10$). The winter of 2001 was a moderate La Niña year with the ONI equal to −0.55; the yearly mean low temperature was 6.72°C and there was 95.4 cm of precipitation. The winter of 2008 was a stronger La Niña with ONI < -1.0; the yearly mean low temperature was 6.89°C and there was 78 cm of precipitation (**Table 2**). Meanwhile, 2003 and 2010 experienced strong El Niño conditions, with ONI >1; the yearly mean low temperature was 7.61 and 7.67°C with 106.1 cm and 119.35 cm of precipitation, respectively (**Table 2**). Over the 10 years of this study winter ONI was strongly positively correlated with mean winter temperature in the Seattle area ($r = 0.73$, $p = 0.016$, $N = 10$), confirming that winter temperatures were warmer in El Niño years.

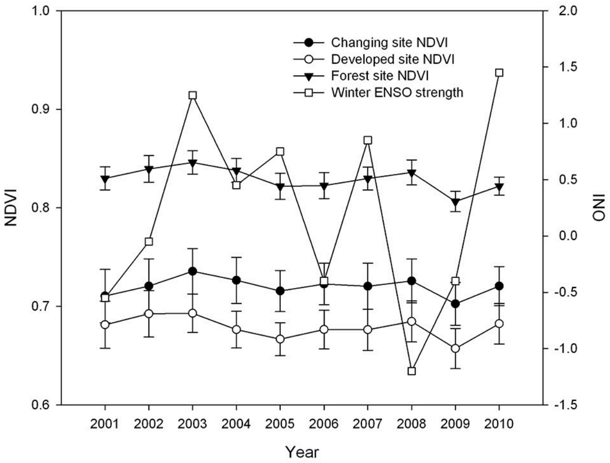

FIGURE 2 | Annual changes in mean NDVI and ONI. NDVI was assessed 23 times yearly at each site from MODIS satellite images, and error free data was averaged to produce yearly data for each site. Error bars represent 95% confidence intervals around the mean. ONI is a measure of ENSO strength; data given here represents ENSO strength of the winter preceding the breeding season.

Annual Variation in Bird Species Richness

Bird species richness differed among site types over time ($F = 6.34$, $p < 0.01$, $df = 2, 18$; **Figure 3**, Table S5). Using yearly means

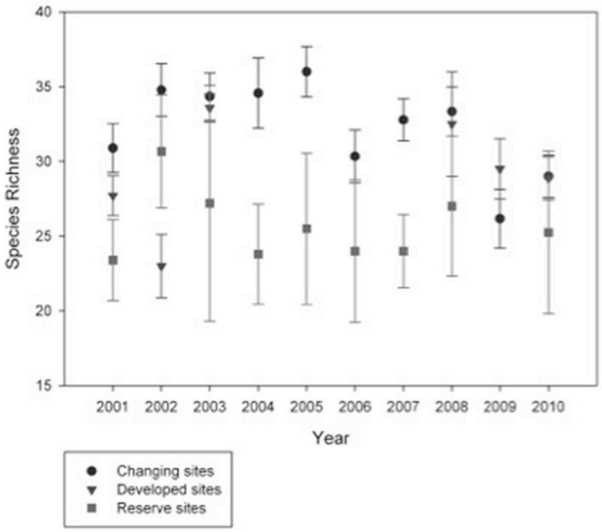

FIGURE 3 | Species richness is greater at Changing sites than it is at Reserve sites. Point counts were conducted during the breeding season and species richness was calculated at the presence of any species in at least one of the point counts. Annual richness for each site was averaged by site type and the error bars are the standard error of those means.

for each site, the mean richness was 32.7 bird species ($S.E. = 1.26$, $n = 9$) at changing sites, 29.3 ($S.E. = 0.89$, $n = 7$) at developed sites and 26.0 ($S.E. = 1.76$, $n = 5$) at reserve sites. Reserve sites had significantly lower mean richness than changing sites (mean difference = 6.67, $q = 5.02$, $p = 0.007$, $n = 6.6$), but changing and developed sites were not statistically different (mean difference = 3.38, $q = 2.55$, $p = 0.15$, $n = 6.6$).

Bird species richness varied by year at changing ($F = 2.74$, $p < 0.01$, $df = 9, 77$) and developed sites ($F = 5.01$, $p < 0.01$, $df = 5, 25$), but was similar across the 10 years at reserve sites, which were not undergoing any landscape changes ($F = 0.97$, $p = 0.481$, $df = 9, 30$; **Figure 3**). Mean richness at changing sites ranged from 36.0 in 2005 to 26.2 in 2009, which was the only significant pair-wise difference (Tukey HSD: mean difference = 9.83, $q = 5.62$, $p = 0.02$); over ten years the mean was 32.4 and the coefficient of variation was 0.185. Mean richness at developed sites ranged from 23.0 in 2002 to 33.57 in 2003, (mean difference = 10.6, $q = 6.00$, $p < 0.01$); the overall mean was 29.23 and the coefficient of variation was 0.159. Mean richness at developed sites ranged from 23.4 in 2001 to 30.7 in 2002, which was not a statistically significant pair-wise difference; the 10-year mean was 25.65 and the coefficient of variation was 0.19.

Does the Relationship of Richness to NDVI and Weather Vary with Site Type as Hypothesized?

Annual variation in richness was correlated with NDVI and weather, but this relationship depended on site type (**Table 5**). At changing sites, richness increased with increasing NDVI (the average of each site's univariate correlation: $r = 0.26 \pm S.E. = 0.10$; the average of each site's partial correlation, holding weather constant: $r = 0.29 \pm S.E. = 0.10$), and, as hypothesized, was very weakly correlated with weather (partial correlation controlling for variable NDVI indicates slightly higher richness during El Niño conditions, $r = 0.03 \pm S.E. = 0.11$). Bird species richness varied with weather at developed sites, being greatest during El Niño conditions (mean $r = 0.44 \pm S.E. = 0.15$; the annual change in NDVI, which was minimal, had little effect as hypothesized; **Figure 2**). Species richness was not significantly correlated with NDVI or weather at reserve sites (**Table 5**). This lack of association was as hypothesized for NDVI, but not for weather.

Variation in Abundance among Years and Sites

There were expected and obvious differences in the total abundance of various guilds in each site type (**Table 4**). Forest species were approximately twice as abundant in reserve sites than developed and changing sites (mean difference = 2.84, 2.50; $q = 5.98, 5.26$; $p = 0.002, 0.005$). Synanthropic species were most abundant in developed and changing sites (significantly greater in developed than reserve sites: mean difference = 2.38, $q = 13.7$, $p = 0.004$). Herbivores were over 60% more abundant at changing sites than reserve sites (mean difference = 1.46, $q = 4.17$, $p = 0.024$). Generalist relative abundance was nearly twice as great at developed sites than reserve sites (mean difference = 1.32, $q = 5.11$, $p = 0.009$), and both site types were similar in abundance to changing sites. Subsidy consumer annual relative abundance was 4.31 at reserve sites, which was significantly less than at changing sites (mean difference = 2.07, $q = 4.16$, $p = 0.025$) and developed sites (mean difference, 2.54, $q = 5.10$, $p = 0.009$). The relative abundance of migrants, residents, insectivores and subsidy avoiders was similar across all site types (Tables S2, S3, S4).

Relative abundance was most variable across years in changing sites and least variable in reserve sites (**Figures 4**, **5**). At changing sites all nine guilds, except synanthropes ($F = 1.54$, $p = 0.15$, $df = 9, 77$), exhibited variable abundance from year to year; for example, forest species ($F = 2.62$, $p = 0.01$, $df = 9, 77$), residents ($F = 4.51$, $p < 0.01$, $df = 9, 77$) and generalists ($F = 2.39$, $p = 0.02$, $df = 9, 77$) were variable over time. There was slightly less annual variability (six of nine guilds) at developing sites than changing sites: residents ($F = 3.17$, $p = 0.02$, $df = 5, 25$), synanthropes ($F = 2.92$, $p = 0.03$, $df = 5, 25$), herbivores ($F = 5.76$, $p < 0.01$, $df = 5, 25$), insectivores ($F = 5.64$, $p < 0.01$, $df = 5, 25$), subsidy consumers ($F = 3.53$, $p = 0.02$, $df = 5, 25$) and subsidy avoiders ($F = 2.93$, $p = 0.03$, $df = 5, 25$) did not have consistent relative abundance from year to year. Unlike changing and developed sites, guild abundance was not significantly different from year to year at reserve sites (**Figures 4**, **5**).

Does the Relationship of Abundance to NDVI and Weather Vary with Site Type as Hypothesized?

Annual change in NDVI was strongly correlated with the abundance of all guild types in changing sites, as hypothesized (**Table 5**). The weakest relationship was with generalists ($r = 0.27 \pm S.E.$ 0.102 and the partial correlation, holding weather

TABLE 4 | Guild abundance varies by site type.

Site type	Forest	Synthropic	Migrant	Resident	Insectivores	Herbivores	Generalists	Subsidy consumer	Subsidy avoider
Changing	2.41 ± 0.33	1.84 ± 0.31	2.57 ± 0.31	5.96 ± 0.60	4.72 ± 0.56	3.86 ± 0.44	2.00 ± 0.25	6.38 ± 0.48	4.19 ± 0.48
Developed	2.07 ± 0.38	2.74 ± 0.82	1.55 ± 0.31	7.01 ± 0.92	3.64 ± 0.61	3.61 ± 0.68	2.85 ± 0.46	6.85 ± 0.96	3.24 ± 0.57
Reserve	4.91 ± 0.51	0.36 ± 0.11	1.70 ± 0.20	5.57 ± 0.56	4.29 ± 0.48	2.40 ± 0.32	1.53 ± 0.21	4.31 ± 0.48	3.90 ± 0.42

Birds were assigned into guilds based on life history traits. Guild abundance was averaged for each site of the study period and then averaged again by site type and is reported as mean abundance ± S.E. For Changing sites N = 87, Developed sites N = 35 and Reserve sites N = 40.

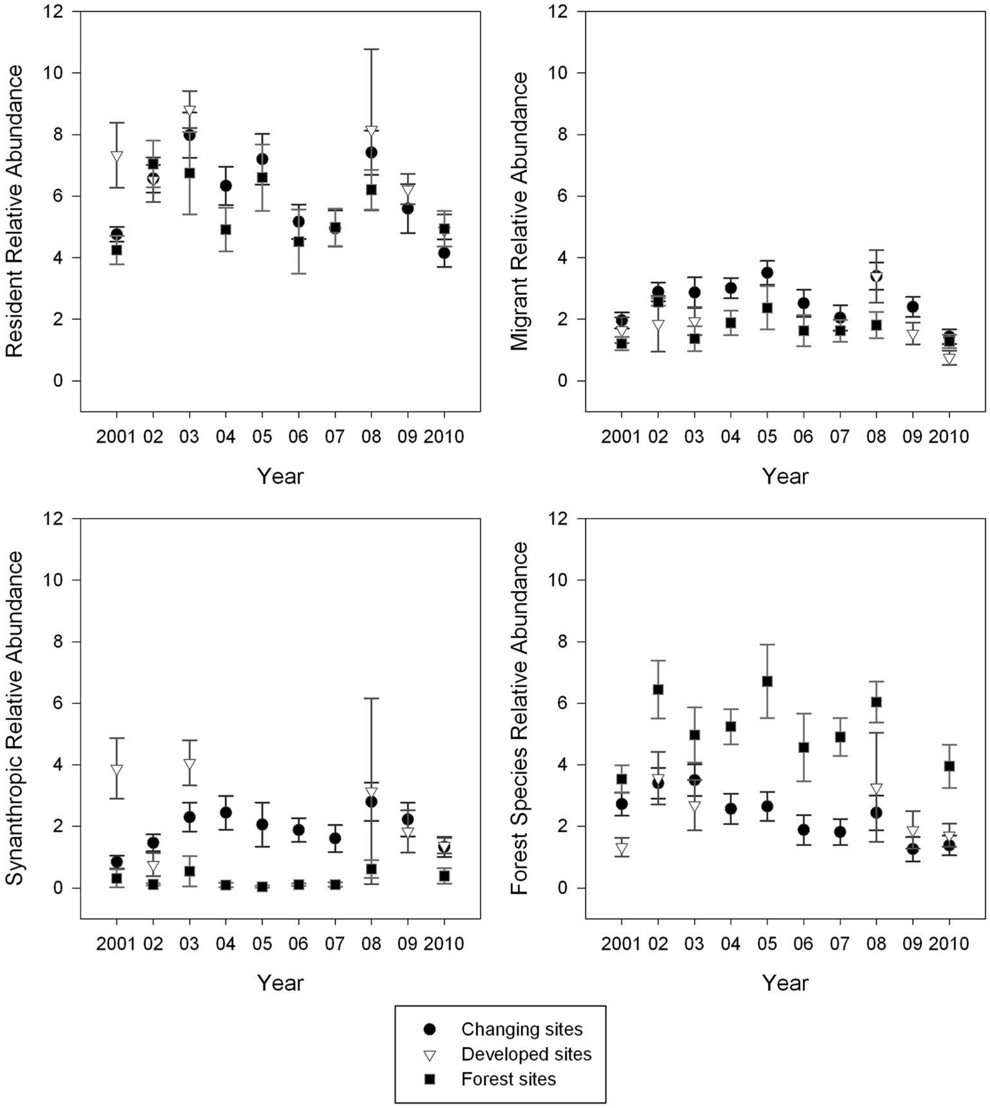

FIGURE 4 | Mean guild abundance was most variable at Changing sites and least variable at Reserve (forest) sites. Point counts were conducted during the breeding season and relative abundance was calculated for each site. Sites were averaged by site type for each year. Those means are presented here with standard error as error bars.

constant, was $r = 0.26 \pm S.E.\ 0.12$). In contrast, as expected in changing sites only 2 of 18 correlations between a guild's abundance and weather were statistically significant. The strongest correlations were negative indicating that migrants, synanthropes, subsidy consumers and herbivores were more abundant following La Niña winters and/or less abundant following El Niño winters.

Variation in abundance within reserve sites was rarely related to NDVI and slightly more correlated with weather as predicted; only 1 of 18 correlations between various guild abundances were

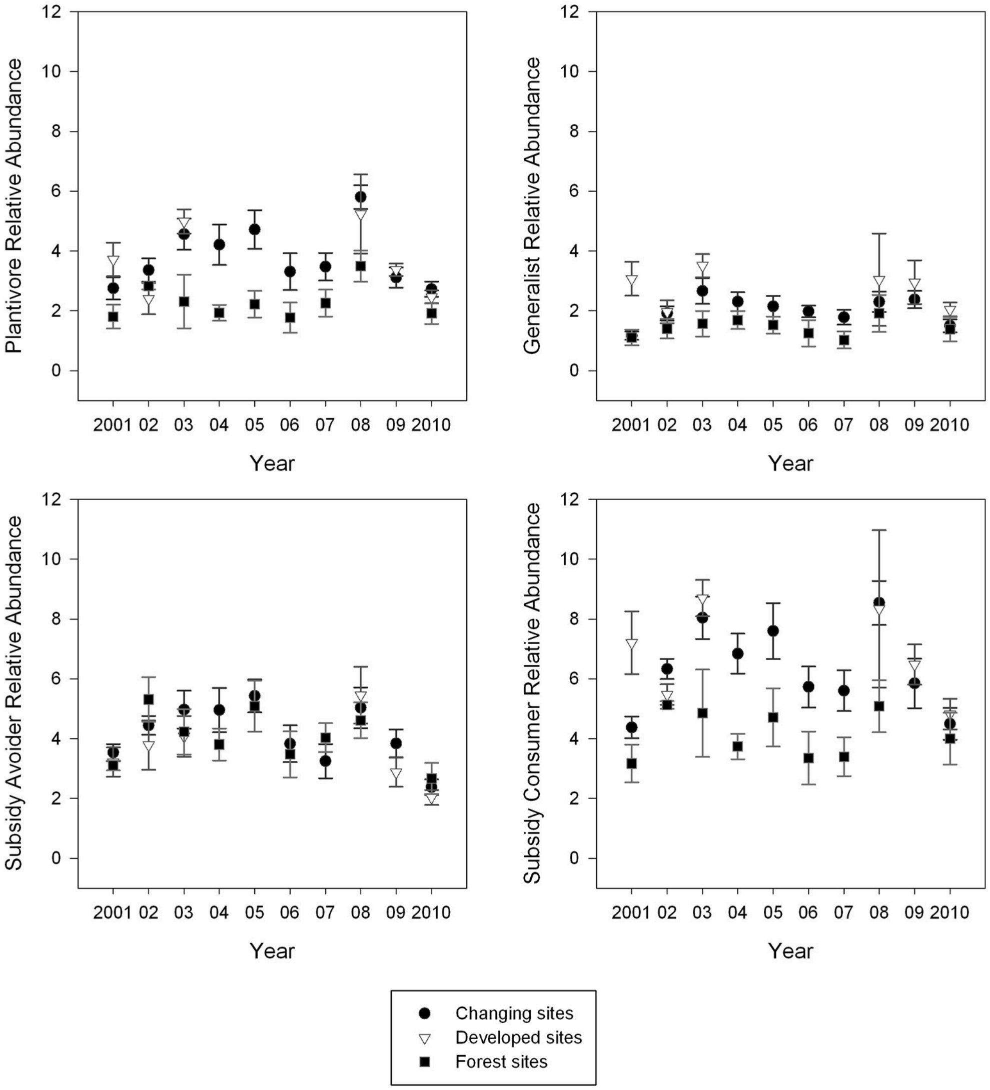

FIGURE 5 | Mean guild abundance was most variable at changing sites and least variable at Reserve (forest) sites. Point counts were conducted during the breeding season and relative abundance was calculated for each site. Sites were averaged by site type for each year. Those means are presented here with error bars representing standard error.

significant for NDVI, while 3 of 18 were significant for weather (**Table 5**). Synanthropic species abundance was correlated with weather (partial correlation holding NDVI constant: mean $r = 0.56 \pm 0.19$). There was also a strong negative correlation between herbivore species abundance and weather (univariate mean $r = -0.36 \pm 0.06$; this improved nearly 15% when NDVI was held constant).

The correlation between bird abundance and weather or NDVI was weakest in developments (1 of 18 guilds correlated with NDVI in partial correlations and 1 of 18 guilds significantly correlated with weather; **Table 5**). Insectivore abundance was positively correlated with NDVI when weather was held constant ($r = 0.52 \pm 0.14$). Insectivores were also strongly correlated with weather ($r = -0.68 \pm 0.19$), albeit negatively; indicating that insectivores were more abundant following La Niña and/or less abundant following El Niño winters.

DISCUSSION

The process of converting native forests to suburban housing developments significantly changes the interplay of forest productivity and weather on bird relative abundance and species richness. In changing sites, where forests are being converted to residential settlements, the relative abundance of various bird guilds and overall species diversity was influenced strongly by changes in NDVI, a proxy for the amount and productivity of forest, and, to a lesser extent, by variation in the winter weather. At changing sites this strong response to loss of native forest cover appears to overshadow typical breeding bird community

TABLE 5 | Bird richness and abundance was primarily correlated with primary productivity at Changing Sites, weather at Reserve Sites, and weakly by both factors at Developed Sites.

	Productivity	Weather Held Constant	Weather	Productivity Held Constant
CHANGING SITES				
Richness	0.256 ± 0.096 ($p = 0.028$)*	0.293 ± 0.100 ($p = 0.019$)*	0.056 ± 0.096 ($p = 0.579$)	0.032 ± 0.111 ($p = 0.777$)
Forest	0.303 ± 0.096 ($p = 0.013$)*	0.337 ± 0.097 ($p = 0.008$)*	−0.024 ± 0.111 ($p = 0.837$)	−0.082 ± 0.120 ($p = 0.514$)
Synthropic	0.294 ± 0.081 ($p = 0.007$)*	0.327 ± 0.094 ($p = 0.008$)*	−0.148 ± 0.087 ($p = 0.129$)	−0.205 ± 0.099 ($p = 0.073$)
Migrant	0.297 ± 0.089 ($p = 0.010$)*	0.357 ± 0.092 ($p = 0.005$)*	−0.200 ± 0.088 ($p = 0.053$)	−0.243 ± 0.105 ($p = 0.049$)*
Resident	0.409 ± 0.079 ($p = 0.001$)*	0.442 ± 0.087 ($p = 0.001$)*	−0.066 ± 0.081 ($p = 0.443$)	−0.135 ± 0.098 ($p = 0.207$)
Generalist	0.269 ± 0.102 ($p = 0.029$)*	0.257 ± 0.115 ($p = 0.057$)	0.023 ± 0.103 ($p = 0.833$)	−0.047 ± 0.112 ($p = 0.683$)
Herbivore	0.317 ± 0.093 ($p = 0.009$)*	0.341 ± 0.117 ($p = 0.019$)*	−0.203 ± 0.097 ($p = 0.069$)	−0.279 ± 0.113 ($p = 0.039$)*
Insectivore	0.355 ± 0.111 ($p = 0.013$)*	0.323 ± 0.086 ($p = 0.006$)*	−0.124 ± 0.094 ($p = 0.222$)	−0.171 ± 0.123 ($p = 0.204$)
Subsidy Consumer	0.371 ± 0.083 ($p = 0.002$)*	0.505 ± 0.121 ($p = 0.003$)*	−0.105 ± 0.081 ($p = 0.228$)	−0.186 ± 0.099 ($p = 0.097$)
Subsidy Avoider	0.310 ± 0.090 ($p = 0.009$)*	0.288 ± 0.062 ($p = 0.002$)*	−0.171 ± 0.116 ($p = 0.178$)	−0.215 ± 0.132 ($p = 0.141$)
	Productivity	**Weather Constant**	**Weather**	**Productivity Constant**
DEVELOPED SITES				
Richness	0.381 ± 0.226 ($p = 0.143$)	0.188 ± 0.466 ($p = 0.701$)	0.439 ± 0.148 ($p = 0.025$)*	0.353 ± 0.244 ($p = 0.199$)
Forest	0.395 ± 0.226 ($p = 0.130$)	0.501 ± 0.240 ($p = 0.082$)	0.600 ± 0.446 ($p = 0.227$)	0.019 ± 0.480 ($p = 0.970$)
Synthropic	0.298 ± 0.223 ($p = 0.230$)	0.318 ± 0.219 ($p = 0.197$)	−0.025 ± 0.175 ($p = 0.893$)	0.003 ± 0.175 ($p = 0.985$)
Migrant	0.263 ± 0.169 ($p = 0.171$)	1.006 ± 0.542 ($p = 0.113$)	−0.359 ± 0.213 ($p = 0.143$)	−0.999 ± 0.566 ($p = 0.128$)
Resident	0.259 ± 0.260 ($p = 0.358$)	0.441 ± 0.373 ($p = 0.282$)	−0.093 ± 0.182 ($p = 0.627$)	−0.380 ± 0.275 ($p = 0.216$)
Generalist	0.109 ± 0.271 ($p = 0.701$)	−0.100 ± 0.417 ($p = 0.818$)	−0.093 ± 0.282 ($p = 0.752$)	−0.099 ± 0.433 ($p = 0.826$)
Herbivore	0.301 ± 0.280 ($p = 0.323$)	0.237 ± 0.341 ($p = 0.513$)	0.038 ± 0.171 ($p = 0.830$)	−0.095 ± 0.243 ($p = 0.709$)
Insectivore	0.047 ± 0.103 ($p = 0.661$)	0.520 ± 0.143 ($p = 0.011$)*	−0.382 ± 0.163 ($p = 0.058$)	−0.681 ± 0.187 ($p = 0.011$)*
Subsidy Consumer	0.242 ± 0.300 ($p = 0.450$)	0.344 ± 0.453 ($p = 0.476$)	−0.110 ± 0.201 ($p = 0.604$)	−0.314 ± 0.327 ($p = 0.374$)
Subsidy Avoider	0.128 ± 0.074 ($p = 0.135$)	0.444 ± 0.202 ($p = 0.070$)	−0.276 ± 0.208 ($p = 0.232$)	−0.559 ± 0.295 ($p = 0.107$)
RESERVE SITES				
Richness	0.245 ± 0.196 ($p = 0.279$)	0.287 ± 0.206 ($p = 0.236$)	0.015 ± 0.195 ($p = 0.942$)	−0.030 ± 0.207 ($p = 0.890$)
Forest	0.278 ± 0.234 ($p = 0.300$)	0.278 ± 0.248 ($p = 0.326$)	−0.098 ± 0.131 ($p = 0.498$)	−0.217 ± 0.124 ($p = 0.157$)
Synthropic	0.176 ± 0.143 ($p = 0.286$)	0.209 ± 0.073 ($p = 0.046$)*	−0.559 ± 0.224 ($p = 0.067$)	−0.560 ± 0.188 ($p = 0.040$)*
Migrant	0.319 ± 0.332 ($p = 0.391$)	0.258 ± 0.325 ($p = 0.472$)	−0.025 ± 0.163 ($p = 0.887$)	−0.049 ± 0.126 ($p = 0.718$)
Resident	0.363 ± 0.169 ($p = 0.097$)	0.296 ± 0.181 ($p = 0.177$)	0.087 ± 0.138 ($p = 0.564$)	0.020 ± 0.124 ($p = 0.878$)
Generalist	0.168 ± 0.122 ($p = 0.240$)	0.141 ± 0.166 ($p = 0.442$)	−0.136 ± 0.125 ($p = 0.338$)	−0.139 ± 0.160 ($p = 0.435$)
Herbivore	0.301 ± 0.256 ($p = 0.305$)	0.364 ± 0.258 ($p = 0.231$)	−0.355 ± 0.064 ($p = 0.005$)*	−0.396 ± 0.093 ($p = 0.013$)*
Insectivore	0.516 ± 0.311 ($p = 0.173$)	0.471 ± 0.333 ($p = 0.230$)	0.200 ± 0.171 ($p = 0.308$)	0.185 ± 0.168 ($p = 0.334$)
Subsidy Consumer	0.223 ± 0.191 ($p = 0.308$)	0.200 ± 0.219 ($p = 0.412$)	−0.049 ± 0.116 ($p = 0.692$)	−0.066 ± 0.145 ($p = 0.675$)
Subsidy Avoider	0.434 ± 0.262 ($p = 0.173$)	0.765 ± 0.611 ($p = 0.279$)	−0.093 ± 0.167 ($p = 0.605$)	−0.592 ± 0.510 ($p = 0.310$)

*We monitored bird richness and abundance for 10 years in the urbanizing fringe of Seattle using point counts and estimated productivity (NDVI) and weather (ONI) ADD WHAT + FOR WEATHER MEAN using remote sensing. In addition to zero−order correlations, partial correlations were computed for each independent variable, while holding the other constant; for example, the Weather Held Constant column is the correlation between bird guild and productivity with weather held constant. Correlations were computed for each site over the study period, Fisher transformed and averaged by site type. Values reported are r ± S.E. (p-value). Mean correlation coefficients significantly different than 0 are designated with a *. For Changing Sites n = 9, Developed Sites n = 7 and Reserve Sites n = 5.*

responses to climate of the preceding winter resulting from El Niño or La Niña. However, even in these dynamic lands, migrants and herbivores were less abundant following warm El Niño winters, suggesting that detrimental reduction in fruit due to a lack of summer soil moisture was compounded by forest loss.

In the typical warm and dry summers of the PNW, water is often a limiting resource to plant growth, leaving plants to compete for moisture stored in the soil from the previous winter. If soil moisture is not replenished during the winter, plants can suffer drought effects, resulting in slower growth (Peterson et al., 2002) and reduced fruit and seed production during the subsequent breeding season (Herrera, 1991; Selås, 2000); changes in precipitation associated with ENSO have been shown to affect soil moisture content (Nott et al., 2002). It appears that the warmer, dryer winters associated with El Niño in the PNW (Thomson et al., 2003) exacerbate soil-moisture deficiencies for the upcoming breeding season and negatively affect summer herbivore abundance. Changes in rainfall associated with ENSO also correlate with avian reproductive success in the PNW (Nott et al., 2002). Thus, reduced herbivore abundance could result from reduced adult populations as well as reduced reproductive success of those adults. In contrast, the cooler and wetter winters associated with La Niña (Thomson et al., 2003) will ensure the soil is adequately moist to allow for greater fruit and seed production, which would likely result in a greater number of nesting birds and nest productivity.

In contrast to actively developing landscapes, in nearby forested reserves or established subdivisions annual variation in NDVI was not strongly correlated with relative abundance or diversity of birds. As in changing landscapes, partial correlations indicated that herbivores were negatively associated with ONI in reserve sites: abundance during the breeding season declined following a strong El Niño winter and increased following a La Niña. Similarly, insectivores in developments did poorly in years with dryer winters and better in years with wetter winters probably because vigorous plant growth throughout the summer supported a greater number of insects (Nott et al., 2002). In reserves, synanthropic species also declined following warm, dry El Niño winters perhaps because forests sites lack human supplements that could buffer the effects of a dry summer. Given our sampling methods, this correlation may be a result of local emigration or actual reduction in productivity.

Given that significant correlations between bird abundance and weather are relatively few compared to correlations between birds and NDVI, climate appears to play a secondary role to vegetative cover in shaping avian relative abundance. This emphasizes the importance of maintaining productive forest cover for birds, especially insectivores and herbivores given that they are also affected by climate. Moreover, in the face of expected changes in the PNW climate, the general finding that guilds fared best following cooler and wetter La Niña conditions (which are expected to be less common in the future) suggests that the composition of northwestern forest bird communities is likely to change. We would expect declines in the abundance of migrants and herbivores and increases in the abundance of permanent residents sensitive to winter cold, such as Bewick's and Pacific wrens. However, if future climate includes prolonged summer droughts that limit primary productivity of the forests that remain, then insectivores, even permanent residents, would likely suffer.

In the developed landscapes of the Pacific Northwest, reserves and developments may work together to buffer birds from some effects of our changing climate. Site preparation that minimizes forest loss during construction and practices that accelerate growth of fruit and seed-bearing shrubs and trees where forests are cleared would increase the resilience of bird populations in developments (Goddard et al., 2010). Supplemental water and food provided by residents would further bolster bird populations during extreme climatic events (Grubb and Cimprich, 1990). While subdivisions now support a great number of species (Marzluff, 2005) and this diversity may increase in a warmer environment (**Table 5**), native forest reserves are required by many species (e.g., Pacific wren; Marzluff et al., 2016). Reserves may also be necessary for a wider diversity of species in the dynamic urban landscape. By providing refuge, reserves enable mobile species to recolonize marginal areas following particularly stressed years (Neuschulz et al., 2013) or during development of neighboring habitat.

Principally homogenous, forested reserves support the fewest number of species over time, but annual mean richness, and abundance of all guilds does not fluctuate from year to year. The lack of correlation with weather was not expected. However, relative stability in reserve bird communities may be a result of the absence of large disturbances and mild weather conditions of the PNW that allow for consistent yearly habitat conditions. Although it may be possible that annual mean NDVI is simply not the correct remote sensing modality for predicting bird abundance in dense forest (Ranganathan et al., 2007), density-dependent factors are likely playing a more important role in shaping bird relative abundance at reserve sites than at changing sites.

The response of most guilds to differences in the landscapes we studied was as expected (more forest specialists in reserves, more synanthropic species association with developments, etc. Marzluff et al., 2016). Interestingly, herbivores were more abundant in changing sites than reserve sites, while being of intermediate abundance in developments. This is likely because reserve sites are dominated by dense overstory trees, with fewer fruiting shrubs in the understory. Developed sites, in contrast, may have fruit and seed producing shrubs included in the landscaping or present as weeds after land clearing. The landscaping at developed sites also includes numerous non-native species, which would increase the plant diversity and lengthen the time during which fruit is available to birds. Additionally, developed sites have more edge that could provide an area for fruiting understory shrubs to thrive. Meanwhile, changing sites may have a greater number of early successional weeds and shrubs that produce fruit or seeds during land clearing and subsequent landscaping with such plants. Particularly in the PNW, Himalayan blackberries (*Rubus armeniacus*) can become dominant in disturbed landscapes, so if the changing areas remain disturbed for an extended period, herbivores may become more abundant. Catterall et al. (1998) saw a similar pattern in some frugivore species and hypothesized that the landscaping, with fruiting and flowing plants, in suburban areas benefits these species.

Limitations and Future Directions

Our use of NDVI as a measure of forest productivity may have reduced our ability to ascertain the true influence of productivity on bird communities. A more direct measure of net primary productivity or an index, such as NDVI, that is derived at a spatial resolution finer than was available to us (250 m) may reveal a tighter link between productivity and birds. Determination of NDVI at a finer resolution may be especially important in urban areas where any annual variation in reflectance from vegetation in large pixels may be masked by annually consistent reflectance from impervious structures within the pixel. However, the seemingly contrary finding that weather and forest productivity, as gaged by NDVI, were not strongly correlated at our study sites, may reflect real patterns in a human-dominated land. For example, perhaps the intense land cover changes at changing sites resulting from anthropogenic forest clearing simply overshadow the changes in forest productivity associated with yearly weather fluctuations. In addition, developed sites, with their manicured landscaping likely receive enough supplemental water and human care to maintain a stable primary productivity through dryer summers of El Niño.

Reserve sites have the greatest and most consistent productivity of the three site types: yet even here the NDVI of established, dense conifer-dominated forests was insensitive to fluctuating weather conditions. This may represent a real limitation of our measure of NDVI. Although water may be limiting during the summer of El Niño years at reserve sites the overstory trees may be able to outcompete the understory for water well enough to maintain a photosynthetically-active canopy which is primarily measured with NDVI. Additionally, by using the yearly mean NDVI rather than a yearly peak NDVI some of the summer drought signals may have been masked. We used yearly mean NDVI because the study area is relatively temperate with a long growing season and there are many resident birds included in this study that may be sensitive to changes in productivity outside the summer months. Additionally, it is known that NDVI can become saturated as the ratio of reflectance approaches 1:1 (Wang et al., 2003), so minor changes that might have been correlated with changes in weather at reserve sites would not be evident due to errors associated with the NDVI measurement. Although beyond the scope of this study, it may be possible to eliminate some of the errors of saturation by utilizing either ground-based or airborne Light Detection and Ranging (LIDAR) to very accurately measure percent canopy cover and understory density, utilizing these variable to improve regressions. Despite these limitations, it has been shown that weather is strongly correlated with primary productivity on a large scale (Box et al., 1989), so partial correlations are appropriate for this study.

Future studies of the relationships between bird population demographics and large-scale variables such as primary productivity or climate should focus on addressing issues of spatial and temporal scales encountered in this study. At a finer scale, site-specific measurements of climatic variables may improve the correlations between productivity and weather and decrease the variability of the correlations between weather and bird abundance. This could be accomplished by installing relatively simple data loggers to record rainfall, temperature and soil moisture at each site. Understanding subtle changes in land cover and productivity at a finer resolution could be improved by utilizing finer spatial resolution imagery or LIDAR to quantify the area and distribution of gardens and small green spaces over time. Work to assess land cover change at very fine spatial resolution is already ongoing at these study sites and will likely yield an increased understanding of the nuanced factors that determine how birds respond to urbanization. Additionally, to assess not only the primary productivity, but also the "bird-friendliness" of these green spaces, on the ground surveying of the gardens for food, cover and nesting locations could be carried out alongside the bird surveys. The garden surveys would be a good opportunity for a partnership with a backyard wildlife group such as the Audubon Society or Backyard Wildlife Sanctuary Program Washington Department of Fish and Wildlife (2013) the partner group could also assess some of the gardens and make recommended improvements for wildlife. A longer-term study could then investigate if the garden improvements change the relationships between productivity and bird populations at developed or changing sites. A finer scale examination of the changes in productivity associated with urbanization may reveal further insights into their impacts on bird abundance and richness.

To understand how larger scale factors influence the correlations between birds, productivity and weather it would be ideal to examine how the productivity of the land surrounding the study sites differs between sites and site types. Collecting NDVI data from the same MODIS products in 2 km^2 concentric around the bird survey sites would provide some insight about the surrounding land, which likely plays an important role in shaping these relationships. Considering weather patterns over a larger time scale could also improve understanding. We did not investigate lags in the effects of weather, though in some cases bird abundance (Pearce-Higgins et al., 2015; Gorzo et al., 2016) is related to the past as well as current year's weather. Future studies should account for possible influences of past weather conditions on birds and consider nonlinear as well as linear responses of birds to weather patterns (Pearce-Higgins et al., 2015).

The objectives of a study invariably drive the methods employed. In our case, our approach to surveying birds was influenced by our aim to understand how a community comprised of abundant as well as rare species responded to environmental conditions across three profoundly different landscapes. To do so, we opted to maximize spatial and annual replicates rather than temporal replicates within a season at a particular site. This decision compromises our ability to estimate occupancy, while increasing our ability to more accurately measure relative abundance. In addition, because distance estimation is difficult and likely biased by undetected movement of birds in forested lands and because developed lands are heterogeneous, we opted to control for imperfect detection in study design rather than with *post-hoc* statistical adjustments (Banks-Leite et al., 2014). We employed several strategies to increase and equalize the detection of birds across our survey plots (see Section Methods). However, even with these adjustments it is likely that we overestimated bird abundance in developed relative to reserve sites and through time at changing sites (Marzluff et al., 2016). Thus, if we found slightly lower abundance in reserves relative to developments or slight increases in abundance over time in changing landscapes, we would be cautious in interpreting this to be a result of ecological change. This was not the case in our study as changes between landscapes and through time were greater than expected based on detectability bias. This potential bias does not apply to our conclusions concerning the influence of weather or forest productivity on richness or abundance within reserves or developments.

ETHICS STATEMENT

All research was conducted in accordance with University of Washington Institutional Animal Care and Use Committee Protocol 3077-01, Washington Scientific Collection Permit 11-359, and US Scientific Collection Permit MB761139-1.

AUTHOR CONTRIBUTIONS

BS conducted research, analyzed data, wrote paper. JM conducted research, analyzed data, designed study, wrote paper. LM analyzed data, wrote paper.

FUNDING

Funding was provided by the U.S. National Science Foundation (DEB-9875041, IGERT-0114351, BCS 0120024, and BCS 0508002), the German Research Foundation (RTG Graduiertenkolleg 780), and the University of Washington (Rachel Wood's Endowed Graduate Program).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00040/full#supplementary-material

Figure S1 | Diagram of the tools utilized in ARCGIS 9.3.3 (ESRI, 2008) to extract the NDVI data at each of the 21 sites from the MODIS product. NDVI data is the first layer of the .HDF file so there is no need to extract it as a subdataset; however, the pixel reliability needed to be extracted because it is the fourth layer. We utilized the Set Null tool to remove any unreliable NDVI data, utilizing the new Pixel Reliability subdataset as the basis for removal; unreliable pixels were classified as "No Data" excluding them from Zonal Statistics calculations. With the reliable NDVI layer prepared, we created a 1 km circle around the center of each site using Mean Center and Buffer tools. The final output, produced with the Zonal Statistics as Table tool, is a table for each date that NDVI was collected by the MODIS satellite in the study area.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 28 July 2017
doi: 10.3389/fevo.2017.00084

Impacts of Urban Areas and Their Characteristics on Avian Functional Diversity

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Urban development is rapidly expanding across the globe and is a major driver of environmental change. Despite considerable improvements in our understanding of how species richness responds to urbanization, there is still insufficient knowledge of how other measures of assemblage composition and structure respond to urban development. Functional diversity metrics provide a useful approach for quantifying ecological function. We compare avian functional diversity in 25 urban areas, located across the globe, with paired non-urban assemblages using a database of 27 functional traits that capture variation in resource use (amount and type of resources and how they are acquired) across the 529 species occurring across these assemblages. Using three standard functional diversity metrics (FD, MNTD, and convex hull) we quantify observed functional diversity and, using standardized effect sizes, how this diverges from that expected under random community assembly null models. We use regression trees to investigate whether human population density, amount of vegetation and city size (spatial extent of urban land), bio-region and use of semi-natural or agricultural assemblages as a baseline modulate the effect of urbanization on functional diversity. Our analyses suggest that observed functional diversity of urban avian assemblages is not consistently different from that of non-urban assemblages. After accounting for species richness avian functional diversity is higher in cities than areas of semi-natural habitat. This creates a paradox as species responses to urban development are determined by their ecological traits, which should generate assemblages clustered within a narrow range of trait space. Greater habitat diversity within cities compared to semi-natural areas dominated by a single habitat may enhance functional diversity in cities and explain this paradox. Regression trees further suggest that smaller urban areas, lower human population densities and increased vegetation all enhance the functional diversity of urban areas. A city's attributes can thus influence the functional diversity of its biological assemblages, and their associated ecological functions. This has important implications for the debate regarding how we should grow the world's cities whilst maintaining their ecological function.

Keywords: bird community, habitat loss, functional diversity, land sparing and land sharing, species trait, urbanization, urban and non-urban comparison

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

***Received:** 15 March 2017*
***Accepted:** 12 July 2017*
***Published:** 28 July 2017*

Citation:
Oliveira Hagen E, Hagen O,
Ibáñez-Álamo JD, Petchey OL and
Evans KL (2017) Impacts of Urban
Areas and Their Characteristics on
Avian Functional Diversity.
Front. Ecol. Evol. 5:84.
doi: 10.3389/fevo.2017.00084

INTRODUCTION

Globally, urban land cover is predicted to triple in extent from 2000 to 2030, with nine-fold rates of increase in some biodiversity hotspots (Seto et al., 2012). Urbanization drives significant habitat loss and fragmentation, pollution (chemical, noise, light, and heat) and other changes in selection pressures that radically restructure biotic communities (Grimm et al., 2008; Pautasso et al., 2011). At local scales this restructuring typically generates a unimodal pattern in native species richness along urbanization gradients, with richness peaking at intermediate levels of urbanization intensity (e.g. Tratalos et al., 2007). Studies documenting these unimodal patterns have primarily been conducted in highly developed regions (Marzluff, 2017). Richness may decline more linearly with increasing urbanization in regions where anthropogenic impact outside cities is more limited, due to a sharper contrast between the occurrence of semi-natural vegetation outside cities and its rarity inside urban areas (Luck and Smallbone, 2010; Norton et al., 2016). Urban development also restructures biotic communities at larger spatial scales, such as the entire urban area. Analyses conducted at city scale suggest that cities typically contain fewer species than equivalent sized rural areas, but such studies are much rarer than those focusing on local scale responses to urbanization and there is limited understanding of factors driving variation in biodiversity across cities (Norton et al., 2016; Lepczyk et al., 2017).

The restructuring of biotic communities in urban areas is strongly associated with the loss of species with limited tolerance to urban development, i.e. urban avoiders, and increased abundances of more tolerant species (Blair, 1996; Sol et al., 2014; Fischer et al., 2015). These shifts in community composition are non-random, with urban environments filtering species according to their ecological and life-history traits, for example, across numerous taxonomic groups, specialized species tend to respond negatively to urbanization (Clark et al., 2007; Evans et al., 2011; Sol et al., 2014). This filtering process occurs across all three stages of the biotic urbanization process, i.e. arrival, adaptation and spread (Evans et al., 2010). These ecological and life-history traits that filter species' responses to urban development also determine how species contribute to and modulate ecosystem processes and services (Luck et al., 2009; Villéger et al., 2010; Dehling et al., 2016). As an example urbanization can alter avian communities in a manner that changes seed dispersal functions which promote invasive plant species over closely related native species (Caughlin et al., 2012). The non-random urban filtering of biotic communities can thus alter the nature and diversity of functions performed by species retained within urban communities. Functional diversity indices are powerful tools that go beyond simple measurements of biodiversity, allowing the quantification and comparison of functional diversity among communities (Pla et al., 2012). Assessing whether observed functional diversity differs from that expected by chance provides a metric of the dispersion of traits across the functional trait space, with increasing clustering or over-dispersion of traits indicating stronger biotic or abiotic filtering of the focal community (Botta-Dukát and Czúcz, 2016). Assessing functional diversity responses to land use changes can thus help to understand community assembly patterns and shifts in community function. Despite this, very little attention has been paid to the functional significance of biotic homogenization in cities, even in well studied groups such as birds (McKinney, 2008; Marzluff, 2017). Initial studies assessing variation in functional diversity at a local scale within a city suggests that functional diversity declines in more urbanized locations, and that habitat composition can influence the functional diversity of avian communities at local scales (Schütz and Schulze, 2015). Negligible work has been conducted, however, to assess how the functional diversity of urban assemblages at the scale of entire cities compares to that of rural areas, and how these patterns are influenced by the characteristics of those cities. Indeed, many characteristics of cities such as their size and amount of green-space, are likely to influence a wide range of biodiversity metrics but such research has until recently been limited by lack of sufficient data (Aronson et al., 2014; Norton et al., 2016).

Our overall goal is to assess how urbanization influences functional diversity, another crucial aspect of biodiversity, which complements the current focus on more traditional measures of biodiversity, such as species richness. In addition, the quantification and investigation of the patterns of trait distribution, allows the observation of non-random compositional shifts that ultimately could affect ecosystem functions and related services therein. We use data from paired urban and rural, i.e. non-urban, assemblages across three continents and focus on assessments at large spatial extents, i.e. city level analyses. In doing so we distinguish between impacts that arise when comparing urban areas to surrounding rural environments, i.e. sites dominated by semi-natural habitats, and those comparing urban areas to agricultural ones in which natural vegetation is more limited. In addition, we assess how key features of urban areas (their size, human population density, and amount of vegetation) modify the impact of urbanization on avian functional diversity. Our analyses thus help to develop further understanding of how urbanization influences trait dispersion, its significance for community function and how to manage urban development to limit adverse impacts on functional diversity.

METHODS

Avian Assemblage Data

Our general approach is to extract data on assemblage composition from peer-reviewed studies that adopt equivalent methods to survey avian assemblages in an urban location and a nearby paired rural site. We do not use data from studies only conducted in urban areas, as whilst the composition of surrounding rural assemblages could be estimated by extracting data from species distribution maps these distributional data are a summary of intensive survey effort accumulated over a substantial time period. Survey effort would thus be much greater for rural sites thus potentially biasing estimates of their functional diversity and increasing it relative to urban sites with less intensive sampling effort. We take habitat definitions from the original papers, but all urban areas are distinguished from rural areas by the dominance of impervious surfaces in the former. We do not place any additional restrictions on the definition of urban areas as one of our objectives is to assess how variation in the characteristics of urban areas influences functional diversity, and we thus wish to maximize variation in the type of urban areas included in our analyses. We distinguish between non-urban areas dominated by semi-natural habitats (such as woodland, referred to as semi-natural assemblages) and those dominated by agricultural habitats.

Our literature search was conducted in April 2012 using Web of Knowledge and combinations of the terms "avian*" "bird∗," "urban,*" "cit*" (truncated to include papers using the term city or cities) and "assemblage*." Additional urban ornithological literature has accumulated since our initial search, but our database is representative of the current spatial distribution of ornithological research (Marzluff, 2017). We excluded studies whose survey design was likely to under-record the urban avian community due to a primary focus on certain species or taxonomic groups. We also excluded studies from bio-regions for which there was only one study available. We did this because the construction of a regional species pool is a crucial step when comparing observed values of functional diversity metrics to null models (see below) and the regional species pool should be composed of a greater set of species than those in a single community. Our criteria were met by 32 papers that described the assemblages of 25 cities (**Figure 1**). Four cities (Montpellier, in the Western Palearctic; and Oxford, Palo Alto, and Tucson in North America) were represented by more than one study and we combined these studies to construct a single urban assemblage for each city, together with its paired natural or agricultural assemblage. In addition, for four urban assemblages (Western Palearctic: Montpellier; South America: La Paz, Esquel, Palermo) we had paired data for both a semi-natural and agricultural assemblage. Our data comprised 529 bird species in 29 paired urban to non-urban assemblages, of which 19 were for natural assemblages (Table S1).

Avian Trait Data

Our functional diversity metrics are based on the approach developed by Petchey et al. (2007) for British birds and subsequently used in other studies of avian functional diversity (e.g. Mendez et al., 2012; Trindade-Filho et al., 2012). We expand the suite of traits beyond those used by Petchey et al. (2007) to ensure applicability to the full suite of species in our much more globally distributed assemblages, e.g. nectar was added as an additional food source. We also included nest site as an additional trait axis. Our trait database (Supplementary Material - Data Sheet 1) thus contains 27 traits that capture inter-specific variation across six trait axes that describe the quantity and type of resources acquired by each species, and the methods used to obtain them (**Table 1**; Table S2). When possible we used data that was specific to the location or region represented in our assemblage composition data (e.g. if a species is distributed across the northern hemisphere but within our data was only present in European assemblages we only used trait data from European populations). We used mean values for continuous traits. Binary traits were classified according to species' typical behaviors, with the primary dietary components classified when taking both adult and nestlings into account (e.g. if a species feeds primarily on seeds as an adult but invertebrates as a nestling, we coded both plants and insects as primary food sources).

Body mass data was obtained from Dunning (2007), and the Handbook of the Birds of the World (hereafter HBW; del-Hoyo et al., 1992-2011) was the primary source for all other traits, although Natuhara and Imai (1996) provided much additional data on nest sites. If data were unavailable from these sources we used regional field guides and handbooks (see Table S3 for list), but data remained unavailable for 140 trait values (0.9% of the total). These missing values were for 20 species for which we used a family mean value calculated only using data for species from the same bio-region, and that occurred at similar latitudes to the location represented in our dataset that contained the species with missing trait data.

FIGURE 1 | The location of 25 study sites for which data were available on the composition of paired urban and rural assemblages.

TABLE 1 | The 6 trait axes and 27 specific traits used to characterize avian functional diversity.

Trait axis	Trait	Variable type
1. Resource quantity	1. Body mass	Continuous
	2. Average clutch size	Continuous
2. Main dietary components	3. Vertebrates	Binary
	4. Invertebrates	Binary
	5. Plants	Binary
	6. Nectar	Binary
3. Main foraging method(s)	7. Pursuit	Binary
	8. Gleaning	Binary
	9. Pouncing	Binary
	10. Grazing	Binary
	11. Digging	Binary
	12. Scavenging	Binary
	13. Probing	Binary
4. Main foraging substrate(s)	14. Water	Binary
	15. Mud	Binary
	16. Ground	Binary
	17. Vegetation	Binary
	18. Air	Binary
	19. Rocks	Binary
5. Main foraging period	20. Nocturnal	Binary
6. Nesting sites	21. Ground (open nesting)	Binary
	22. Cavities in cliffs, rocks or the ground	Binary
	23. Tree cavity	Binary
	24. Other vegetation	Binary
	25. Artifacts	Binary
	26. On water surface in vegetation	Binary
	27. Obligate brood parasite	Binary

Foraging behavior and nesting site definitions are provided in Table S2.

Functional Diversity Metrics

We measured functional diversity using three metrics that require information on species presence/absence; alternative metrics that use data on species' relative abundances could not be used as such data were not provided sufficiently frequently by our focal studies. Our two continuous traits (body mass and clutch size) were standardized to vary from zero to one and thus match the range of values of the binary traits. Trait matrices for each community were converted into distances matrices using the Gower distance method, which can deal with both continuous and binary data to calculate pair-wise dissimilarities (Gower, 1971). Using these distances matrices, we calculated (i) functional diversity (FD; Petchey and Gaston, 2002), (ii) mean nearest taxon distance in functional space (MNTD; Webb, 2000), and (iii) convex hull volume (CHV; Cornwell et al., 2006). We select these metrics due to their complementary nature and recommendations of Trisos et al. (2014). FD describes the functional relationships shared by species, and theoretically can reveal the net effects of major assembly processes (Mayfield and Levine, 2010; Trisos et al., 2014). FD and MNTD are particularly good at detecting over-dispersion in trait distribution across an assemblage, a pattern that is compatible with strong effects of inter-specific competition. In contrast, CHV is more sensitive to detecting trait clustering, a pattern that is more indicative of strong filtering generating a community with highly similar trait values (Mouchet et al., 2010; Aiba et al., 2013). We also use MNTD as it is one of the most commonly used measures of community relatedness, thus aiding comparison with other studies (Kraft et al., 2007). Full details regarding the calculation of functional diversity metrics are provided in Supplementary Material. All analyses were done in R *v.3.3.1* (R Core Team, 2015).

We compare observed functional diversity with that of random assemblages, to assess how functional diversity varies between our focal assemblages when taking into account differences in their species richness. The spatial definition of the pool of species on which null models are based can strongly determine conclusions regarding community assembly processes (Chalmandrier et al., 2013; Herben et al., 2013). Therefore, we constructed a specific trait matrix for the regional pool of species occurring within each of our main bio-regions (the Western Palearctic, North America and South America). The comparison of the patterns of observed and expected functional diversity was performed by calculating a standardized effect index (SES) as proposed by Mendez et al. (2012).

$$\mathrm{SES} = \frac{Observed - Expected}{\sigma_e}$$

Where *Observed* is the observed value of functional diversity, σ_e is the standard deviation of 1,000 simulations of expected functional diversity for the focal community, and *Expected* is the mean of the functional diversity of the simulations. This standardized effect index allows comparison between the differences of observed and expected functional diversity across studies and facilitates the visualization of outlier assemblies. The standardized effect size is a z-score of the absolute functional diversity values and measures the number of standard deviations that the observed index is above or below the mean index of the simulated communities. SES values lower than zero are compatible with environmental filtering (trait convergence) influencing community structure, whilst SES greater than zero are compatible with effects of limiting similarity (i.e. trait divergence, Figure 1 in Petchey and Gaston, 2007). Nevertheless, in an analysis of species relatedness patterns in communities, Mayfield and Levine (2010) suggest that competitive exclusion could generate clustering, over-dispersion or no pattern on species traits. Other mechanisms can also contribute to trait clustering or over-dispersion, complicating inferences regarding assembly processes. Resource mismatch and specialization, for example, can generate signals of trait over-dispersion (Pontarp and Petchey, 2016). Consequently, our focus is on how trait distribution responds to environmental variables.

Our 27 traits are separated into six axes: resource quantity, main dietary components, foraging methods, foraging substrates, foraging periods and nesting sites (**Table 1**). We calculate our three functional diversity metrics using all 27 traits in combination. However, we also calculate our three functional diversity metrics separately for the five trait axes that contained multiple traits, i.e. resource quantity, dietary components, foraging method, foraging substrate and nesting site. This enables us to test how urbanization separately influences different functional diversity axes. This decreases the likelihood of not detecting an effect of urbanization on overall functional diversity due to opposing effects on different trait axes that cancel each other out, and thus mask an effect on functional diversity calculated across all trait axes (Trisos et al., 2014). This analysis of separate trait axes also provides additional information to test specific hypotheses derived from the literature regarding how urbanization filters avian communities, e.g. that urbanization promotes birds with particular foraging niches or nest site attributes (Evans et al., 2011; Sol et al., 2013).

We also conduct two additional checks to explore factors that may influence estimates of urban functional diversity. It is plausible that urban bird survey data incorporate some species that migrate through cities but are not an established part of the avifauna, despite being established in nearby non-urban areas. This could create a bias that increases estimates of urban functional diversity. We assess this by calculating our three functional diversity metrics with and without including migratory species (coded per city based on range maps from HBW Alive - www.hbw.com). These two sets of functional diversity metrics were strongly and positively correlated (FD $r = 0.91$, MNTD $r = 0.86$, CHV $r = 0.83$; $P < 0.0001$ in all cases), and we thus consider this potential bias to have negligible influence on our results. Whilst urban areas can contain a number of migratory species that do not breed there we expect that the studies from which we extract data were typically conducted in a manner to avoid incorporating such species. In addition, cities often contain water-bodies that support a range of wetland birds, whilst our focal non-urban areas are typically dominated by a single terrestrial habitat type. There is thus also the potential for the greater diversity of waterbirds in urban areas to enhance estimates of urban functional diversity. We conducted a second robustness check by assessing the correlation between functional diversity metrics including and excluding waterbirds. These metrics are strongly and positively correlated (FD $r = 0.99$, MNTD $r = 0.98$, CHV $r = 0.96$; $P < 0.0001$ in all cases) suggesting that increased species richness of waterbirds in urban areas is not driving the patterns in functional diversity that we report.

City Characteristics

We use three metrics that describe key features of the urban landscape that are likely to influence the composition of avian assemblages. The spatial extent of an urban area can influence assemblages by changing selection pressures, such as the magnitude of the urban heat island effect and contributing to large scale habitat fragmentation impacts by changing the degree of connectivity between central urban locations and surrounding rural areas (Norton et al., 2016). City size is difficult to measure from an ecological perspective as a city's administrative boundaries often do not coincide with the boundary between areas dominated by built up land and less urbanized locations. We thus defined the spatial extent of our focal cities using an urban cluster methodology that manually delimited a polygon that encompassed all areas dominated by impervious surfaces within each focal city using Google Earth Pro software, and using aerial imagery from as close as possible to the year(s) in which avian data were collected for that urban area (Figure S1). The defined urban area could thus be much smaller or larger than administrative city size and more accurately captured the spatial extent of a city than other commonly used metrics of city size, such as the extent of administrative boundaries or human population size.

We use human population density as an alternative measure of urbanization intensity (Gaston, 2010). This metric captures the potential for human disturbance, and is directly relevant to addressing policy questions regarding how to meet the growing need to house people in urban areas whilst minimizing impacts on biodiversity. Indeed, human population density is often associated with assemblage species richness and composition, including at fine spatial scales within urban areas (Evans et al., 2006; Fontana et al., 2011). We thus obtained data on human population density within each of our urban areas for the year 2000 using data at the 1km resolution from the Global Human Settlement Layer (GHSL) project, supported by European Commission [European Commission, Joint Research Centre (JRC); Columbia University, Center for International Earth Science Information Network (CIESIN), 2015]. The density values were obtained by a bilinear interpolation of values stored at the raster image provided by the dataset for each study point.

Numerous studies have documented strong associations between the structure and composition of urban bird assemblages and the amount of green-space within the survey location, which provides an additional measure of urbanization intensity (Marzluff, 2017). Indices derived from remote sensing images that measure the proportions of reflected light in different wavelengths (such as the Normalized Difference Vegetation Index, NDVI, and Enhanced Vegetation Index, EVI) provide robust estimates of the amount of vegetation and correlate positively with avian species richness in urban areas (Bino et al., 2008; Silva et al., 2015). We use EVI rather than NDVI as the former provides a better correction for bare ground and residual atmospheric contamination effects and more accurately captures spatial variation when vegetation is abundant (Xiangming et al., 2003). We used EVI data collected at a 30 × 30 m pixel resolution from Moderate Resolution Imaging Spectroradiometer (MODIS) satellite images, retrieved from the Oak Ridge National Laboratory data server (ORNL DAAC, 2011). Within each city, we use EVI data from a 16.25 km^2 grid that is centered on the ornithological survey area used in each study, and use the MODIS landmasks to correct for the presence of water features (Table S4). We calculate EVI as the mean of all EVI estimates during spring and summer (defined as March to August for studies in the northern hemisphere, and September to

February in the southern hemisphere) for each city using remote sensing data from the year closest to that of the ornithological surveys.

Statistical Analysis of Urbanization Effects

All analyses were conducted in R 3.3.1. We start by using paired tests to compare species richness and functional diversity metrics of our paired assemblages. We conduct separate tests for urban assemblages paired with semi-natural sites and those paired with agricultural sites. Species richness and observed FD, MNTD, and CHV data met the assumptions of parametric tests and we thus analyzed these data using paired *t*-tests. Paired data on SES values from FD, MNTD and CHV data were, however, analyzed using a Wilcoxon signed rank test as the data did not meet the assumptions of parametric tests. We conduct these tests using our global data-sets but also conduct separate tests for data from each of our three regions in case heterogeneity in the direction of effects across regions influences the detection of patterns at a global scale, i.e. positive and negative effects in different regions canceling each other out in a global analysis. We interpret the results of these regional tests cautiously due to low sample sizes in some cases. Similarly, to assess if heterogeneity in the impacts of urbanization on different trait axes influences the detection of a global pattern in functional diversity measured across all six trait axes in combination we conduct separate paired tests for each functional diversity trait axis. These single trait axes analyses are only conducted for FD and MNTD functional diversity metrics as the code for calculating CHV does not perform well when only using binary traits that comprise most of our single trait axes (**Table 1**). Our heterogeneity analyses provide robustness tests but find negligible impact of heterogeneity in the direction of effects across regions or different trait axes (see Section Results). Consequently, our further analyses (regression trees, see below) only focus on global patterns using functional diversity metrics constructed across all six trait axes.

We then explore how the magnitude of divergence in the functional diversity of our global paired urban and rural assemblages is associated with key features of the paired assemblages using a regression tree approach. Regression trees deal better with non-linearity and interactions between explanatory variables than regression based generalized linear or additive models; regression trees can also find interactions missed by other methods (Zuur et al., 2007). We calculate an index of divergence in functional diversity between paired urban and non-urban assemblages by subtracting the non-urban functional diversity metric from the urban functional diversity metric. We do this using (i) observed FD, MNTD, and CHV (which does not take species richness of the assemblages into account), and (ii) SES values based on FD, MNTD and CHV (which focuses on how observed functional diversity varies relative to expected functional diversity given the number of species present in each assemblage). We use five predictor variables: EVI as a measure of the amount of urban vegetation, urban human population density (km^{-2}), city size (km^2), non-urban habitat type (agriculture or semi-natural habitat), and region (Western Palearctic, North America and South America). Prior to analysis city size and human population density were $\log_{10}$ transformed to reduce the skew in their distributions. Regression trees were constructed using the package "Tree" (Ripley, 2016). The tree fitting function adopts a pre-pruning strategy which has a number of control settings which limit how much the tree grows. Our trees were pre-pruned using the default settings, i.e. a minimum number of 5 observations per branch and the within-node deviance being at least 0.01 times that of the root node for the node to be split (Ripley, 2016). The pre-pruning approach can lead to over-fitting with terminal branches explaining relatively little additional variation (Zuur et al., 2007). We thus adopt a conservative approach that also uses a post-pruning strategy and only retain final branches if they contribute an additional 5% explanatory capacity. Whilst this threshold is somewhat subjective, when sample sizes are limited it is more appropriate than alternative *P*-value based post-pruning rules (Alvarez-Iglesias et al., 2017). The explanatory capacity was calculated using McFadden's pseudo R^2 (McFadden, 1974) for trees with and without each terminal branch. This post-pruning strategy led to the removal of one branch from each of two trees (SES FD and SES CHV).

RESULTS

Our data comprise a total of 529 species, of which 141 species occurred at sites in the Western Palearctic (urban sites: 106 species; non-urban sites: 135 species), 199 species in North America (urban: 163; non-urban: 172) and 207 in South America (urban: 130; non-urban: 169). The majority of species co-occurred in urban and non-urban sites, but species also occurred exclusively in each habitat type. A greater number of species were restricted to non-urban areas than urban ones (globally: 142 exclusively non-urban species, 66 exclusively urban species; Western Palearctic: 35 exclusively non-urban, 6 exclusively urban; North America: 36 exclusively non-urban, 27 exclusively urban; South America: 77 exclusively non-urban, 38 exclusively urban). Species that, within our dataset, were exclusively found in urban sites included a small number of classical urban exploiters, such as feral pigeon *Columba livia*, but primarily comprised a wide range of native species: e.g. mallard *Anas platyrhynchos*, peregrine *Falco peregrinus*, burrowing owl *Athene cunicularia*, hooded crow *Corvus cornix*, bronzed cowbird *Molothrus aeneus* and black-and-rufous warbling finch *Poospiza nigrorufa*. Non-native species were rare in urban assemblages (mean species richness ± standard error: 1.4 ± 0.3).

Paired Analyses

Urban assemblages tended to comprise fewer species than assemblages at natural sites, which also tended to support more species than agricultural sites (**Figure 2**). However, there were no significant differences in species richness when comparing paired urban and natural assemblages, or paired urban and agricultural assemblages at the global scale or for individual regions, i.e. the Western Palearctic, North America and South America (**Table 2**).

When comparing observed functional diversity of paired urban and non-urban assemblages the only significant difference was between CHV of urban and natural assemblages in North America, with higher CHV in urban assemblages ($P = 0.04$;

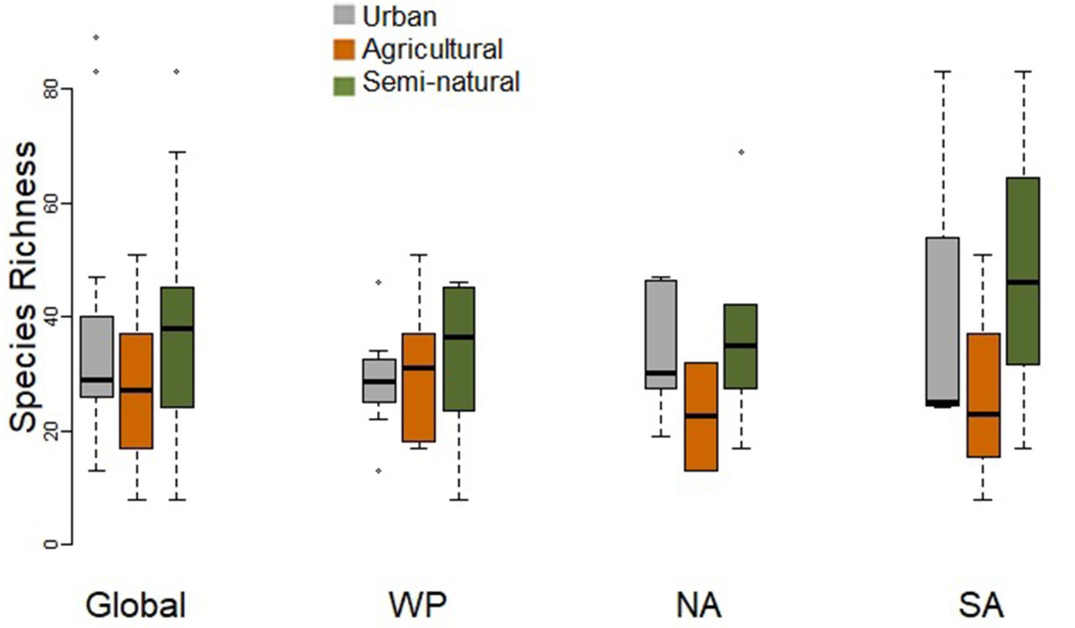

FIGURE 2 | Box-plot comparing species richness of paired urban and non-urban assemblages (in either natural or agricultural habitats) at the global scale and for the Western Palearctic (WP), North America (NA), and South America (SA). Paired *t*-tests did not find any significant differences (**Table 2**).

Table 2; **Figure 3**). The same pattern was observed, but with *P*-values just above the significance threshold ($\alpha = 0.05$) in North America for FD ($P = 0.06$). There was no evidence that the lack of significant differences in observed functional diversity at the global scale was due to significant heterogeneity in the direction in which each single trait axes responded to urban development (**Table 3**; **Figure 4**).

When comparing standardized effect sizes (SES) of functional diversity, i.e. taking species richness into account, there was a globally statistically significant pattern of higher functional diversity in urban assemblages than ones in natural habitats (FD $P = 0.005$; CHV $P = 0.004$; MNTD $P = 0.04$; **Table 2**; **Figure 4**). Identical and statistically significant patterns occurred in North America (**Table 2**; **Figure 5**). A similar and marked trend occurred in South America (**Figure 5**), but the sample size was small and the trend was not statistically significant (**Table 2**). Analyses of single trait axes SES metrics suggested that for FD the higher functional diversity of urban assemblages was driven by three single trait axes: dietary components ($P = 0.02$), foraging methods ($P = 0.02$) and foraging substrates ($P = 0.01$; **Table 3**; **Figure 4**). Similar patterns were exhibited by single trait axes for resource quantity and nesting sites, albeit well above the $\alpha = 0.05$ significance threshold (**Table 3**; **Figure 4**). Global comparisons of single trait MNTD metrics did not find any evidence of heterogeneity in the responses of single trait axes between paired urban and non-urban assemblages, either natural or agricultural ones (**Table 3**; **Figure 4**).

Regression Trees

We used regression trees to assess how the difference in functional diversity of urban and non-urban assemblages is related to the features of each city (amount of vegetation, size, and human population density), the nature of the non-urban assemblages (semi-natural or agricultural sites) and region. These regression trees explained large amounts of the variation in the divergence in observed functional diversity (FD 54%, CHV 40%, MNTD 43%) and SES metrics of functional diversity (FD 47.6%, CHV 28.4%, MNTD 50.7%). The regression trees of observed and standardized effect size functional diversity metrics varied with the functional diversity metric that was used. All six trees provide evidence, however, that the characteristics of urban areas were associated with the magnitude of divergence between the functional diversity of urban and non-urban avian assemblages (**Figure 6**).

The regression tree for observed FD only retains EVI as a predictor and splits urban assemblages into three groups with contrasting amounts of vegetation. Cities with the highest EVI values (>0.275) have bird assemblages with functional diversity metrics that are closest to those of non-urban assemblages. The influence of EVI on differences in the observed functional diversity of urban and non-urban assemblages is, however, not consistent as urban avian functional diversity is higher than non-urban ones in cities with the lowest EVI values (<0.25), and cities with intermediate EVI values (0.25–0.275) have lower functional diversity than non-urban sites.

The regression tree for observed CHV shows that observed functional diversity in urban bird assemblages is lower than that in paired assemblages from agricultural sites (left hand branch). When urban assemblages are compared to those in natural sites (right hand branch) the direction of the differences between urban and natural sites depends on the human population density within the city. Cities with lower human population

TABLE 2 | Results of paired tests for differences in species richness, observed functional diversity metrics, and standardized effect size (SES) functional diversity metrics that take species richness into account between paired urban avian assemblages and those in natural habitats, and between paired urban and agricultural assemblages.

Response	Region	Urban vs. natural	Urban vs. agricultural
Species richness	Global	$P = 0.72; n = 19$	$P = 0.80; n = 10$
"	WP	$P = 0.29; n = 8$	$P = 0.58; n = 5$
"	NA	$P = 0.44; n = 8$	$P = 1.00; n = 2$
"	SA	$P = 1.00; n = 3$	$P = 0.10; n = 3$
Observed FD	Global	$P = 0.41; n = 19$	$P = 0.92; n = 10$
"	WP	$P = 0.35; n = 8$	$P = 0.63; n = 5$
"	NA	$P = 0.06; n = 8$	$P = 1.00; n = 2$
"	SA	$P = 1.00; n = 3$	$P = 0.75; n = 3$
Observed CHV	Global	$P = 0.09; n = 19$	$P = 0.62; n = 10$
"	WP	$P = 0.25; n = 8$	$P = 0.46; n = 5$
"	NA	$\mathbf{P = 0.04; n = 8}$	$P = 1.00; n = 2$
"	SA	$P = 0.50; n = 3$	$P = 1.00; n = 3$
Observed MNTD	Global	$P = 0.16; n = 19$	$P = 0.85; n = 10$
"	WP	$P = 0.93; n = 8$	$P = 1.00; n = 5$
"	NA	$P = 0.11; n = 8$	$P = 1.00; n = 2$
"	SA	$P = 0.75; n = 3$	$P = 1.00; n = 3$
SES FD	Global	$\mathbf{P = 0.005; n = 19}$	$P = 0.38; n = 10$
"	WP	$P = 0.84; n = 8$	$P = 0.81; n = 5$
"	NA	$\mathbf{P = 0.008; n = 8}$	$P = 1.00; n = 2$
"	SA	$P = 0.25; n = 3$	$P = 0.25; n = 3$
SES CHV	Global	$\mathbf{P = 0.004; n = 19}$	$P = 1.00; n = 10$
"	WP	$P = 0.38; n = 8$	$P = 0.63; n = 5$
"	NA	$P = 0.07; n = 8$	$P = 1.00; n = 2$
"	SA	$P = 0.25; n = 3$	$P = 0.50; n = 3$
SES MNTD	Global	$\mathbf{P = 0.04; n = 19}$	$P = 0.92; n = 10$
"	WP	$P = 0.25; n = 8$	$P = 0.31; n = 5$
"	NA	$\mathbf{P = 0.016; n = 8}$	$P = 1.00; n = 2$
"	SA	$P = 0.25; n = 3$	$P = 0.50; n = 3$

*Comparisons are conducted globally and also, but note the small sample sizes for some comparisons, for paired assemblages in the Western Palearctic (WP) North America (NA) and South America (SA). Results in bold indicate those with $P < 0.05$, and those in gray font ones with $P \leq 0.10$. In all highlighted comparisons, functional diversity is higher in urban assemblages (see **Figures 2, 3**).*

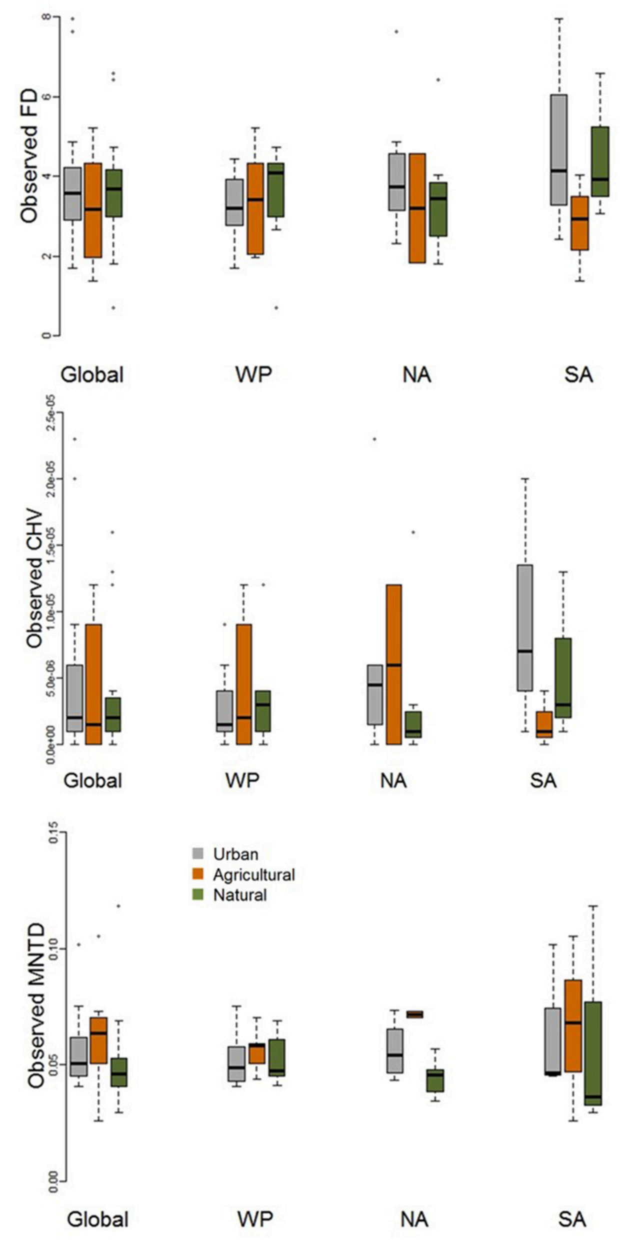

FIGURE 3 | Boxplot comparing observed functional diversity metrics (FD, MNTD, and CHV) for urban avian assemblages and their paired natural and agricultural assemblages at the global scale and for the Western Palearctic (WP), North America (NA), and South America (SA). See **Table 2** for statistical results.

density (log10 population density <3.66) have higher observed functional diversity than assemblages in semi-natural sites, but in more densely populated cities observed functional diversity is lower than in semi-natural sites.

The regression tree for observed MNTD shows that when cities have high human population densities (log10 population density > 3.61) the differences between the functional diversity of their avian assemblages and those in non-urban assemblages depends on the amount of vegetation. In cities with less vegetation (EVI < 0.265) observed functional diversity is lower in urban areas than paired non-urban assemblages. In contrast, when cities contain more vegetation (EVI > 0.265) observed functional diversity is higher in urban areas than non-urban sites.

Regression trees using standardized effect sizes (which takes species richness into account) of the FD and CHV metrics find a number of splits which reveal that differences in the functional diversity of urban and non-urban assemblages are influenced by complex interactions between city size and EVI. The first branch

TABLE 3 | Results of paired tests for differences in species richness, observed functional diversity metrics, and standardized effect size (SES) functional diversity metrics that take species richness into account between paired urban avian assemblages and those in natural habitats, and between paired urban and agricultural assemblages.

Response	Axis	Region	Urban vs. natural	Urban vs. agricultural
Observed FD	Resource quantity	Global	$P = 0.559$; $n = 19$	$P = 0.695$; $n = 10$
″	Dietary components	″	$P = 0.102$; $n = 19$	$P = 0.100$; $n = 10$
″	Foraging methods	″	$P = 0.129$; $n = 19$	$P = 0.635$; $n = 10$
″	Nesting sites	″	$P = 0.244$; $n = 19$	$P = 0.625$; $n = 10$
″	Foraging substrates	″	$P = 0.118$; $n = 19$	$P = 0.498$; $n = 10$
Observed MNTD	Resource quantity	Global	$P = 0.383$; $n = 19$	$P = 0.492$; $n = 10$
″	Dietary components	″	$P = 0.755$; $n = 19$	$P = 1.000$; $n = 10$
″	Foraging methods	″	$P = 0.151$; $n = 19$	$P = 0.375$; $n = 10$
″	Nesting sites	″	$P = 0.459$; $n = 19$	$P = 0.322$; $n = 10$
″	Foraging substrates	″	$P = 0.276$; $n = 19$	$P = 0.625$; $n = 10$
SES FD	Resource quantity	Global	$P = 0.352$; $n = 19$	$P =0.375$; $n = 10$
″	Dietary components	″	**$P = 0.016$; $n = 19$**	$P = 0.131$; $n = 10$
″	Foraging methods	″	**$P = 0.018$; $n = 19$**	$P = 0.232$; $n = 10$
″	Nesting sites	″	$P = 0.134$; $n = 19$	$P = 0.846$; $n = 10$
″	Foraging substrates	″	**$P = 0.014$; $n = 19$**	$P = 0.131$; $n = 10$
SES MNTD	Resource quantity	Global	$P = 0.293$; $n = 19$	$P = 0.769$; $n = 10$
″	Dietary components	″	$P = 0.225$; $n = 19$	$P = 0.695$; $n = 10$
″	Foraging methods	″	$P = 0.312$; $n = 19$	$P = 0.375$; $n = 10$
″	Nesting sites	″	$P = 0.890$; $n = 19$	$P = 0.322$; $n = 10$
″	Foraging substrates	″	$P = 0.168$; $n = 19$	$P = 1.000$; $n = 10$

*Comparisons are conducted globally for paired assemblages in the Western Palearctic (WP) North America (NA) and South America (SA). Results in bold indicate those with $P < 0.05$, and those in gray font ones with $P \leq 0.10$. In all highlighted comparisons, functional diversity is higher in urban assemblages (see **Figures 2**, **3**).*

of the regression tree for both these metrics show that urban functional diversity is higher than that of non-urban assemblages when cities are small. The subsequent interactions between EVI and city size do not reveal any consistent patterns. The regression tree for the standardized effect size of MNTD show that the higher functional diversity of urban avian assemblages, compared to non-urban assemblages, is greater for cities in North and South America than the Western Palearctic. The length of this branch indicates that this split contributes greatly to the tree's overall explanatory power.

DISCUSSION

Our results are only applicable to cities with similar characteristics to those within our dataset, which is dominated by urban clusters with spatial extents between 4 and 3,500 km^2 (52% of cities lower than 100 km^2 and 16% above 1,000 km^2), with low to intermediate population densities, between 875 and 16,300 people km^{-2} (80% of the cities between 1,000 and 10,000 people km^{-2}). Whilst our results thus cannot inform discussions of the impacts of megacities on functional diversity, they are relevant to the small and medium sized urban areas in which most of the projected future urban growth is predicted to occur (United Nations Human Settlements Programme, 2006).

Within each of our bio-regions fewer species occurred exclusively in urban assemblages than non-urban assemblages. This is compatible with the ecological literature demonstrating that some species are excluded from urban areas, and thus urbanization is a potent driver of species loss at regional and global scales (Blair, 2001; McKinney, 2006; Ibáñez-Álamo et al., 2016). Although there was a trend toward lower urban species richness, the difference was not statistically significant perhaps due to relatively small sample sizes, especially within some regions. A number of studies show that species richness can be maintained even in highly modified landscapes as specialized species are replaced by a similar number of more generalist and less sensitive species (Mayfield et al., 2010; Litteral and Shochat, 2017). Indeed, the urban locations in our study contain diverse avian assemblages and their species richness at our focal city level scale could be maintained by persistence of rarer species within remnants of high quality habitats within the urban area.

Globally, urban and non-urban assemblages did not exhibit significant differences in their observed functional diversity, and there was no evidence that this was due to heterogeneity of responses between different regions or between different functional trait axes. These results were consistent when comparing urban assemblages with those in semi-natural or agricultural areas. Notably, however, when taking into account the number of species within an assemblage, we found consistent evidence across each of our three functional diversity metrics that avian functional diversity is higher in cities than areas of natural habitat. At first sight this seems a remarkable

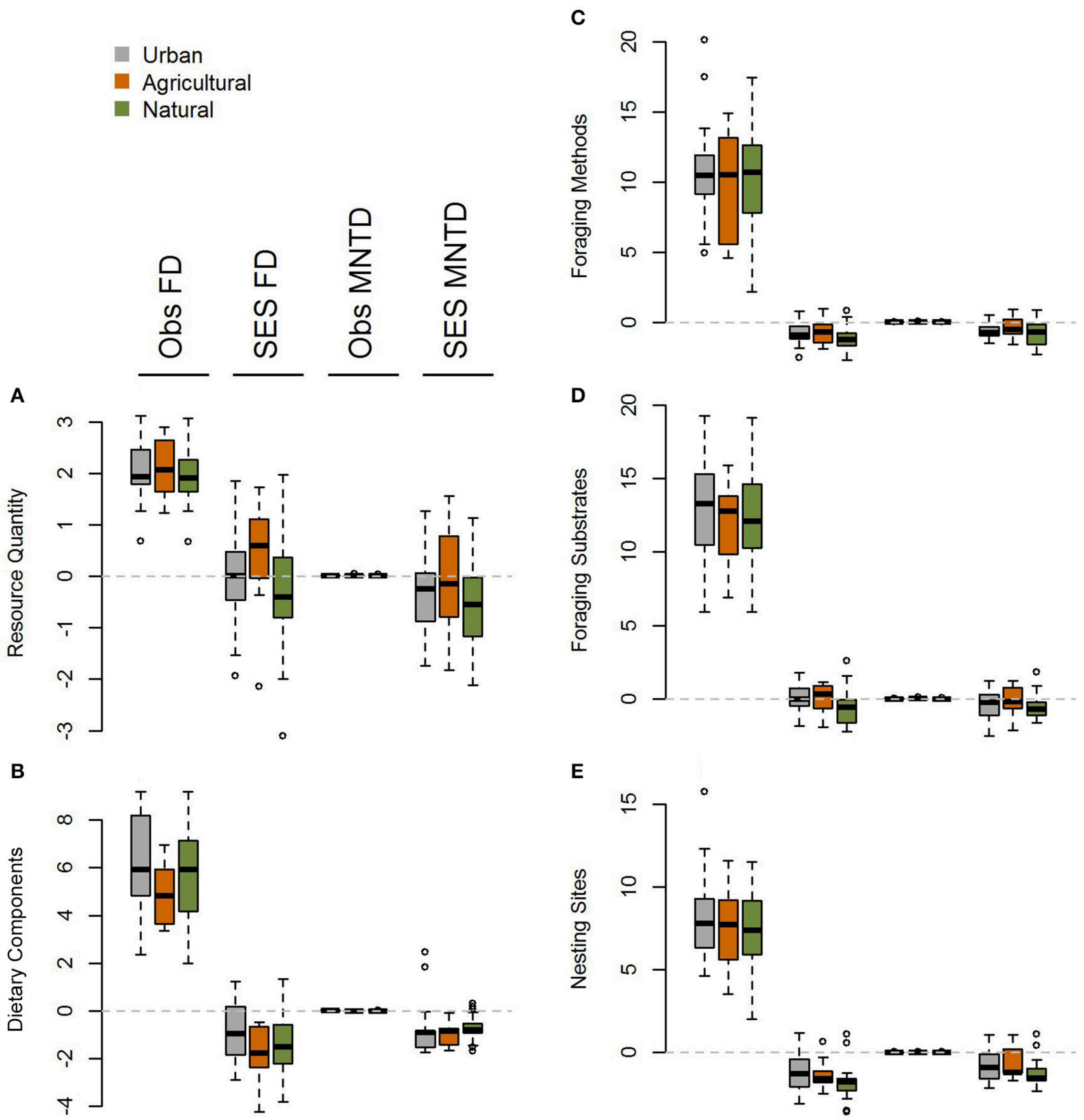

FIGURE 4 | Boxplot comparing single trait axis functional diversity metrics at the global scale for **(A)** resource quantity, **(B)** dietary components, **(C)** foraging methods, **(D)** foraging substrates, and **(E)** nesting sites for observed and standardized effect size FD and MNTD metrics in urban assemblages and paired natural and agricultural assemblages. There is no evidence for heterogeneity in the impacts of urbanization across different functional trait axes.

result that is highly unexpected given the plethora of studies showing that urbanization acts as a strong filter of biotic communities favoring species with a particular set of ecological and life history traits (Blair, 1996; Evans et al., 2011; Sol et al., 2014; Fischer et al., 2015) and negative effects of urbanization on avian taxonomic and evolutionary diversity (e.g. Marzluff et al., 2001; Chace and Walsh, 2006; McKinney, 2008; Ibáñez-Álamo et al., 2016) the other two major components of biodiversity. One would thus expect urban assemblages to be characterized by functional trait clustering that reduces functional diversity. Divergent responses of different biodiversity metrics to environmental change are, however, not uncommon (e.g. Devictor et al., 2010) including in an urbanized context (Morelli et al., 2016).

One potential explanation for enhanced functional diversity in urban areas is greater numbers of non-native species, that contribute unique traits—but this seems unlikely given their rarity in our focal urban assemblages. Our analysis focuses at a large spatial scale, i.e. entire urban areas which typically contain a wide diversity of different habitat types, primarily as a consequence of human planting and management decisions (Forman, 2014). We compare urban avian assemblages with those from rural semi-natural or agricultural areas that are typically dominated by a single habitat type, which is likely to

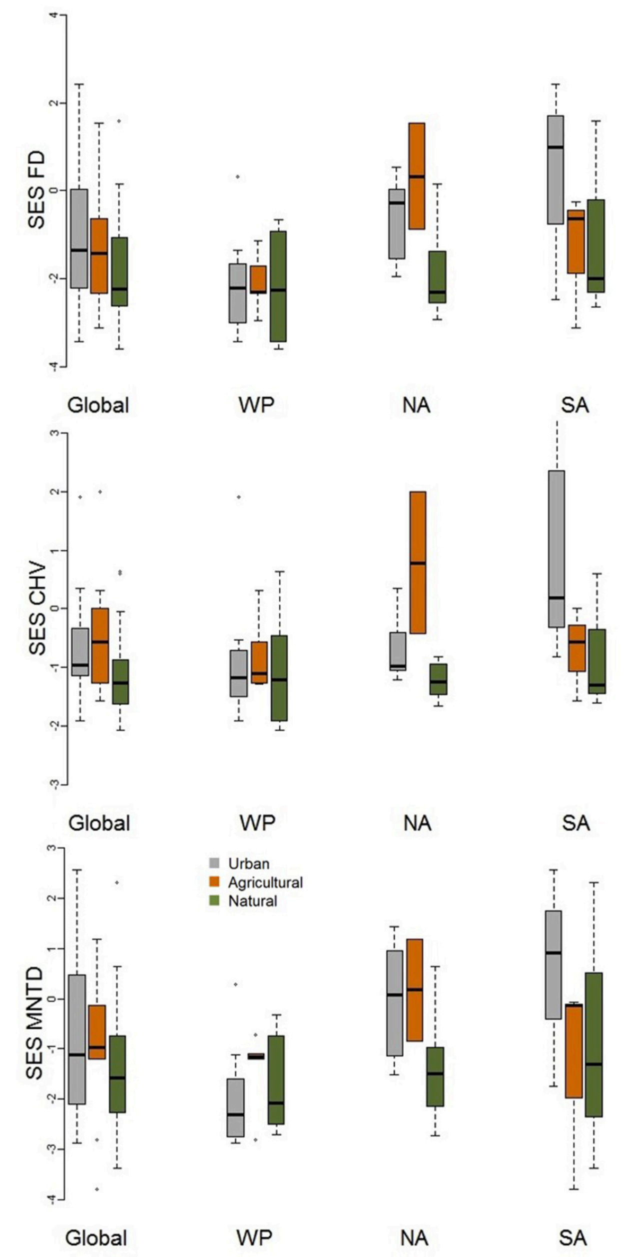

FIGURE 5 | Boxplot comparing standardized effect size functional diversity metrics (FD, MNTD, and CHV) that take species richness into account for urban avian assemblages and their paired natural and agricultural assemblages at the global scale and for the Western Palearctic (WP), North America (NA), and South America (SA). See **Table 2** for statistical results.

support species that are specialized on that single habitat type and thus exhibit stronger clustering of their functional traits. Consequently, greater habitat diversity within cities compared to similarly sized areas of semi-natural or agricultural vegetation may explain the apparent paradox of higher functional diversity, when taking species richness into account, in urban areas. Whilst more work is required to test this hypothesis it suggests that maintaining high levels of habitat diversity across urban areas will be important for maximizing their functional diversity.

Increases in urban functional diversity, when taking species richness into account, appears to be driven by traits related to foraging behavior as we only found significant differences for the three functional axes related to such function (i.e. dietary components, foraging methods and foraging substrates; **Table 3**). Whilst this finding contrasts with a global comparison in which diet type was not significantly different between urban exploiters and avoiders (Sol et al., 2014), several more local studies have found that urbanized and non-urbanized species differ in their diet or foraging behavior (e.g. Blair, 1996; Kark et al., 2007). This finding is also consistent with studies demonstrating that urbanization induces shifts in ecological functions linked to foraging, such as seed dispersal (Caughlin et al., 2012). Direct or indirect provision of anthropogenic food sources (such as garden bird food, garbage or nectar from ornamental plants) may also influence the distribution of foraging traits within urban bird assemblages, as such sources can supply additional resources that help to retain some (occasionally specialized) species in urban areas (Pauw and Louw, 2012).

Our results provide strong evidence that cities' characteristics determine how avian functional diversity compares to that of paired non-urban assemblages. The most notable pattern that emerges when taking species richness into account is the effect of city size, with elevated functional diversity being much more marked in smaller cities. This pattern does not arise when using observed functional diversity metrics, presumably because of species-area effects which increase the total number of species in larger urban areas (Norton et al., 2016). Regression trees that use observed functional diversity metrics provide evidence that cities with lower human population densities, and that contain more vegetation, have higher functional diversity. These results provide a novel extension of previous work on factors influencing urban biodiversity (e.g. Pellissier et al., 2012; Aronson et al., 2014; Bellocq et al., 2017; Chen and Wang, 2017) and extends the work of a study conducted within a single city at a finer spatial scale that found higher avian functional diversity in locations with greater vegetation cover (Schütz and Schulze, 2015). Crucially we demonstrate that less densely populated cities with a greater amount of vegetation will enhance functional diversity at the city scale. This information can guide policy makers and city planners who seek to increase avian functional diversity and associated ecological functions.

CONCLUSIONS

We demonstrate that urban areas can retain significant amounts of functional diversity, adding to previous work indicating that they can support a large and diverse range of species including endangered ones (Müller et al., 2013; Aronson et al., 2014; Threlfall et al., 2016). Remarkably, urban areas can contain significantly higher avian functional diversity than natural habitats, a pattern that is probably a reflection of greater habitat diversity in the urban areas facilitating the occurrence of species with a greater range of functional types than more specialized

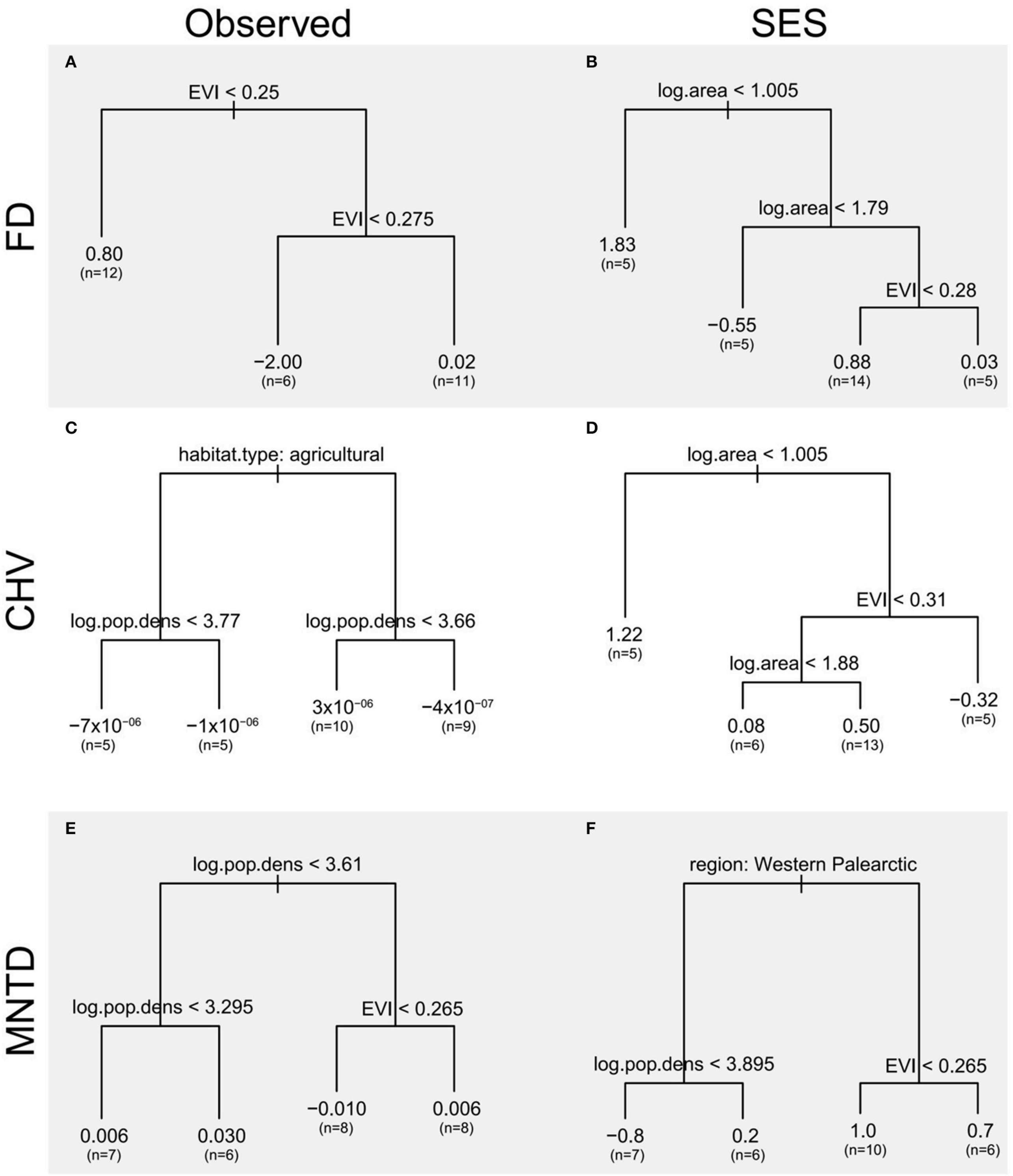

FIGURE 6 | Regression trees of differences in the functional diversity between urban and non-urban avian assemblages as a function of city traits EVI, a measure of the amount of vegetation, log area, $\log_{10}$ of the spatial extent of the urban area (km^2) and log.pop.density, $\log_{10}$ of the human population density (km^{-2}), nature of the non-urban assemblages (natural habitat or agricultural land) and bio-region. Regression trees are presented for **(A,B)** FD, **(C,D)** CHV, and **(E,F)** MNTD functional diversity metrics using observed values and standardized effect size metrics (SES) that take species richness into account. The left-hand branch of each tree is followed when the condition stated at the top of the branch is met. The length of each branch is proportional to the deviance explained by each split, the values at the end of each branch are the group mean and (in parentheses) the number of observations contributing to each branch.

species that occur within a single natural habitat. Crucially, we provide some preliminary evidence that smaller cities, with lower human population densities and more green space can support greater avian functional diversity. This has important implications for the debate regarding how we should grow the world's cities.

AUTHOR CONTRIBUTIONS

KE designed the study with assistance from EO, JI, and OP. EO and JI conducted literature reviews and EO trait data collection, with JI and KE contributing to checking the accuracy of the trait matrix. EO, OP, and OH conducted the analysis. EO and KE wrote the paper with assistance from all authors.

FUNDING

The University of Zurich supported the research during the Master studies of EO. KE was funded by a Biodiversity and Ecosystem Service Sustainability (BESS) programme grant (NE/J015369/1) funded by the UK Natural Environment Research Council (NERC) and the Biotechnology and Biological Sciences Research Council (BBSRC) as part of the UK's Living with Environmental Change (LWEC) programme. JI was funded by a postdoctoral contract (TAHUB-104) from the program "Andalucía Talent Hub" (co-funded by the European's Union Seventh Framework Program Marie Skłodowska-Curie actions-COFUND- and the regional Government of Andalucía).

ACKNOWLEDGMENTS

We thank the University of Zürich for the financial and scientific support during the Masters of EO. We also thank the Swiss Ornithological Institute—Vogelwarte for the access to their library during the trait sampling phase. In addition, we thank Cristopher Trisos for the helpful inputs that greatly improved the analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00084/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 15 June 2017
doi: 10.3389/fevo.2017.00063

Dispersal in the Urban Matrix: Assessing the Influence of Landscape Permeability on the Settlement Patterns of Breeding Songbirds

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Specialty section:
This article was submitted to
Urban Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *20 February 2017*
Accepted: *31 May 2017*
Published: *15 June 2017*

Citation:
Evans BS, Kilpatrick AM, Hurlbert AH and Marra PP (2017) Dispersal in the Urban Matrix: Assessing the Influence of Landscape Permeability on the Settlement Patterns of Breeding Songbirds. Front. Ecol. Evol. 5:63. doi: 10.3389/fevo.2017.00063

The ability of organisms to disperse across urban landscapes is theorized to be constrained by habitat fragmentation. While previous research has shown the distribution of forest patches is a determinant of dispersal patterns among forest-obligate bird species, the impacts of habitat distribution on the dispersal of "urban-adapted" species, has yet to be examined. Here, we use capture-reencounter data of birds banded over a 9-year period at six banding stations in greater Washington, DC to assess dispersal in four species of songbirds and a translocation experiment to examine the influence of land cover on movement. Point count and land cover data were used to construct habitat suitability and landscape permeability surfaces, with the latter representing potential travels costs from the capture location to the surrounding landscape. To assess how dispersal processes are affected by urban land cover, we searched for previously banded birds at sampling locations within 1.5 km of each banding station and compared the distribution of sampling locations with and without observations of previously-banded birds. We found evidence that settlement of two of four focal species, the Northern Cardinal (*Cardinalis cardinalis*) and Gray Catbird (*Dumetella carolinensis*), was more likely in sites with high relative permeability. To experimentally explore the consequences of the urban matrix habitat on movement, we attached radio transmitters to male Cardinals, translocated individuals 1.5 km across high-intensity urban, suburban, and forested landscapes, and recorded the time to return to their territory. Return time was dependent on land cover with Cardinals translocated across suburban habitats returning significantly faster than those moved across the other two land use classes. Combined, our findings suggest that, even among some "urban-adapted" species, dispersal within urban environments may be influenced by landscape structure and composition.

Keywords: landscape ecology, urban ecology, avian dispersal, movement ecology, avian ecology

INTRODUCTION

Habitat fragmentation is hypothesized to be one of the primary mechanisms through which the expansion of urban environments has led to a global decline in biodiversity (McKinney, 2002). As environments are modified by urbanization, the area associated with available high quality habitat is often reduced for many species (i.e., habitat loss). While often treated synonymously in the literature, habitat loss describes the conversion of habitat by anthropogenic land use practices while habitat fragmentation is the physical breaking apart of once contiguous habitats (Wilcove et al., 1986). Fragmentation may physically isolate patches of habitat, which decreases the structural connectivity of a landscape and may impede the movement and dispersal of individuals (Fischer and Lindenmayer, 2007). Habitat modification due to urbanization therefore reduces habitat quality for many species and functionally isolates individuals and populations from portions of the landscape (Andren and Delin, 1994; Moilanen and Nieminen, 2002). The movement of individuals between birthplace and first breeding location (natal dispersal) or between successive breeding locations (breeding dispersal), is critical for metapopulation persistence through linking local populations and permitting gene flow across a landscape (e.g., Levins, 1969; Hanski, 1999; Clobert, 2001). As human-built habitats likely affect patterns of dispersal in many urban regions, determining how organisms disperse within such environments provides an important tool for understanding the impact of urbanization on wildlife populations (Crooks and Sanjayan, 2006).

Dispersal is described as a three-step process involving emigration from breeding or natal sites, movement between sites, and settlement to new breeding locations in a subsequent year (Weisser, 2001; Bowler and Benton, 2005). This process is more complex for migratory species, as conditions on wintering grounds have been found to determine the condition of individuals and the degree of natal dispersal in subsequent years (i.e., carry-over effects, e.g., Studds et al., 2008). Even among resident species, the costs associated with one stage of dispersal affect subsequent stages because the condition prior to emigration, the movement trajectory to new breeding locations, and the success of an individual following settlement are interdependent (Stamps et al., 2005; Clobert et al., 2009). For dispersal to be advantageous, the benefits of establishing new territories must outweigh the costs associated with increased predation risk, stress, and energy depletion for dispersing individuals (Greenwood and Harvey, 1982). Although both emigration and settlement are thought to be a function of multiple factors (e.g., conspecific density and patch size), movement is primarily dependent on an organism's perception of the landscape and the ability of an individual to travel through the inter-patch matrix (Baguette and Van Dyck, 2007). As such, the physical arrangement of habitat patches, the life history traits of the associated species, and the species-specific quality of the inter-patch matrix are expected to be determinants of whether individuals can successfully disperse between patches (Opdam et al., 1985; Urban and Keitt, 2001; Bélisle, 2005).

While the influence of habitat modification on the movement of organisms has often been considered largely a function of the physical distance between resource patches and the arrangement of those patches within a given landscape, the habitat encountered during transit is likely critical for dispersing birds (Haila, 2002; Ewers and Didham, 2006). The extent to which the movement of individuals may be facilitated or impeded by the resources and conditions encountered is described as landscape permeability, which is a continuous metric that is a determinant of the structural and functional connectivity of landscapes (e.g., Taylor et al., 1993; Kupfer et al., 2006; Lindenmayer and Fischer, 2006). The ability of organisms to move across landscapes fragmented by urbanization, and thus access patches of resources, is contingent both on the quality of the landscape matrix and species-specific response to landscape features (Wiens, 1976; Kotliar and Wiens, 1990; Pearson et al., 1996). For example, in the study of a single landscape, Bunn et al. (2000) found that their study landscape had high permeability for the American Mink (*Mustela vison*) and low permeability for the Prothonotary Warbler (*Protonotaria citrea*) due to their different dispersal abilities.

An organism's response to the landscape during dispersal is expected to be dependent on its life history traits as well as the landscape-specific costs and benefits associated with movement and settlement decisions (Stamps et al., 2005; Bonte et al., 2012; Burgess et al., 2012). Dispersal events that incorporate exploratory behaviors (e.g., as a consequence of foraging activities) are expected to minimize dispersal costs as exploration increases the efficiency by which individuals settle in suitable habitat, thus reducing the risk of unsuccessful dispersal (Baguette et al., 2013). For dispersal events that include exploratory behaviors, it is expected that the path taken between previous natal or breeding sites and settlement locations will be strongly affected by the distribution and quality (i.e., permeability) of habitat encountered during dispersal (Van Dyck and Baguette, 2005). As a result, patches of suitable habitat that are located within a matrix of low habitat quality are expected to have a lower probability of settlement. If the distance between patches exceeds the perceptual range of a species or patches are embedded within a low quality matrix, directed dispersal events (i.e., those that involve long distance straight-line movements) are expected to predominate and intervening land cover will have little effect on the probability that a location will be settled (Wolff et al., 1997; Mennechez et al., 2003). Directed dispersal events may be less energetically costly for dispersing individuals but may increase the risk of unsuccessful dispersal (Zollner and Lima, 1999; Bonte et al., 2012).

Direct studies of animal movement within fragmented landscapes demonstrate that land cover features are critical to constraining or facilitating travel. For example, previous research using simulated territorial intrusion (Awade and Metzger, 2008), simulated predation threat (Bélisle and Desrochers, 2002), and radio-telemetry (Bayne and Hobson, 2001) have shown that forest bird species exhibit behavioral avoidance when crossing open habitats (i.e., gap-crossing decisions). At larger spatial scales, patterns of seed dispersal and translocation experiments have provided proximate evidence that birds utilize corridors of habitat when moving across fragmented landscapes (e.g., Haddad et al., 2003; Carlo et al., 2013) and that avian movement is impacted by the degree of human land use intensity (e.g., Kennedy et al., 2010). At still larger scales, graph models, which are network models in which landscapes are delineated into interconnected habitat patches, have provided a link between bird dispersal behavior and the structure and composition of landscapes (Bunn et al., 2000; Urban and Keitt, 2001; Minor and Urban, 2008). The applicability of graph models, however, is dependent on the patchiness of the landscape and may be difficult to apply in regions with large contiguous habitat patches or for species that utilize the inter-patch matrix (Minor E.S., personal communication). Despite evidence that urban land cover mediates movement, there remain comparatively few studies that empirically link movement and land cover to dispersal events in urban environments (but see Delgado et al., 2010) and none to our knowledge that have assessed the impacts of land cover on the dispersal of species common to urban and suburban habitats.

Here, we consider the breeding dispersal syndromes of four songbird species within urban landscapes of varying intensity in metropolitan Washington, DC, USA. Our study species, which include the American Robin (*Turdus migratorius*), Gray Catbird (*Dumetella carolinensis*), House Sparrow (*Passer domesticus*), and Northern Cardinal (*Cardinalis cardinalis*), co-occur across the rural-to-urban gradient but represent distinctly different life history strategies (e.g., resident species and short-distance and Neotropical migrants) and are thus expected to have a differential response to the composition and structure of landscapes. We address the breeding dispersal of these species using two levels of inference—a mark-recapture study to assess the influence of landscape permeability on settlement patterns and a translocation experiment to evaluate the effect of rural, suburban, and high-intensity urban land cover on movement. We test the hypothesis that landscape permeability affects settlement patterns by assessing the spatial distribution of previously marked individuals at sampling locations within a 1.5 km radius of six banding stations. We predicted that the likelihood of settlement of a sampling location is positively correlated with landscape permeability due to exploratory movements during the dispersal process. Because events outside of the breeding landscape have been found to strongly affect the settlement patterns of migrant species (e.g., Studds et al., 2008), we expected that permeability will be a greater determinant of settlement patterns for resident (Northern Cardinal and House Sparrow) than migrant birds (American Robin and Gray Catbird) in urban environments. We further examined the impact of land cover on the movement phase of dispersal by conducting a translocation experiment across landscapes of differing urban intensity with one of our focal species, the Northern Cardinal.

MATERIALS AND METHODS

To assess patterns of breeding dispersal, birds color-banded at six banding stations in metropolitan Washington DC between the years of 2003 and 2011 were resighted during the breeding seasons of 2011 and 2012. Banded birds were aged by plumage and skull ossification (see Pyle, 1997)—because hatch year birds are expected to have dispersal kernel extents that exceed the spatial extent of our sampling, only adult birds banded in previous years were maintained for this analysis. Banding stations were representative of rural (Rock Creek Park, Maryland), suburban (Spring and Opal Daniels Parks Takoma Park, Maryland, Bethesda Maryland), and high-intensity urban (Foggy Bottom and the National Mall in Washington, DC) landscapes. For each banding station, the surrounding area, hereafter referred to as the study landscape, was binned into four distance classes (the banding location, 150–500 m, 500–1,000 m, and <1,000–1,500 m) and four directional classes, representing each cardinal direction. Sampling locations were selected at random from each directional and distance bin with the number of samples per bin stratified by distance such that an equal proportional area was sampled within each bin. The sampling extents for Spring and Opal Daniels Parks were partially overlapping—because color-banded birds that were banded at either station can be resighted, these landscapes contain more sampling locations within the area of overlap than do the other study landscapes. As our study region is predominantly composed of privately-owned land, sampling locations were adjusted when possible to the nearest accessible area and a minimum distance of 100 m was maintained between samples. For each sampling location, a minimum of 20 min, when possible, was spent searching for color-banded birds within an area of 50 m from the center of the sample using playback of conspecific song and mobbing calls. Time spent resighting averaged 36 min per sampling location and ranged from a minimum of 13 min to a maximum of 70 min. Resighted birds were identified by color bands and their location was recorded using a handheld GPS.

To evaluate how landscape permeability affects settlement patterns, we estimated habitat suitability using fixed radius (50 m) point count data and land cover metrics expected to be representative of habitat preference. Point counts were conducted during the breeding season between 2009 and 2012 by technicians as a part of the Neighborhood Nestwatch program at 102 sites across the rural-to-urban gradient within the Washington DC metropolitan area (for full description of point count methodology, see Evans, 2015). Proportional canopy cover and impervious surface (30 m resolution, Xian et al., 2011) were used as proxy variables in constructing habitat suitability models. While we acknowledge that these variables do not represent the totality of habitat variation along the rural-to-urban gradient, these land cover metrics have been shown to be predictive of avian nest success (canopy cover and impervious surface, Ryder et al., 2010), adult survival (impervious surface, Evans et al., 2015), and community composition (impervious surface, Evans, 2015). All spatial analyses were carried out in R using packages *raster* (Hijmans, 2015) and *sp* (Pebesma and Bivand, 2005). We calculated the proportional cover of impervious surface and canopy within a 3 × 3 cell neighborhood moving window (for R code used throughout this manuscript, see Supplementary Material). Because several of our species are considered to be edge specialists, we also calculated the standard deviation of canopy cover values for each 3 × 3 cell neighborhood (root mean squared, RMS). This metric is considered a continuous analog of categorical habitat heterogeneity as high standard deviation in canopy cover values relates to habitats associated with forest edges (see McGarigal et al., 2009).

We used the N-mixture models of Royle et al. (2004) in the R package *unmarked* (function *pcount*; Fiske and Chandler, 2011) to model the habitat suitability for each species as a function of land cover metrics. Point count data were subset such that only sites with a minimum of three counts were evaluated. Because detection likely varies by observer, we included observer within the detection parameter of abundance models. As species may show a differential response to canopy cover at different levels of development (impervious surface) and degrees of heterogeneity (standard deviation of canopy cover), interaction terms were included between these variables and proportional canopy cover as well as an interaction term between impervious surface and heterogeneity. Akaike's Information Criteria, adjusted for small sample size (AIC_c), were used to assess models (Burnham and Anderson, 2002). To evaluate the explanatory power of models, we used Nagelkerke's (2004) R-squared index. To avoid overemphasizing potentially spurious relationships between explanatory variables and avian abundance, and because the purpose of constructing these models was to develop habitat suitability surfaces rather than determining which explanatory variables best explain avian distributions, we used model averaging of our symmetrical model set to predict habitat suitability across each sample landscape (**Figure 1**; see Burnham and Anderson, 2002; Lukacs et al., 2010; Mazerolle, 2015). Habitat suitability values were estimated for each focal species and scaled from zero to one for each landscape surrounding banding stations, with zero representing unsuitable habitat, and one for suitable habitat.

Habitat suitability models were used to estimate the permeability from the banding station to surrounding raster cells for each study landscape using the package *gDistance* (functions *transition*, *geoCorrection*, and *accCost*; Van Etten, 2015). We calculated a transition matrix between adjacent raster cells (queen's case) weighted by the inverse of the habitat suitability rasters—thus suitability provides a measure of conductance between cells (see McRae et al., 2008). Transition matrices were used to develop a cost distance raster representing the walk distance, in meters, between the banding station and all surrounding cells with the probability of an individual moving between cells weighted by habitat suitability. Because cost distance increased and the prevalence of resighted birds decreased as a function of the Euclidean distance from the banding station, we defined the permeability for cells within study landscapes as the ratio of the cost distance and random walk distance between the banding station and each raster cell. Thus, permeability rasters represent the functional distance between capture and all cells within a given study landscape after removing the effect of Euclidean distance. Because there was considerable variation among study landscapes in suitability and permeability, rasters were scaled (z-scores) such that cell values are provided relative to all other values within a given study landscape.

To test our prediction that habitat permeability impacts settlement patterns, we compared models of dispersal in which sampling locations were occupied by banded birds as a function of the habitat suitability of the settlement location and the permeability between the banding station and sampling location for each study landscape. Crucially, the level of observation for this study is the sampling locations rather than resighted birds. Therefore, while a single sampling location may include several resighted individuals, each location is only representative of one positive sample per species resighted. Sampling locations within each landscape were subset such that samples within 150 m of the center of the banding station were not considered to have dispersed. This distance, while conservative, may be representative of within-territory movement of birds maintaining the same territories in subsequent years—thus patterns of observations of previously banded birds in this distance range likely result from biological processes other than dispersal. Additionally, not all species were resighted across all study landscapes—only sampling locations within a landscape with a resight for a given species were included in our analysis. Permeability and habitat suitability raster values were extracted to each sampling location and the influence of these variables were assessed in a mixed effect logistic regression framework in the R package *lme4* (Bates et al., 2015). We developed a candidate set of models that included study landscape as a random effect and the fixed effects of habitat suitability, permeability, and the additive and multiplicative effects of these predictors. We evaluated support for the effect of permeability on settlement locations by comparing ΔAICc scores and evidence ratios of models with and without this variable (see Arnold, 2010; Burnham et al., 2011).

To further assess dispersal across matrices of varying urban intensity, we explored the influence of land cover on movement by conducting a translocation experiment across landscapes representing rural, suburban, and high-intensity urban land use classes. This experiment provides us with proximate evidence of how dispersal patterns in human-dominated landscapes might be shaped by variation in the movement of birds in response to human land use practices. We conducted the experiment on eight male Northern Cardinals captured at the banding stations during the breeding seasons of 2011 and 2012. Northern Cardinals were chosen because they were the most frequently re-encountered birds among our focal species and exhibit the highest degree of territoriality. On each bird, we attached a 0.39 g VHF radio transmitter (Advanced Telemetry Systems, Isanti, MN) using a backpack harness of elastic thread (Rappole and Tipton, 1991). Birds were translocated 1.5 km from the capture location across one of eight pre-determined landscapes, and tracked for a period of no less than 0.5 h twice per day following release. The location of the bird was determined using triangulation and, when possible, visible identification of the individual. To assess the effect of intervening land cover on movement, we then calculated the latency (days-to-return) for each individual. Behavioral latency was evaluated as a function of the three land use classes within a generalized linear model framework (Poisson distribution) and assessed using AICc as above. We were unable to observe a return for two of the eight

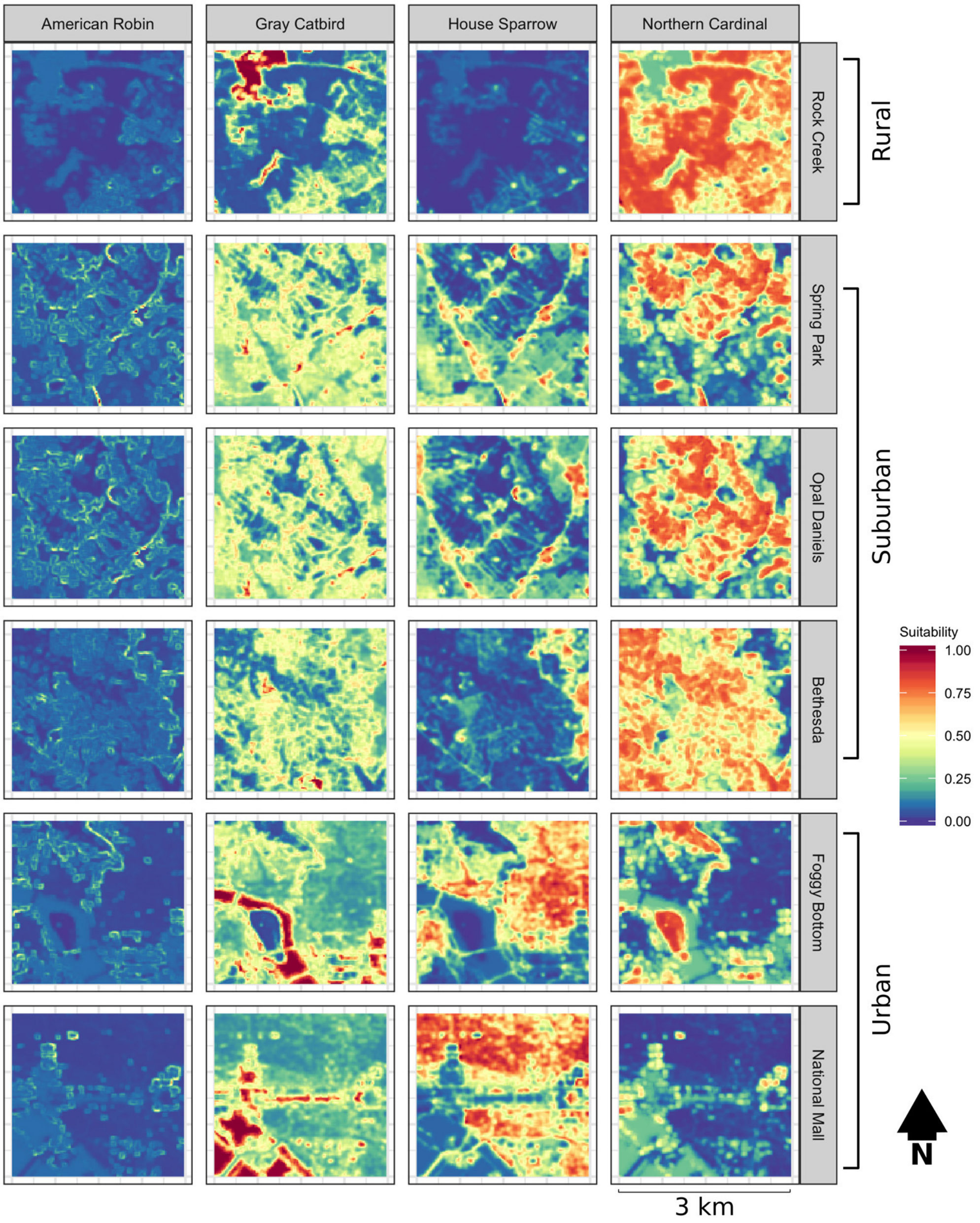

FIGURE 1 | Habitat suitability estimates obtained from point count data, scaled from 0 to 1, for sample landscapes encompassing rural, suburban, and urban habitat types.

individuals, one translocated across a forested habitat matrix and the other across a suburban matrix. Only three of the eight individuals were able to be tracked while moving to their capture location.

RESULTS

Habitat suitability models developed from point count data show species-specific differences in suitability across study landscapes. The fit of abundance models was highest for the House Sparrow and Northern Cardinal (pseudo-$R^2 = 0.97$ and 0.72, respectively) and lowest for the American Robin (0.50) and Gray Catbird (0.65, see **Table 1** for abundance model selection). Raster predictions of American Robin habitat suitability exhibited considerably lower variability than that of the remaining species (**Figure 1**). These results suggest that, given the environmental variables used to develop these models, the Northern Cardinal and House Sparrow are found in a narrower portion of environmental niche space within our study landscapes than are the Gray Catbird and American Robin.

Among 2,706 identifiable adult birds that were color-banded at the six banding stations, a total of 127 birds were

TABLE 1 | Samples of focal species across each of the six banding stations.

Species	Samples	Spring park	Opal daniels	National mall	Foggy bottom	Rock creek	Bethesda	Total
American robin	Banded	139	65	63	141	6	129	504
	Resights	8	9	3	9	0	2	31
	Resights > 150 m	2	5	2	4	0	2	15
	Resight locations > 150 m	2	3	1	2	0	2	10
Gray catbird	Banded	172	109	78	67	10	122	558
	Resights	18	11	3	6	0	4	40
	Resights > 150 m	9	7	1	4	0	3	24
	Resight locations > 150 m	6	7	1	1	0	2	17
House sparrow	Banded*	235	177	302	330	1	168	1212
	Resights	5	0	8	6	0	0	19
	Resights > 150 m	3	0	2	4	0	0	9
	Resight locations > 150 m	3	0	2	3	0	0	8
Northern cardinal	Banded	129	22	9	17	195	60	432
	Resights	21	6	0	2	6	2	37
	Resights > 150 m	12	6	0	2	2	1	23
	Resight locations > 150 m	8	5	0	1	2	1	17

Banding totals include only adult birds with identifiable band combinations. Resights represent the total banded birds encountered across distance classes. Only birds resighted at a distance of greater than 150 m from the banding station were considered to be dispersed and used in dispersal analyses.

**Banding counts for House Sparrow represent the number of uniquely color-banded individuals with color combinations distinguishable from juvenile birds rather than total captures.*

re-encountered in this study, with 70 individuals observed at a distance of greater than 150 m from the banding station (**Table 2**). This yielded a total of 53 sampling locations greater than 150 m from the initial capture location with resighted color-banded birds. Observed settlement patterns highlight species-specific differences in how landscape permeability and habitat suitability affect dispersal, with supportive evidence for our prediction that patterns of settlement would reflect the permeability between capture and resight locations for two of our four focal species (**Table 3**, **Figures 2**, **3**). There was model support that the probability of settlement was higher in sites with high landscape permeability for the Northern Cardinal and Gray Catbird, with evidence ratios of 17.0 and 17.5, respectively, relative to the best performing model that excluded this variable. These results support the hypothesis of dispersal as a consequence of exploratory movements. There was no evidence that American Robin and House Sparrow settlement patterns were impacted by permeability, as the null and permeability models received equivalent support for these species. As such, there was no support for our hypothesis that intervening land cover is a determinant of American Robin and House Sparrow dispersal behavior. Additionally, as Northern Cardinal and House Sparrow are resident species, and Gray Catbird and American Robin are migrants, our results did not fit our expectation that residents would be more likely to be influenced by landscape permeability.

Our translocation experiment supported the prediction that movement patterns of the Northern Cardinal are influenced by the degree of urban intensity between capture and release locations. Cardinals translocated 1.5 km over suburban landscapes returned in 1 and 3 days, whereas those that were moved over forested landscapes returned in 5 and 7 days and those moved over high-intensity urban landscapes returned in 9 and 14 days. Despite sample size limitations, the model that included land cover variables as a predictor of days-to-return received considerably more support than the null model (ΔAICc = 7.24). We tracked movements over a portion of the route for both of the suburban Cardinals and one of the urban Cardinals, while the remaining individuals were not able to be observed until they returned to their respective capture locations. Movements of suburban Cardinals predominantly followed forest edges, when available, as they returned to the capture location. The high-intensity urban Cardinal moved a distance of roughly 0.5 km on the day following release and remained at that site for 8 days until traveling back to the capture location in a single flight.

DISCUSSION

This study addresses the impact of urbanization on the dispersal syndromes of birds through environments of varying urban intensity—we assess patterns of settlement of migrant and resident birds one or more years after banding to evaluate dispersal, and the return time of Northern Cardinals translocated across rural, suburban, and high-intensity urban environments to explore the influence of urban land cover on movement. The settlement patterns of banded Gray Catbird and Northern Cardinal in relation to the habitat suitability and permeability of the landscape provide support for our prediction that landscape permeability impacts the dispersal of individuals within urbanized environments. In conjunction with the results of our translocation experiment, these findings provide evidence

TABLE 2 | Model selection table for distance models used to estimate the abundance of four focal species at sites throughout the Washington D.C. metropolitan region.

Species	Model	*K*	AICc	ΔAICc	*w*	-LogLik
American robin	∼OBS ∼IMP*RMS + CAN	19	1,347.3	0.00	0.36	654.7
	∼OBS ∼IMP*CAN	18	1,348.7	1.20	0.20	656.3
	∼OBS ∼IMP*CAN + RMS	19	1,348.9	1.40	0.18	655.4
	∼OBS ∼IMP*RMS + CAN*RMS	20	1,351.3	1.97	0.13	654.7
	∼OBS ∼IMP*RMS	18	1,352.7	3.82	0.05	657.7
Gray catbird	∼OBS ∼IMP*CAN + RMS	19	1,015.5	0.00	0.84	488.4
	∼OBS ∼IMP*CAN	18	1,019.1	3.60	0.14	488.9
	∼OBS ∼IMP*RMS + CAN*RMS	20	1,024.7	9.19	0.01	469.6
	∼OBS ∼CAN	16	1,026.9	11.4	0.00	238.6
	∼OBS ∼IMP*RMS + CAN	19	1,027.7	12.2	0.00	465.1
House sparrow	∼OBS ∼IMP*CAN	18	1,300.5	0.00	0.72	632.3
	∼OBS ∼IMP*CAN + RMS	19	1,302.5	2.00	0.26	632.3
	∼OBS ∼IMP*RMS + CAN	19	1,309.6	9.13	0.01	635.8
	∼OBS ∼IMP*RMS + CAN*RMS	20	1,310.0	9.46	0.01	635.0
	∼OBS ∼IMP + RMS + CAN	18	1,311.2	10.84	0.00	637.7
Northern cardinal	∼OBS ∼IMP*CAN	18	1,292.6	0.00	0.24	628.3
	∼OBS ∼CAN*RMS	17	1,293.1	0.19	0.19	629.5
	∼OBS ∼CAN	16	1,293.8	1.26	0.13	630.9
	∼OBS ∼IMP*CAN + RMS	19	1,294.4	1.81	0.10	628.2
	∼OBS ∼CAN*RMS	18	1,294.9	1.92	0.09	629.2

All models include observer (OBS) as a predictor variable for the detection parameter. Predictor variables for abundance include canopy (CAN), impervious surface (IMP), and the standard deviation of canopy cover (RMS) within 100 m of the point count location. The top five models for each candidate set are shown. Full model selection table is located in Supplementary Material.

for a link between the structural and functional connectivity of urban landscapes (Tischendorf and Fahrig, 2000; Schooley and Wiens, 2003; Kindlmann and Burel, 2008).

Our observation that the behavioral latency of translocated Northern Cardinal is affected by urban intensity is comparable to those of previous translocation experiments. For example, Kennedy et al. (2010) found the return time and success of translocated American Redstart (*Setophaga ruticilla*) and Jamaican Todies (*Todus todus*) was dependent on human land use practices, with the days-to-return for individuals increasing with the intensity of anthropogenic habitat modification. In our study, Northern Cardinals were observed to have the shortest return time when moved across suburban habitats, habitats which likely represent high suitability for this species (see Evans, 2015). Cardinals exhibited intermediate return times across forested habitats and the longest return time across the most heavily urban landscapes, which reflect moderate and low estimated habitat suitability, respectively. The influence of forested habitat on Cardinal return time especially underscores the species-specific nature of landscape permeability as Northern Cardinal tend to occupy forest edge and open or shrub-dominated at comparatively high densities relative to intact forest (see Leston and Rodewald, 2006). It would therefore not be expected that a forested landscape would be highly connected for this species (see Wiens, 1989; Pearson et al., 1996). Indeed, for the Northern Cardinal in our study region habitat "patches" largely represent suburban environments fragmented by forests. An important caveat to our results, however, is that the movement patterns observed in a translocation experiment may not be representative of movement during breeding dispersal events, as the motivation and behavior during transit may differ considerably between dispersing and translocated individuals (see Betts et al., 2015). Despite this limitation, translocations provide proxy information regarding how animals move through urban landscapes—used in conjunction with patterns of settlement, the results of our translocation experiment support our hypothesis that urban land cover shapes Northern Cardinal dispersal patterns. Inference from our translocation analysis, however, is constrained considerably by low sample size and our inability to track birds throughout their return to their breeding territories (see Zeller et al., 2012).

The permeability of urban landscapes may be a determinant of dispersal patterns if individuals exhibit behavioral avoidance of some habitat features (i.e., matrix resistance and boundary effects, Haddad, 1999; Ricketts, 2001) or if the travel phase of dispersal occurs as a consequence of routine behavior, such as during foraging activities (Baguette and Van Dyck, 2007). Observations of gap-crossing behavior suggest that Northern Cardinal do not avoid gaps and thus are not expected to exhibit behavioral avoidance of the urban matrix (Grubb and Doherty, 1999). Likewise, translocated Northern Cardinals within the current study were observed to travel greater distances when

TABLE 3 | Model selection table for logistic mixed effects models describing the probability that settlement will occur between the banding station and a sampling location as a function of habitat suitability and permeability between the two points.

Species	Model	*K*	AICc	ΔAICc	*w*	-LogLik
American robin	Permeability + Suitability	4	75.5	0.00	0.44	33.6
	Suitability	3	76.9	1.45	0.21	35.4
	Null	2	77.0	1.55	0.20	36.5
	Permeability	3	77.8	2.32	0.14	35.8
Gray catbird	Permeability	3	85.6	0.00	0.70	39.7
	Permeability + Suitability	4	87.8	2.18	0.24	39.6
	Null	2	91.4	5.77	0.04	43.6
	Suitability	3	92.4	6.77	0.02	43.0
House sparrow	Null	2	55.3	0.00	0.39	25.6
	Permeability	3	55.7	0.38	0.32	24.7
	Suitability	3	57.0	1.70	0.17	25.3
	Permeability + Suitability	4	57.6	2.30	0.12	24.5
Northern cardinal	Permeability	3	69.1	0.00	0.70	31.3
	Permeability + Suitability	4	71.4	2.29	0.22	31.3
	Null	2	74.4	5.30	0.05	35.1
	Suitability	3	75.4	6.36	0.03	34.5

intervening land cover was of low suitability, even when locations were available to minimize the flight distances (e.g., shrub or tree perches). This may suggest that, while landscape conductance and resistance are mathematically reciprocal, and thus either would yield equivalent settlement patterns, behavioral motivation during transit is more likely representative of exploratory behavior rather than avoidance. The influence of landscape permeability on the settlement patterns of the migratory Gray Catbird may also be a consequence exploratory movement. Because territories are established soon after arrival to the breeding grounds, and the post-breeding period has been found to involve considerable landscape exploration during foraging activities (e.g., Rappole and Ballard, 1987; Heise and Moore, 2003), it is likely that the influence of intervening land cover on dispersal reflects exploratory movement rather than behavioral avoidance of matrix habitat.

While our data support the hypothesis that dispersal is influenced by habitat between banding and settlement locations for two of our study species, behavioral motivation (e.g., exploratory movements) for habitat selection during the movement phase of dispersal events could not be directly assessed. Motivation for landscape resistance or conductance is dependent on the behavioral state of dispersing individuals. For example, in their study of the movement of pumas in southern California, Zeller et al. (2014) found that pumas exhibited strong behavioral avoidance of agricultural and urban areas during resource use (e.g., foraging), as evaluated by the path tortuosity of radio-tagged animals. Settlement locations provide insufficient evidence for behavioral motivation and, because we were largely unable to follow translocated Cardinals throughout their return to their respective banding locations, we were unable to assess the behavioral state of radio-tagged individuals. Moreover, we acknowledge that behaviors, such as conspecific attraction, impact movement and settlement decisions and these behaviors are not observable with settlement data alone. It therefore remains uncertain which mechanisms best explain the observed settlement patterns, especially as the path taken between breeding patches cannot be known within this context. Likewise, low reencounter rates may suggest that our study was necessarily biased toward short-distance dispersers, which are expected to be more responsive to landscape pattern (Van Dyck and Baguette, 2005). Thus there may be multiple behavioral strategies driving movement within our sampled population. These caveats highlight a need to track individuals during the movement phase of dispersal, assess dispersal events across the annual cycle of birds, and expand the spatial extent of sampling in order to develop a better understanding of the dispersal syndromes of our study species.

Though we failed to observe a direct effect of habitat suitability on settlement patterns, the low explanatory power of suitability within our dispersal models is representative only of resighted birds, not of the occupancy distribution as a whole. Indeed, while sample locations spanned a range of suitability values, samples with and without resighted birds were often occupied by unbanded individuals of each species. As dispersing birds typically encounter other territory-holders when attempting to establish a new breeding territory, it is therefore unsurprising that we observed no influence of suitability on patterns of settlement. Alternatively, lack of observed influence of habitat suitability may also be representative of limitations in the use of point count data to estimate suitability within our study region. Because point counts, especially those conducted in residential areas where private lands predominate (see Evans, 2015), are able to sample only a subset of a given landscape, the accuracy of estimates may be limited in un-sampled and under-sampled portions of the evaluated environmental gradients. This limitation is exemplified by suitability values for the Gray Catbird in the Foggy Bottom study landscape, in which open water (the Potomac River) was estimated to have much higher suitability than the surrounding region (see **Figure 2**).

Dispersal syndromes for the House Sparrow and American Robin were not shown to be influenced by habitat permeability. Indeed, we found no evidence that American Robin settlement patterns were affected by either landscape permeability or habitat suitability. The low explanatory power of American Robin dispersal models may have been driven in part by low variation in predicted habitat suitability of our study landscapes (**Figure 1**). For House Sparrow, however, the observed landscapes had low suitability overall, and this species was only observed at sampling locations with considerable urban development. It is expected the overall degree of suitability of a landscape is a determinant of whether differences in permeability may impact dispersal. Conversely, the lack of an observed effect of landscape permeability for these species may also be a

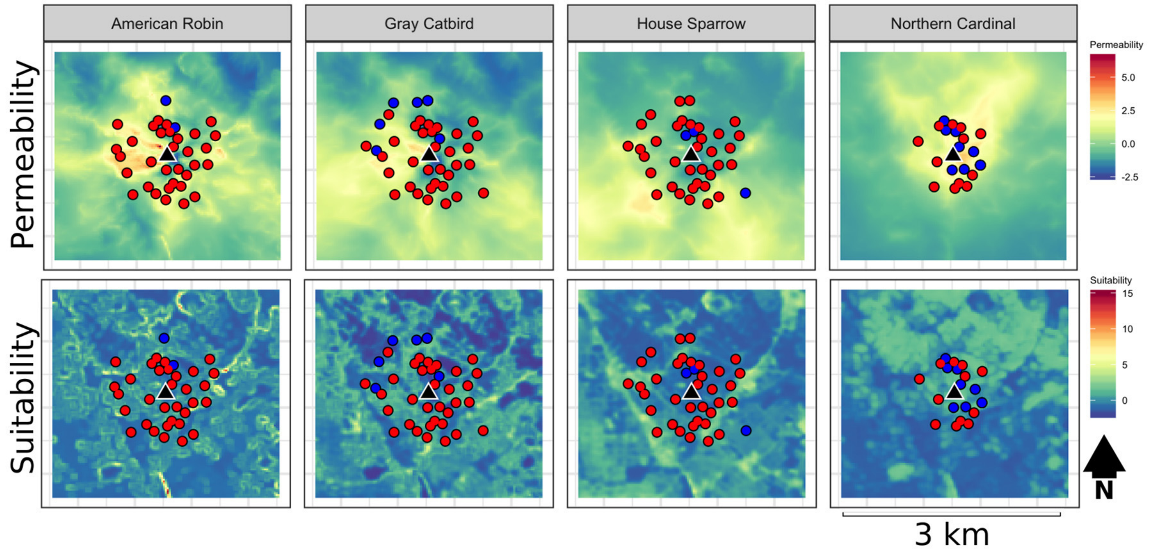

FIGURE 2 | Example habitat suitability and permeability raster maps, in addition to sampling locations with (blue) and without (red) resighted individuals for each of our four focal species surround the suburban banding station located at Spring Park in Takoma Park, Maryland. See **Supplementary Figure 1** for suitability and permeability maps across banding stations.

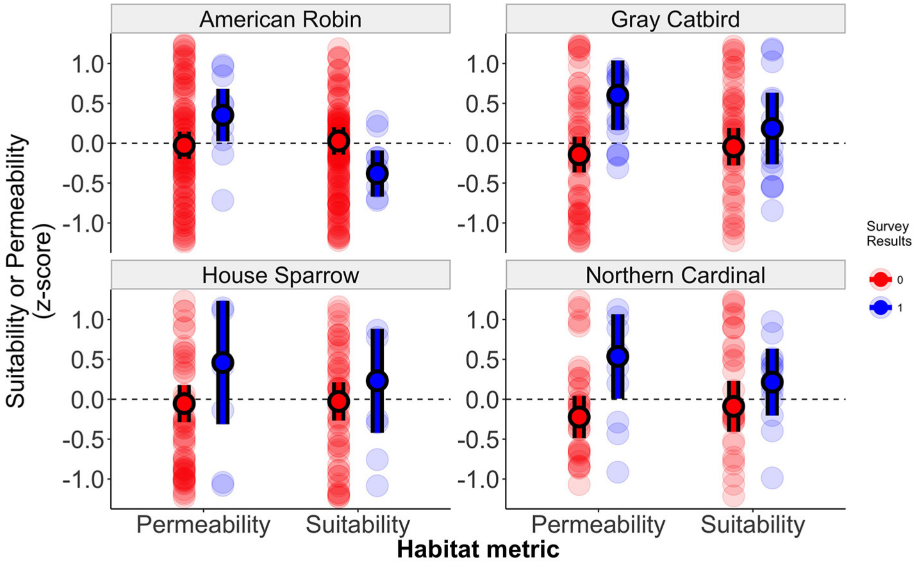

FIGURE 3 | Habitat connectivity and suitability of sites in which birds were resighted or in which no banded birds were observed. Standardized effect sizes represent habitat connectivity and suitability values for each banding station, with values above zero representing samples with high suitability or connectivity. Solid points represent mean values for each category with error bars representing 95% confidence intervals about the mean. Semi-transparent points represent the observed data.

consequence of a differential behavioral response to the urban environment, such as low predator avoidance behavior (observed in House Sparrow, Tsurim et al., 2008). Our results may also represent a response to the landscape that occurs at a larger spatial grain and extent than accounted for in this study. For example, the breeding home range size of the resident House Sparrow is estimated to encompass several kilometers (Bennett, 1990) and previous findings have shown low rates of dispersal for this species (Altwegg et al., 2000; Pärn et al., 2009). Therefore, the distribution of resighted House Sparrows may represent observations within individuals' home ranges rather than patterns of dispersal. Likewise, settlement patterns observed during the breeding season reflect components of dispersal (i.e., emigration, movement, and settlement) that may occur at varying spatial scales across birds' annual cycles. For example, although the American Robin typically holds territory sizes roughly equivalent to the Northern Cardinal and Gray Catbird during the breeding season, subsequent to breeding this species often forms wide-ranging foraging flocks (Janousek et al., 2014; Vanderhoff et al., 2014; Rodewald, 2015). Therefore, movement outside of the breeding period may increase an individual's perceptual range of its environment. Finally, the sample size for these species was limited relative to that of the Gray Catbird and Northern Cardinal—this may suggest a dispersal kernel that is considerably larger than the 1.5 km radius sampled in this study. Cumulatively, the results for American Robin and House Sparrow emphasize that determining the influence of habitat composition and structure on dispersal necessitates investigating the scale by which species respond to their environment (Wiens, 1989).

CONCLUSION

With the spread of urbanization and associated fragmentation of urban landscapes, the maintenance of functional connectivity is increasingly recognized as critical for supporting biodiversity (Wiens, 1997). To date, few have addressed patterns of dispersal across urban environments (see LaPoint et al., 2015) and research on how habitat fragmentation influences bird dispersal has reflected a strong bias toward forested environments and forest specialist species (Bayard and Elphick, 2010). As ecological patterns in forested habitats may not be applicable across habitat types (see Rudnicky and Hunter, 1993), the current study provides an important step toward understanding the impact of humans on ecological processes that are shaping bird populations and communities with the expansion of urban landscapes. Overall, for two of our four focal species, our results support the hypothesis that the permeability of landscapes is a determinant of dispersal in urban environments and thus structural components of urban habitats likely govern the functional connectivity of these landscapes. While we acknowledge that patterns of settlement are not directly representative of the path taken between capture and settlement location and, likewise, translocation experiments may not adequately reflect the behavior of organisms during dispersal, our results offer key evidence that land cover associated with urban environments affect avian dispersal syndromes. Despite the unprecedented spatial distribution of resighting effort in this study, we were constrained by the number of banded birds across species, we were able to re-encounter—this suggests limited applicability of using resighting data to identify short-distance dispersal events. With recent technological advancements in the study of birds, it is now becoming feasible to track individuals throughout a bird's annual cycle—including the processes of emigration, movement, and settlement that comprise dispersal events (see Bridge et al., 2011; Hallworth and Marra, 2015). Such advances will greatly increase our understanding of the influence of landscape structure and composition on dispersal across scales.

ETHICS STATEMENT

This research project was approved by the Smithsonian Conservation Biology Institute's Institutional Animal Care and Use Committee. All precautions were taken to minimize any potential stress during handling, including checking nets at a minimum frequency of once per 15 min, handling birds for as short a time as possible, and banding only under optimal weather conditions (e.g., temperatures of less than 32°C). We observed no mortality while conducting this study.

AUTHOR CONTRIBUTIONS

BE: Primary author, conducted field work (banding and resight), sampling design for resight activities, and statistical analysis. AK: Second author, provided major edits to manuscript and its proposal, developed sampling design for banding activities, conducted field work (banding). AH: Third author, provided major edits to each draft of the manuscript and its proposal, including additional text, statistical advising, and sample design advising. PM: Fourth author, provided major edits to each draft of the manuscript and its proposal, including additional text, and sample design advising.

FUNDING

BSE was awarded a fellowship from the Smithsonian Fellowship Program for resighting activities and statistical analyses. Banding data was funded by National Institute of Allergy and Infectious Disease contract NO1-AI-25490, grant 2003–0209–000 from the National Fish and Wildlife Foundation, and by core funding to the Consortium for Conservation Medicine at Wildlife Trust from the V. Kann Rasmussen Foundation.

ACKNOWLEDGMENTS

We thank the many field researchers who contributed to this project. We also thank Thomas B. Ryder and manuscript reviewers whose helpful comments on earlier versions of this work greatly improved our manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00063/full#supplementary-material

Supplementary Figure 1 | Boxplot displaying the distance between the initial banding location and the locations of resighted birds for each of our four target species.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 19 June 2017
doi: 10.3389/fevo.2017.00058

Changes over 26 Years in the Avifauna of the Bogotá Region, Colombia: Has Climate Change Become Important?

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High Neotropical mountains are among the most threatened ecosystems by climate change and this problem could be accentuated in cities where temperatures are higher. However, there are few data of long-term avifaunal changes in Neotropical cities, and the potential impact of climate change has yet to be addressed. Using data from 26 years of Audubon's Christmas Bird Counts (CBCs) in the Bogotá area (ca. 2,600–3,100 m in Colombia's eastern Andes), we analyze long-term changes of the avifauna using linear regressions corrected for species' habitat preferences and compared urban and rural sites. For the CBC we followed the National Audubon Society's methodology in which birds are counted over a 24-h period within a fixed circle 24 km in diameter. We recorded 235 species in the Bogotá circle with an average of 122 spp/year, including 46 boreal migrants, three endemic species, and four endemic subspecies, two globally and four locally threatened species. Species richness was higher in rural than in urban areas and most species were associated with native forest and scrub and wetlands. Among the species that were analyzed for changes in time 31% increased, 20% decreased, and 49% did not change. Strong fluctuations or changes in abundance were more frequent in urban than rural environments. Many of the species that increased or became established during the CBC interval came from lower elevations or the warmer, drier parts of the region beyond the count circle. By contrast, the lower elevational limits moved to higher elevations in several species that decreased. Climate change with its related effects represented the factor associated with the most changes in abundance. Other potential causes were direct human actions, mostly associated with urbanization, and "natural" successional changes in vegetation; predation by feral dogs and cowbird parasitism also affected several species negatively. Observations indicate that these factors will continue into the future, and the effects of climate change and urbanization in particular will probably intensify. The Bogotá CBC has provided much valuable information for Neotropical urban ornithology and its practice should be encouraged in other Latin American cities and should definitely continue in Bogotá for many years to come.

Keywords: Andes, conservation, Audubon's Christmas Bird Counts, neotropical mountains, global warming, land use change, urban ornithology

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to
Urban Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *10 March 2017*
Accepted: *19 May 2017*
Published: *19 June 2017*

Citation:
Stiles FG, Rosselli L and De La Zerda S (2017) Changes over 26 Years in the Avifauna of the Bogotá Region, Colombia: Has Climate Change Become Important? Front. Ecol. Evol. 5:58. doi: 10.3389/fevo.2017.00058

INTRODUCTION

Climate change is presently a growing threat to biodiversity (Root et al., 2003). It is affecting a variety of organisms by shifting elevational distributions (Chen et al., 2011), causing decoupling of phenological relationships (Visser and Both, 2005), changing times of reproductive events (Both et al., 2004), and changing composition and structure of ecosystems (Grimm et al., 2013), among others. High mountain neotropical ecosystems such as those of the Andes are among the most threatened, because of the progressively smaller areas available as elevation increases (Sekercioglu et al., 2008; Chen et al., 2011; Chaverri et al., 2016). Organisms that shift their altitudinal limits upwards, as predicted by global warming, will therefore suffer reductions in their populations and eventually may be driven to extinction at the highest elevations (Larsen et al., 2011; Feeley et al., 2012); tropical mountain systems are particularly vulnerable to climate change because organisms are adapted to more stable climates than temperate organisms at similar elevations and many populations may not have the ability to withstand temperatures not previously experienced (Janzen, 1967; Ghalambor et al., 2006). Moreover, such species often occupy narrow elevational ranges (Anderson et al., 2011). Birds represent an ideal study group for monitoring elevational shifts because they are more easily identifiable in the field than other vertebrate groups and often exhibit strong habitat preferences, especially in montane areas. There are few long-term studies along elevation transects that repeat surveys made decades earlier to evaluate altitudinal movements. One by Forero-Medina et al. (2011) in Perú and another by Freeman and Class-Freeman (2014) in New Guinea detected upward shifts in dozens of bird species. Another approach is to monitor species composition of birds over several decades at a single locality. Pounds et al. (1999) compared data over nearly two decades for a mid-elevation site in Costa Rica, and found that the bird community changed due to an increase of species moving from lower elevations.

There are still relatively few studies of urban birds in Latin America (Ortega-Álvarez and MacGregor-Fors, 2011; Delgado-V and Correa-H, 2013), and these focus mainly on urban gradients, ecology and community composition but few have been developed over a long-term and none have focused on the effects of climate change on birds. Climate change in cities is of particular interest due to their increased tendency to warming, forming "heat islands" due to high carbon dioxide emissions and aerial contamination (Foley et al., 2005; Wilby and Perry, 2006). The city of Bogotá has a population of nearly eight million and is located on an Andean plateau (the Sabana de Bogotá (Montañez et al., 1994) at an elevation of 2,600 m with adjacent mountain ridges reaching over 3,000 m. The city contains a heat island over most of its extension with a temperature 3°C above the perimeter and surrounding areas (Angel et al., 2010). This combination of features makes Bogotá an ideal site for studying the combined effects of climate change and urbanization on birds at high elevations because it is an expanding city at a high altitude, and because few—if any—Andean sites with these characteristics have long-term observations similar to those we report for Bogotá.

Long-term studies of bird communities and populations have a long history in Europe and North America and have contributed greatly to knowledge in science and ecology (e.g., Holmes, 2011; Perrins and Gosler, 2011), but have mostly concentrated on particular forest sites. Another type of long-term monitoring is the annual North American Audubon's Christmas Bird Counts (CBCs), involving the participation of professionals and amateur observers that count birds during 1 day in 24 km diameter circles chosen by compilers. The CBCs in North America now involve thousands of bird watchers and more than 1,286 count circles monitored over periods of up to a century or more (National Audubon Society, 2017; http://www.audubon.org/conservation/science/christmas-bird-count) and have provided fundamental information on changes in distribution and abundance of birds (e.g., Johnsgard, 1967, 2015; Bonney, 1979; Monahan and Koenig, 2006; Soykan et al., 2016). However, such long-term bird monitoring is in its early stages in Latin America due to the lack of a tradition of popular bird watching, largely reflecting the lack of well-illustrated bird guides until the 1980s. In Colombia the authors started a CBC circle in the Sabana de Bogotá in 1986 which became established in 1989 with the foundation of the Bogotá Ornithological Association (ABO). This CBC has continued uninterrupted for more than 25 years, becoming the longest monitoring of any vertebrate group in the country. Our objective here is to use the data of the CBC from 1989 to 2014 to evaluate the changes in the bird community of the city of Bogotá and its periphery and their potential causes, including their possible association with climate change.

Prior to the initiation of this CBC, there was no systematic program of bird observation in Bogotá and no reliable, updated list of the birds of the Sabana. However, the important work by Olivares (1969) permits an earlier summary of the birds of this area, updated through the 1960's. Despite being based nearly exclusively on specimen records (sometimes with vague localities like "around Bogotá"), this work provides a basis for evaluating changes in the two decades prior to the CBC; especially useful in this regard are Olivares's comments on the local abundances of a number of species. Also providing data that help in interpreting the changes in the avifauna of the CBC is a concurrent study by the authors (Rosselli et al., in press) of an area of high Andean forest at 2,800–3,300 m ca. 25 km east of the CBC circle and involving two inventories of its birds in 1991–1992 and 2015–2016.

METHODS

Study Area

The area of the count circle includes the northern part of the city of Bogotá and adjacent rural areas, as well as several mountain ridges reaching elevations between 2,800 and 3,100 m. Its center at 4°48′N, 73°11′W was chosen to include a variety of habitats in an area that we predicted would be affected by the expansion of urbanization in future decades (**Figure 1**).

Mean annual temperature in the city ranges from 12 to 16°C and mean annual rainfall shows a gradient from 800 mm in the south to more than 1,000 mm on the northeastern border (Angel et al., 2010). The rainfall pattern of the count area is

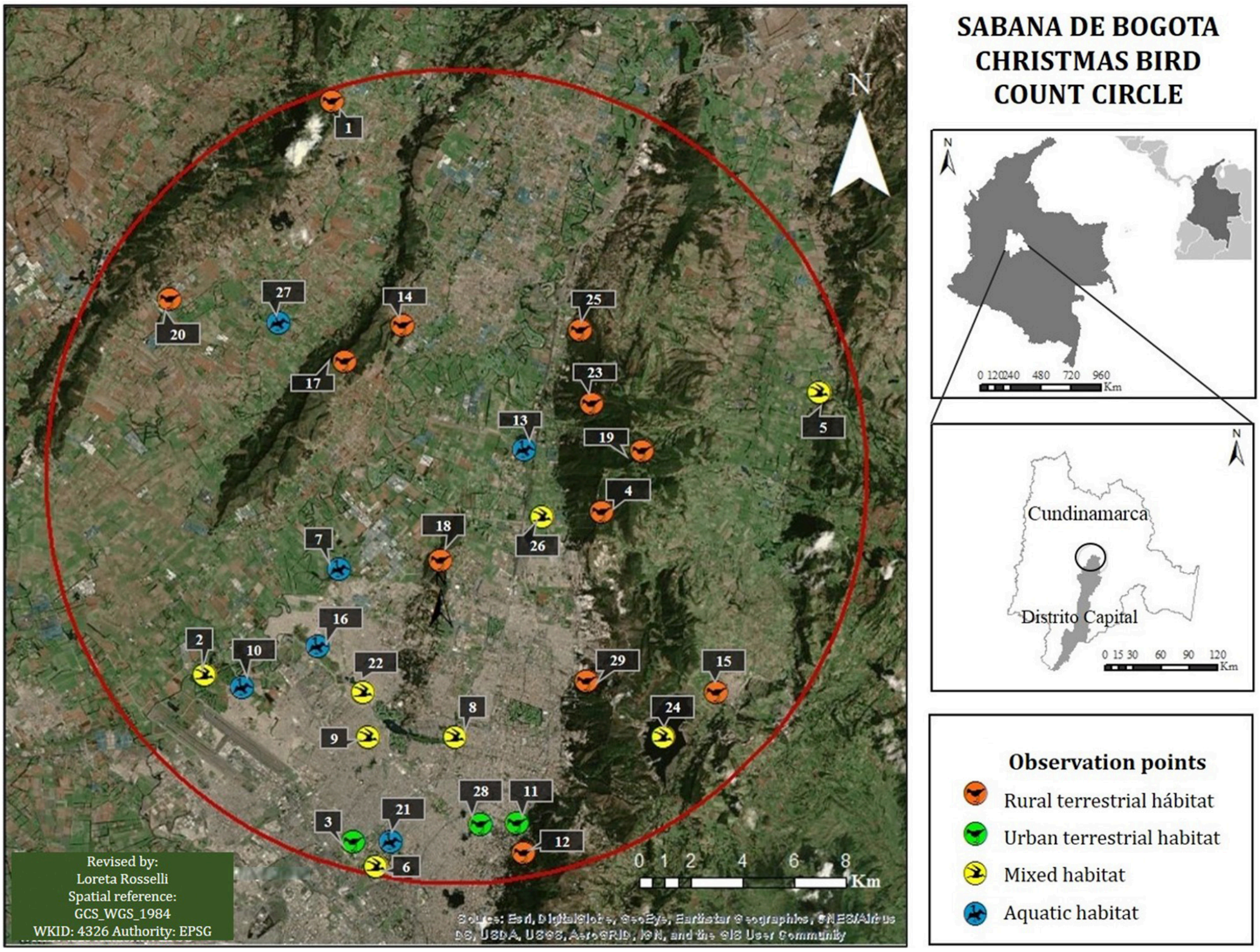

FIGURE 1 | Study area. Location of the Sabana de Bogotá Audubon's Christmas Bird Count circle and the 29 locations visited between 1989 and 2014. Habitat types were assigned according to dominant land covers in each place (more details about habitats in Section Audubon's Christmas Bird Count Methods and Circle).

bimodal, with the CBCs occurring in the transition between the October–November rainy season and the January–March dry season. There has been an increase of 0.2–0.4°C per decade in the mean maximum temperature in the region (Rojas et al., 2010) and a particularly high increase in recent decades in the city itself due to the formation of a heat island that is expanding toward the northwest (Angel et al., 2010), and now includes 9 of the 11 CBC localities within the urban matrix of Bogotá.

The flat Sabana was the bottom of a large lake that was naturally drained and the original habitats of the now dry Sabana were probably native grasslands, wetlands that varied according to rainfall and patches of forest along streams and around wetlands. Most of the city of Bogotá lies on the flat part of the Sabana, which beyond the urban areas includes mostly pastures and croplands. The Sabana is bordered eastward by a branch of the Colombia's eastern Andes reaching elevations of up to 3,200 m, parts of which are included in the count circle, as are several small chains of mountains with elevations between 2,800 and 3,100 m that run in a north-south direction within the Sabana. The slopes of the mountains support native forests and scrub as well as portions of exotic forest plantations. The Teusacá River drains into the Sabana through a gap in the eastern mountains; part of the flat lower valley includes pastures, croplands and small artificial wetlands, which are also included within the count circle.

The transformation of the natural habitats of the Sabana began over 10,000 years ago with the arrival of indigenous peoples who cultivated areas near the wetlands and rivers. Transformation of terrestrial habitats to urban areas, pastures and croplands became more intense during the colonial period. The draining of the wetlands increased especially following the independence from Spain in the nineteenth century and reached a peak in the latter half of the twentieth century (van der Hammen et al., 2008), by the end of which only 3% of the original wetlands remained (Andrade, 1998). The growth of the city of Bogotá also produced the repeated deforestation of most of the adjacent mountains for construction and firewood. Coal and more recently, oil and gas replaced firewood for cooking in the latter part of the twentieth century leading to succession of native forest and growth of exotic tree plantations (mostly *Eucalyptus globulus*) on most of the mountain slopes. The accelerated displacement of rural pastures and croplands by the city's growth over the flat areas of the Sabana continues through the present day. The introduction of the exotic kikuyu grass in the early twentieth century to improve pastures (Pérez-Arbeláez, 1935) resulted in the eradication of native grasses and herbs over most of the Sabana. Between 1995 and 2015 Bogota's population increased by 38% to nearly 7,878,783 (Secretaría Distrital de Planeación, 2016), and its urban area by 33.8% to ca. 38,430 ha between 1993 and 2010, with this growth concentrated on the northern, western and southern edges of the city (Romero, 2010) including some of the CBC points. The reduced remaining urbanizable area in Bogotá has also pushed urbanization to the neighboring municipalities west and north of the city (Martínez-Herrera, 2015) where some of the CBC observation points are located. To evaluate the local changes in our observation points we estimated the changes of 1 km radius circles around the center of each locality using available high resolution historic images from Google Earth (mostly from 1999 to 2005 to the present). We also made comparisons of the amount of urbanized and rural areas in the complete CBC circle comparing such images dating from 1980, 2005, and 2016.

The status of the wetlands is of special concern, due to the number of endemic and threatened bird species they support. Although, in 2000 the city's authorities declared 12 wetlands as ecological parks for conservation, the pressure from urbanization continues, including increased pollution, encroachment of the borders and further reduction of wetland areas, and increasing presence of domestic dogs (Rosselli and Stiles, 2012a).

Audubon's Christmas Bird Count Methods and Circle

The methodology of the CBC was standardized in North America in the 1980s (Arbib, 1981, 1982). For a period of 24 h between approximately December 14 and January 5, parties (groups) of bird watchers identify and count all birds they can detect in a 15 mi diameter circle (24.1 km) with a fixed center. Each party counts birds in one or more specific sites representing some of the habitats in the circle. The distance traveled is recorded (by foot, car, etc.), as are the starting and ending times and the weather conditions. Party coordinators need to have the knowledge and ability to ensure that the identifications of the reported species are correct. Every group's data is revised and compiled by the circle's coordinator. To correct for differences in sampling effort all data for the circle are divided by a global measurement such as party-hours or distance traveled. The method is designed to ensure as far as possible that the data gathered by different groups in different years are comparable and enable assessment of long-term changes of species and populations in the circles. Bock and Root (1981), Butcher and McCulloch (1990), Dunn et al. (2005) and others have addressed the standardization and comparability of data since observers in the circle may change from year to year. Some of these difficulties include identification capabilities of individuals, number of bird watchers in the groups, weather and times of observation, travel distances, hours of observation, extent coverage of different sites and habitats, and use of bird song recordings to attract the birds.

The CBCs have proven reliable to register presence-absence of species and abundance tendencies and have shown correlations with various other surveys in North America (Dunn et al., 2005; Soykan et al., 2016). Over the 26 years of the Sabana de Bogotá counts, group coordinators were familiar with their areas and birds, and as far as possible coordinated in the same locations year after year to reduce variations due to human factors. Since 2000 we designated 13 representative sites with at least 8 years of data as high priority to be counted every year to reduce variability of coverage and to be able to compare the sites year after year. The center and diameter of the Bogotá circle have remained constant over the 26 years, thus ensuring comparability between years.

The CBC Circle

The circle includes a variety of habitats including urban parks, rural areas with crops and pastures, natural and artificial wetlands of different sizes and surroundings, natural and planted forests (mostly *Eucalyptus*) and secondary scrub. We did not intend to count birds in completely urban areas without vegetation due to the limited presence of birds in them; neither did we include the only naturalized species in the area: the rock dove (*Columba livia*); its status as a wild bird is uncertain outside of urban areas because many farmers keep a pigeons that fly freely during the day but sleep and are mostly fed in dovecotes.

We used a simple system to classify the four principal kinds of vegetation cover found in the circle:

NFS- native forests and secondary scrub (locally called matorral; **Figure 2a**): because such scrub represents natural succession to forest and there is a gradation in the ages and heights of the shrubbery, it was better to combine them into a single category. We include exotic forests in this category because their avifauna is basically a subset of those adjacent or intermixed native forests.

ORA- open rural areas: pastures, crops, some scrub and living fences of different types that divide or define a property, farm buildings, and constructions (**Figure 2b**).

WET: Wetlands of various types, from marshes and natural lagoons to artificial ponds, the Bogotá and Teusacá rivers, and seasonal puddles (**Figure 2c**). Species considered as wetland birds include those that depend on the water of the wetland and not vegetation from surrounding areas with different types of terrestrial vegetation.

UPT: Urban parks with trees, with an arboreal stratum of exotic or native trees and underneath areas of lawns and gardens, which sometimes may include patches of dense vegetation such as ornamental shrubs or natural vegetation at the edges (**Figure 2d**). Such parks may be within the urban matrix or adjacent to some wetlands within the city. It is common for parks to attract birds from adjacent urban areas, as well as a greater variety of species that depend on the greater complexity of their vegetation.

TER: A general term for terrestrial vegetation, including NFS, ORA, and UPT, when we make comparisons with wetland birds and vegetation (WET).

URB, RUR: general terms for urban (within Bogotá's urban perimeter) or rural (outside the city) for other comparisons (see **Figure 1**).

Counts were made in a total of 29 sites in the circle during the 26 years analyzed (**Table 1**). Some localities were changed

FIGURE 2 | Main habitats of Audubon's Christmas Bird Counts in the Sabana de Bogotá from 1989 to 2015. **(a)** Native forests and secondary scrub (NFS). **(b)** Open rural areas (ORA). **(c)** Wetlands (WET). **(d)** Urban forested parks (UPT).

in subsequent years in favor of others more representative or accessible. Sometimes permission was no longer granted to visit a site and we tried to replace it with a similar one to maintain continuity of the habitats censused. Beginning in 2001, 13 localities, identified as prioritary, were visited every year. Additional localities were added to these 13 as coordinators and participants were available.

Analysis

To analyze the structure and composition of the birds registered in the 26 years of the CBC, we first defined the status of all recorded species (**Table 2**) as residents or visitors; for visitors, we determined their most probable origin in terms of elevation and region. This classification was based on the count data and on our experience of decades in the Sabana and in other parts of the eastern Andes region. We then identified four important parameters for each species: (a) regularity, in terms of number of years in which it was recorded; (b) its distribution in terms of the number of sites in which it was recorded; (c) its abundance calculated as the average of individuals counted in the years it was registered; and (d) changes in abundance and status of the species over the 26 years, here correcting for habitat preferences (see below). To simplify analysis, we divided these numerical parameters into a smaller number of classes (**Table 2**). Initially, we compiled all general data from the counts: number of sites, party-hours, kilometers/party, number of species, and individuals for each species and site. We obtained the species accumulation curve and analyzed the frequency distributions of species according to their regularity, distribution, and abundance using the numbers of species in each status class of these parameters.

We standardized sampling effort by dividing the abundances of every species by party-hours in the preferred habitat of each one to take into account habitat preferences and yield a more precise assessment of long-term population changes; this is an adaptation of the most commonly used effort adjustment (Raynor, 1975). To begin with, we arbitrarily calculated linear regressions of the corrected numbers/count for all species recorded in 10 or more years but examining the data we found that clear tendencies were evident for some species with 6–9 records that were also analyzed. We excluded from the analysis all species with 5 or fewer records because we considered them insufficient to permit reliable analysis. We also analyzed data for a few species with 6–9 years (data points) where these appeared to show a clear tendency and did not have many years without data between the data points to evaluate tendencies in populations. For several species we calculated regressions with and without outliers (atypical high or low counts in a year, Gotelli and Ellison, 2004) because these can have a disproportionate influence on the regression results, especially when occurring at the beginning

TABLE 1 | Characteristics of the 29 sites censused during the 26 years of the Sabana de Bogotá Audubon's Christmas Bird Count (1989–2014).

A. Numbers of years a site was censused: in parentheses, the number of sites designated as priority (*) and the number of sites requiring substitution due to problems of access (†).

No. years	1–5	6–10	11–15	16–20	21–26
No. sites	7	9 (2*, 2†)	3 (1*,1†)	4 (4)	6 (6)

B. Numbers of sites in which the four principal habitats were represented by 20% or more of the area in a given site.

Numbers of habitats	1	2	3	4
Numbers of sites	10	18	1	0

C. Numbers of sites with the four principal habitats represented (see below for abbreviations of habitats).

Habitats	NFS	ORA	UPT	WET
As principal habitat: 40% or more of site area (in parentheses, two equally represented habitats)	9(3)	2	6(2)	10(2)
Habitats represented by 20% or more of area of site	12	14	10	13

D. Numbers of sites with different combinations of habitats represented by 20% or more of the site areas.

Combinations of habitats	NFS-ORA	WET-UPT	WET-ORA	NFS-WET	ORA-UPT	WET-UPT-ORA
Numbers of sites	8	4	4	1	1	1

NFS, native forests and scrub; ORA, open rural areas; WET, Wetlands; UPT, Urban forested parks.

or end of the interval of time considered. We considered that a species had become established in the circle if it was present for at least 10 consecutive or nearly consecutive (with few isolated missing years in the series) years, preceded by 5 or more years without records; extinction of a species in the circle was inferred if no record of its presence was obtained through the end of the study period following at least 5 continuous years in which it was present. We accepted as valid changes in the numbers of a species when the p-value for the regression was ≤ 0.05; if p was between 0.05 and 0.10, we considered changes as slight increases or decreases and we considered regressions with $p > 0.10$ as indicators of stable populations. We compared the number of the species in rural vs. urban areas, and terrestrial localities (TER) vs. wetland localities (WET) using Chi-square goodness of fit tests.

We used a non-parametrical Mann–Kendall Trend Test to evaluate possible temperature increases with the Sen method for slope calculation. This method is appropriate for time series with missing values and outliers and is oriented to detect and calculate tendencies in time series of climate data (Salmi et al., 2002; He and Zhang, 2005).

RESULTS

Land Cover and Temperature Changes

There were relatively few pronounced changes in the CBC observation points: more than half of the sites showed no change or <5% increase in urbanization (**Figure 3**). These sites with little or no change were mainly (75%) rural and native forest and secondary scrub in which a few houses were added. Three were parks embedded within the city. Management efforts by the city's authorities increased open water habitat in some of the wetlands (**Figure 3**) whilst in a rural locality a 3.5 ha pond was destroyed. The highest percentages in urbanization (10–25%) occurred on the borders of the city and in one of the sites in a neighboring municipality. In some of the urban parks that were established at the beginning of the CBC counts, the trees grew and densified in 5–12% of the area. Therefore, changes at the local level in the observation points were not large but reflect general changes at a broader scale. The urban and conservation areas that were present in late eighties have changed little; the main changes have occurred along the borders of the city in and around the CBC circle and mostly consist of replacement of pastures with housing and commercial developments as well as floriculture greenhouses. In the circle as a whole, we found that the proportion of rural areas, especially pastures, had been decreased by 30–40% by 2005, and by 60–75% by 2016; two neighboring towns have also reduced pastures and crop fields by a similar amount in the last 10–15 years (Supplementary Image 1).

There was a significant trend of temperature increase at the Eldorado airport between 1962 and 2015 in mean maximum, minimum and mean annual temperatures (**Figure 4**). The strongest tendency occurred in the minimum annual mean (Kendall's Tau = 0.62, $p < 0.0001$) with an increase of 0.043°C/year (**Table 3**). The increase rate in temperature was higher and significative for the minimum and mean annual means during the CBC years, but not for the maximum mean (**Table 3**).

General Ornithological Results

In the 26 years of counts, we recorded 235 species of birds in the Sabana de Bogotá Audubon's Christmas Bird

TABLE 2 | Classification of species registered in the Audubon's Christmas Bird Counts in the Sabana de Bogotá circle, 1989–2014.

A. SITUATION IN THE CIRCLE	
PR	Permanent Resident. Present all year long, reproduces in the circle or nearby áreas. Rare or difficult to detect species are classified as residents if there is no evidence of migration or regular movements away from the circle for part of the year. Includes species that were residents at the beginning of the study but eventually disappeared from the counts and others that became established as residents and reproduced in the years included in the analysis.
SR	Seasonal Resident. Present for several months around the date of the CBC although not necessarily all years; almost all these species are boreal migrants that spend the northern winter in the circle. At this time of year austral migrants are almost never found.
MV	Mountain Visitors. Residents from other areas in Colombia's Eastern Andes or "Cerros Orientales" but not within the circle where they appear in low numbers and not regularly in some years.
HV	Highland Visitors. Visitors from higher elevations that appear sporadically in the circle, mostly in low numbers and for which there is no evidence of reproduction in the circle.
LV	Lowland Visitors. Visitors from lower elevations that appeared in a few years but also in low numbers without evidence of settling.
RV	Reproductive Visitors. Visitors in some years with evidence of reproduction attempts but that eventually disappeared without settling.
TV	Transient Visitors. Visitors that pass through the circle, generally flying, in the course of migration or regional movements. Includes a record of a late austral migrant and a large flock of another species that disappeared after the count.
IV	Introduced Visitor. Resident in distant areas of the country that may have been set free or has escaped from captivity.
B. REGULARITY IN THE CIRCLE*	
7	Recorded in 24–26 years
6	Recorded in 19–23 years
5	Recorded in 13–18 years
4	Recorded in 8–12 years
3	Recorded in 4–7 years
2	Recorded in 2–3 years
1	Recorded only in 1 year
C. DISTRIBUTION IN THE CIRCLE*	
6	Recorded in 19–26 localities
5	Recorded in 11–18 localities
4	Recorded in 7–10 localities
3	Recorded in 4–6 localities
2	Recorded in 2–3 localities
1	Recorded in only one locality
D. ABUNDANCE IN THE CIRCLE**	
8	Average $\geq$250.00
7	Average 100.00–249.99
6	Average 50.00–99.99
5	Average 25.00–49.99
4	Average 12.50–24.99
3	Average 5.50–12.49
2	Average 2.50–5.49
1	Average 1.00–2.49
E. ABUNDANCE TENDENCIES IN THE CIRCLE***	
Significant increase	Regression coefficient (slope) positive, $p \leq 0.05$
Slight increase	Regression coefficient (slope) positive $0.10 \leq p \geq 0.05$
No change	Regression coefficient positive or negative but $p > 0.10$
Slight decrease	Regression coefficient (slope) negative, $0.10 \leq p \geq 0.05$
Significant decrease	Regression coefficient (slope) negative, $p \leq 0.05$
X	Insufficient data to be able to determine tendency

**In categories B and C, we arbitrarily established classes according to the distribution of numbers of species trying to leave similar numbers of species in each class to make comparison between species easier in the analysis.*

***Since the averages of numbers recorded for each species ranged over three orders of magnitude and most species have small abundances, for category D we established classes in an approximately exponential scale, with similar numbers in the majority of classes. We did not include the years in which the species were not recorded in the averages*

****To evaluate the tendencies in Category E, we used linear regressions, especially for species recorded during 10 or more years and some with less (but always above five) that became established or went extinct during the period of the CBCs. Classes were defined according to the p value of the regression. Each number was divided by the party-hours spent in the species preferred habitat to correct for differences in sampling intensity between years.*

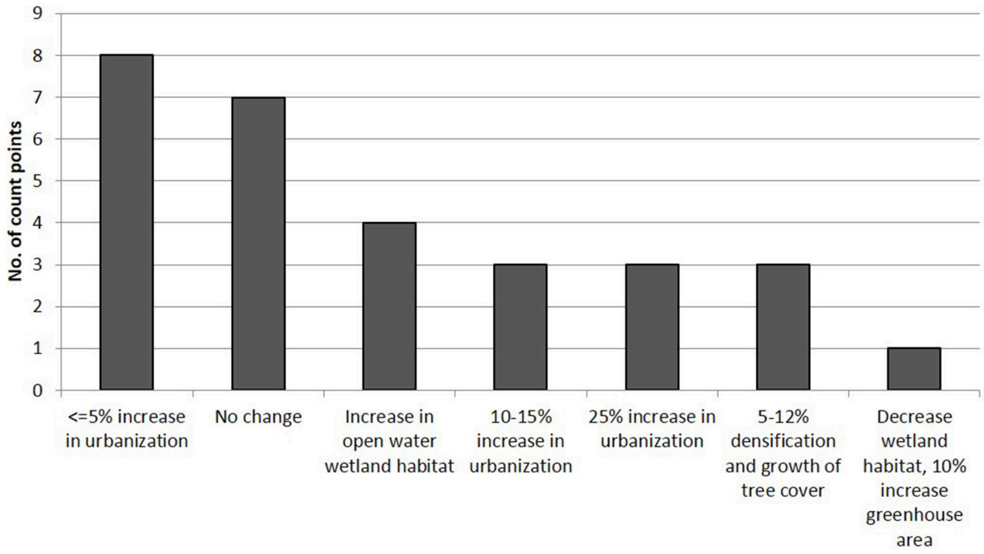

FIGURE 3 | Land cover changes in the last ~16 years in 1 km radius circles around the 29 count points in the Sabana de Bogotá Audubon's Christmas Bird Count circle.

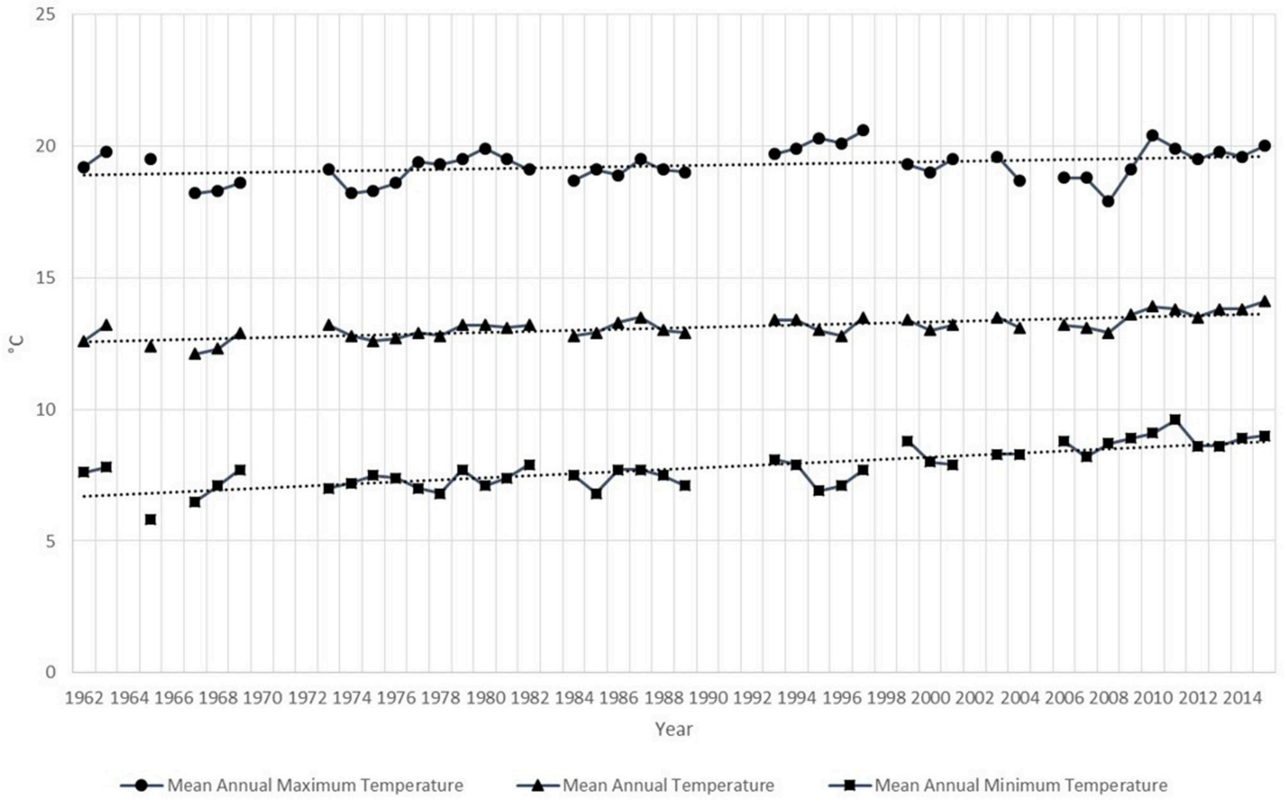

FIGURE 4 | Mean annual maximum, mean and minimum temperatures, and tendency lines between 1962 and 2015. Data from Eldorado airport located in the CBC circle.

Count circle (Table S1). The accumulated number of species increased notably in the first 16 years of counts (1989–2003) from 103 to 215 species and stabilized thereafter but always increasing, reaching the maximum in 2014 (**Figure 5**). The average number of species/count was 122.1 ± 12.4 *s.d.* increasing from 103 in 1989 to a maximum (142) in 2001

TABLE 3 | Results of the non-parametrical Mann–Kendall trend test for temperature increase in Bogotá's airport between 1962 and 2015 and during the 26 years of Audubon's Christmas Bird Counts (1989–2014).

	1962–2015			**1989–2014**		
	Minimum mean annual temperature	**Mean annual temperature**	**Maximum mean annual temperature**	**Minimum mean annual temperature**	**Mean annual temperature**	**Maximum mean annual temperature**
Kendall's Tau	0.622	0.547	0.266	0.582	0.370	−0.148
p	<0.0001	<0.0001	0.017	0.001	0.034	0.400
Temperature (°C) increase rate/year	0.043	0.02	0.017	0.075	0.025	

and remaining more or less constant in the following years (**Figure 5**).

Families with most species recorded in the counts were Thraupidae (33), Tyrannidae (28), Trochilidae (18), and Parulidae (15). Fifteen of the families are represented by only one species (Table S1). Permanent residents comprised the largest category, with 187 species. Forty-six of the species were wintering boreal migrants, mainly Parulidae and Tyrannidae (Table S1). In terms of geographical distribution, three species and four subspecies are endemic to the northern part of Colombia's eastern Andes (*Rallus semiplumbeus, Synallaxis subpudica, Cistothorus apolinari, Ixobrychus exilis bogotensis, Porphyriops melanops bogotensis, Eremophila alpestris peregrina,* and *Chrysomus icterocephalus bogotensis*; Table S1). Seven species are Range Restricted according to BirdLife International's criteria (global breeding range below 50,000 km^2; Stattersfield et al., 1998). *R. semiplumbeus* and *C. apolinari* are globally threatened (IUCN, 2017) and four other species are locally threatened in Colombia (*Oxyura jamaicensis, Porphyriops melanops, Pseudocolopteryx acutipennis,* and *Eremophila alpestris*; Renjifo et al., 2014, 2016; Table S1).

Most species (84) were associated with native forest and scrub, or wetlands (56) whereas there were only 15 species exclusive to open rural areas (**Figure 6**). Nearly all of the species (91%) that were restricted to urban parks with trees (UPT) correspond either to visitors from lower elevations (57%) or wintering boreal migrants (34%). Some of the species from lower elevations could also have been cage birds escaped from captivity (e.g., *Pteroglossus castanotis, Cyanocorax affinis, Icterus icterus*) while others had more likely shifted from lower surrounding areas (e.g., established species such as *Elaenia flavogaster, Pitangus sulphuratus,* and *Thraupis palmarum,* or visitors like *Todirostrum cinereum, Columbina talpacoti,* and *Saltator striatipectus*; Table S1).

Urban vs. Rural Areas

The total number of species registered was significantly higher in rural than in urban areas (209 vs. 167 species, $\chi^2 = 4.691$, $p = 0.03$). Sixty-eight species were exclusive to rural areas, 26 were exclusive to urban areas and 141 were registered in both urban- and rural-areas (Sorensen similarity Index = 0.7446, Table S2). The number of species in terrestrial localities (TER) was higher for rural areas than for urban areas ($\chi^2 = 5.442$, $p = 0.02$), and there was no significant difference in the total number of aquatic species between rural and urban areas for wetland localities (WET; Table S2).

Forty-two species that were registered both in rural and urban areas had a significant difference in corrected abundance. Eighteen species were more abundant in the urban areas, among them *Gallinula galeata*, *Phimosus infuscatus, Vanellus chilensis, Zenaida auriculata*, *Colibri coruscans, Molothrus bonariensis, Tyrannus melancholicus, Thraupis episcopus* and the boreal migrants *Tringa melanoleuca, T. solitaria, T. flavipes, Leiothlypis peregrina, Piranga rubra,* and *Parkesia noveboracensis.* Among the species that were more abundant in the rural areas were *O. jamaicensis, Ardea alba, Bubulcus ibis,* and *Gallinula melanops* and NFS species like *Penelope montagnii, Patagioenas fasciata, Grallaria ruficapilla, Scytalopus griseicollis, S. subpudica, Elaenia frantzii, Mecocerculus leucophrys,* and 4 species of hummingbirds. The only boreal migrant more abundant in rural areas was *Setophaga fusca* (Table S2).

Twenty-six species were registered only in urban areas, among them residents or visitors that came from the warmer lowlands, like *P. sulphuratus, C. affinis, T. palmarum, Cacicus cela,* and *Gymnomystax mexicanus.* Most of the 68 species that were registered only in rural areas were found only in natural habitats (NFS) (Table S2).

Long-Term Changes in Birds in the Bogotá Area

Sixty-six percent (155) of the species were analyzed for changes over the 26 year interval; of these 31% increased, 20% decreased, and 50 did not change significantly (**Table 4**). Thirty-six permanent residents increased their abundance and 22 decreased at least slightly ($p \leq 0.10$). Two species that did not reach the minimum number of records for the regression analysis (*I. exilis* and *E. alpestris*) should be added to the latter since they did change but disappeared from the counts in the initial years; this would add a total of 60 (47%) permanent residents that showed changes along the study period; 53 species did not change their abundance (42%) and the remaining 14 not analyzed or without evident tendencies added only 11% (Table S3). Among species with significant increases ($p \leq 0.05$), six arrived in the circle area, started to reproduce and continue to increase: *P. infuscatus, V. chilensis, Rupornis magnirostris, Elanus leucurus, I. icterus, Quiscalus lugubris*; *Asio clamator* became established but did not increase thereafter. Several other species showed significant increases:

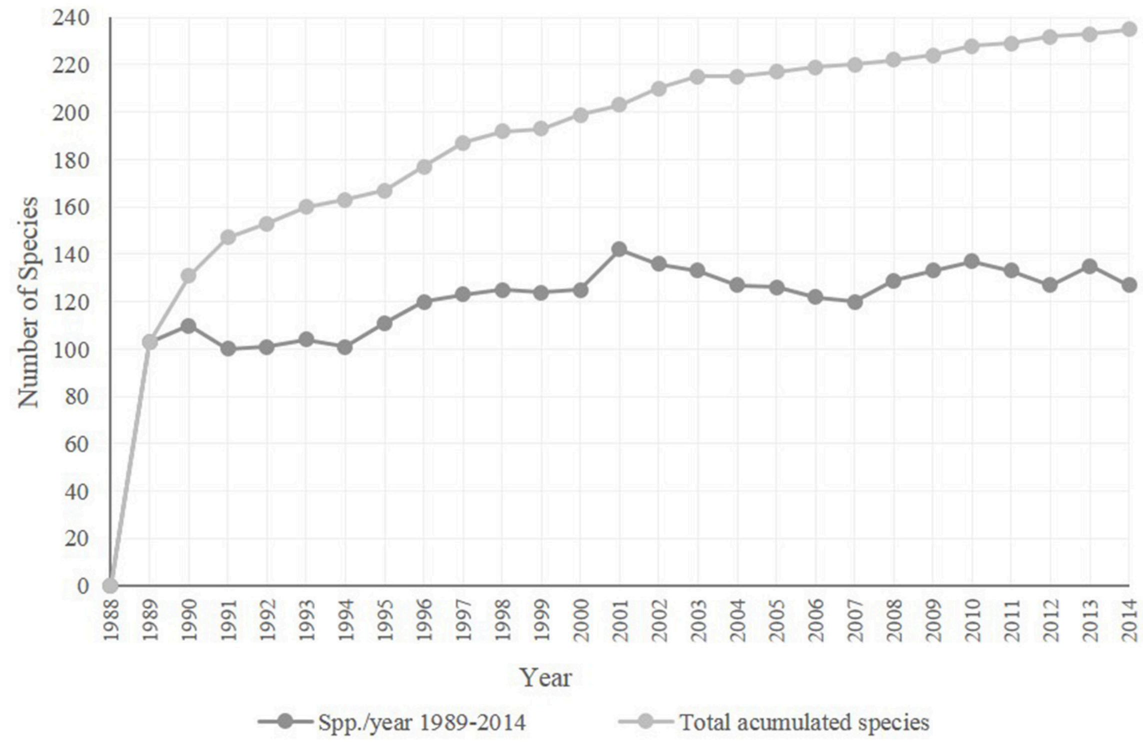

FIGURE 5 | Species accumulation curve and number of species/year registered at the Sabana de Bogotá Audubon's Christmas Bird Count circle between 1989 and 2014.

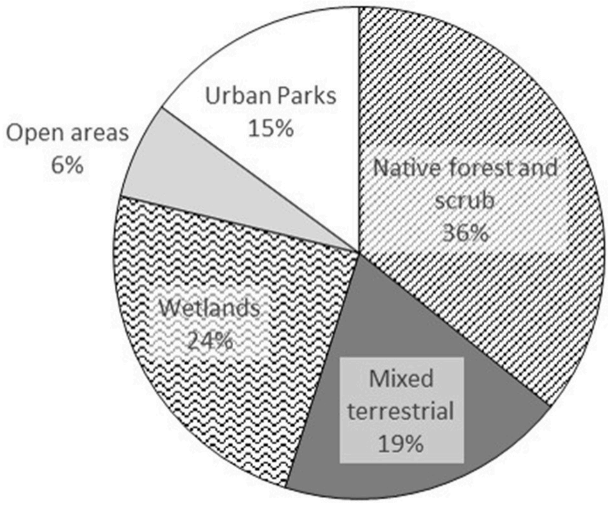

FIGURE 6 | Percentages of habitats preferred by the 235 bird species recorded in the Sabana de Bogotá Audubon's Christmas Bird Count circle between 1989 and 2014.

TABLE 4 | Summary of abundance changes of bird species due to the different factors affecting them in the Sabana de Bogotá Audubon's Christmas Bird Counts circle in 26 years (1989–2014).

Situation	**Types of abundance changes**				**Total**
	I	**D**	**NC**	**NA**	
PR-Permanent resident	36	24[†]	53	14	127
SR-Seasonal resident	11	3	18	10	42
MV-Mountain Visitors	0	0	0	8	8
HV-Highland Visitors	1	3	3	8	15
LV-Lowland Visitors	0	0	1	30	31
RV-Reproductive Visitors	0	0	1	2	3
TV-Transient Visitors	0	0	0	4	4
IV-Introduced Visitor	0	0	1	4	5
Total	48	30	77	80	235

I, increase; D, decrease; NC, no change; NA, not analyzed (see text).

[†] *Includes two species (Ixobrychus exilis, Eremophila alpestris) that disappeared from the circle in the first years of the CBC without having accumulated 5 years of records; they were not analyzed with regressions but were included as decreases.*

P. montagnii, Coragyps atratus, C. coruscans, two species of *Grallaria, S. subpudica, T. melancholicus, M. leucophrys, Troglodytes aedon, Turdus fuscater, Hemispingus superciliaris*, two species of *Diglossa, Atlapetes schistaceus, Cacicus chrysonotus*, and *Icterus chrysater*. On the other hand, the regression results for several species of permanent residents showed significant decreases: *Butorides striata, Tyto alba, Eriocnemis cupreoventris, Ochthoeca fumicolor, C. apolinari, Diglossa lafresnayii*, two species of *Catamenia* and *Spinus spinescens* (**Figure 7**). The species that either increased or decreased significantly or slightly ($0.05 < p < 0.10$; Table S3) were ecologically and

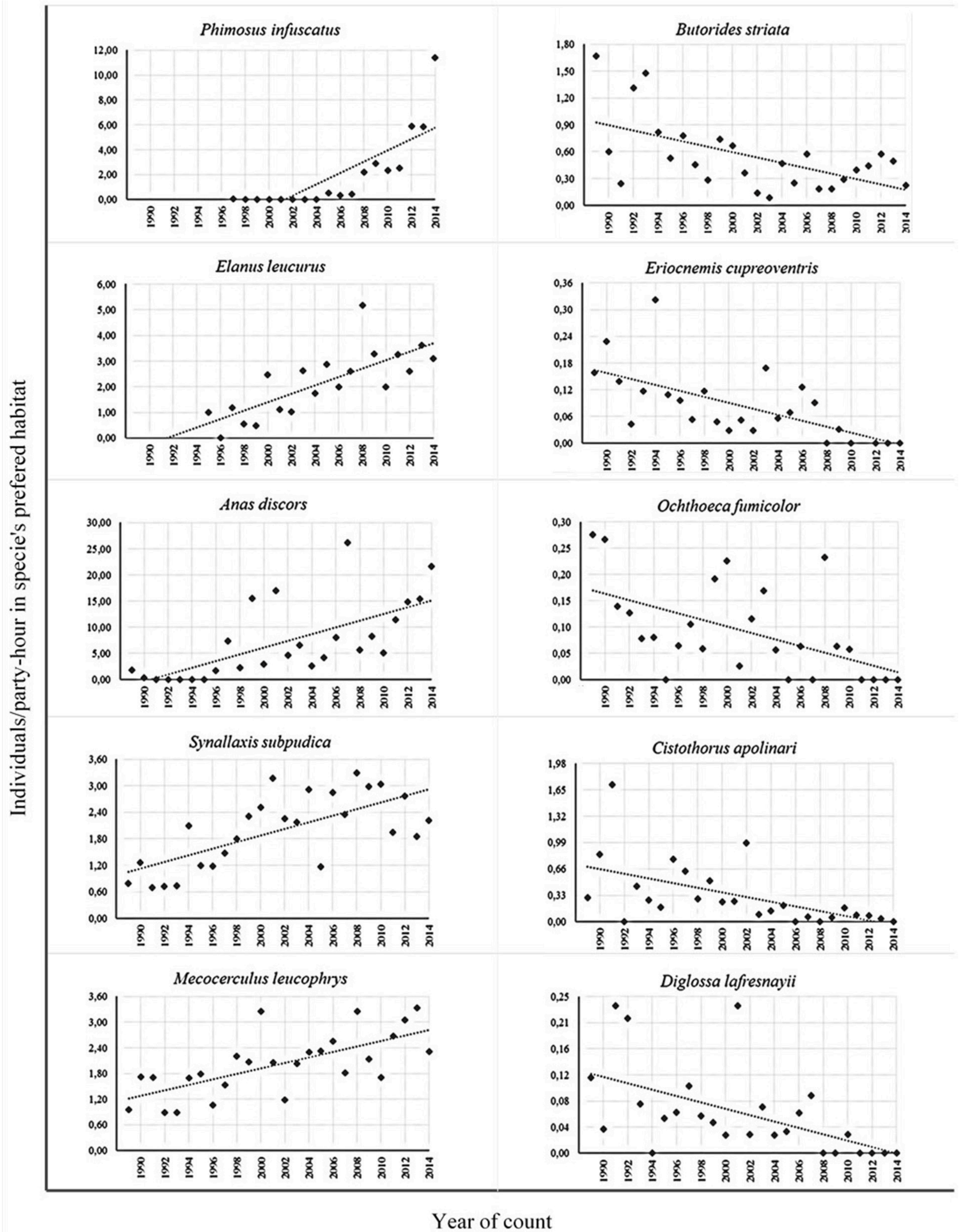

FIGURE 7 | Examples of bird species that increased or decreased significantly between 1989 and 2014 in the Sabana de Bogotá Audubon's Christmas Bird Count circle.

taxonomically diverse, indicating multiple factors involved in these changes.

In addition to these unidirectional changes, in seven species of permanent residents the direction of changes varied within the count period. For most, a period of 10 years of increases (*B. ibis, Z. auriculata, T. episcopus,* and *palmarum*) or stability (*Elaenia frantzii, Zonotrichia capensis*) was followed by several years of decreases (**Table 5**). *M. bonariensis* was an exception: its abundance increased significantly at the beginning of the study (not including two "outliers" that corresponded to flocks overflying the area), then was followed by several years with a marginally slight increase (**Table 5**, **Figure 8**).

Among the 46 seasonal residents (wintering boreal migrants), 11 increased their abundance at least slightly (26%) whereas only three (7%) decreased and 18 (43%) did not change according to regression results (**Table 4**). Since the species that increased were mainly known as passage migrants, it is evident that several of them were becoming winter residents during this period. In fact, regressions could be underestimating this change; when we compared the number of years in which several of these species were recorded in the first vs. the second half of the study period, five showed more frequent records in the last 13 year even though their regressions did not detect numeric changes in abundances (e.g., *Cardellina canadensis* and *Contopus cooperi,* Table S3); three additional species not analyzed with regressions were also recorded more frequently during the second half of the study (*Ardea herodias, Butorides virescens,* and *Myiarchus crinitus*). We may conclude therefore that 19 species (45%) increased as winter residents during the study (Table S3), reinforcing this unidirectional tendency of changes. The records of *Gallinago delicata* and *Hirundo rustica* decreased during the second half of the study. By contrast, among the visitors from higher elevations one species apparently increased in abundance (*Myiotheretes striaticollis*) but three species of hummingbirds decreased; for three more, regressions did not show significant changes and the other eight species did not have enough years of records to perform the analysis. For the other types of visitors very few species (7%) could be analyzed with regressions; in all such cases (one lowland visitor, one reproductive visitor, and one introduced visitor) there were no changes; the remaining 93% were not analyzed due to insufficient years of records (**Table 4**, Table S3).

The abundance tendencies between rural and urban localities for two of the most common species in the study (*Z. auriculata* and *T. fuscater*) were very different: in both cases the situation outside the city was very stable (slope $= -0.0003$ for *Z. auriculata*, $p = 0.99$ and 0.0044 for *T. fuscater*, $p = 0.81$) whilst in the urban localities the slope was 2.365 ($p = 0.005$) between 1986 and 2003 and -1.915 ($p = 0.078$) between 1994 and 2014 for *Z. auriculata* and 0.105 ($p = 0.203$) for *T. fuscater* (**Figure 9**).

DISCUSSION

The most difficult and important aspect of this study is to assign possible causes to the abundance changes found in the 85 species (55%) analyzed with regressions or frequency of occurrence. This task is made more difficult given that many of these changes could have been due to a combination of factors that operate simultaneously and that the same type of factor could benefit some species and affect negatively others. To begin, we classify the factors that could cause the changes observed in bird abundance in five general groups and then analyze their effects in further detail.

Lack of Continuity in Observations in Particular Sites

This factor has been a problem throughout the study period, largely reflecting the representation of different habitats in the final results. The most evident example is the representation of well-preserved forest in the mountain slopes, the habitat with the highest species richness, in which many species occur naturally in low densities. Only one locality with this kind of forest had continual observations over almost all the study period but in 2000, we added a second site. The duplication of forest sites may have caused the apparent increases in regressions of some forest species (e. g., *Glaucidium jardini, Margarornis squamiger, Coeligena helianthea, C. chrysonotus*), which therefore might not indicate real increases in their populations. Also, the increase in some nocturnal species (e.g., *Megascops choliba*) may reflect more time invested in nocturnal auditory surveys in the last years, more than real changes. Finally, during these years the availability of recordings of several forest species in particular helped in their detection and identification. Nevertheless, when we checked the data we did not find major differences in the amount of auditory vs. visual detections along the period.

Direct Human Actions

The reduction in hunting of *P. montagnii* in several forest localities could have well caused its increase. The reduction also of the intense hunting of *Z. auriculata* reported by Olivares (1969) could explain partially its marked increase in the first half of the study. Another positive change could have been the diminution of trapping of *I. chrysater* as a cage bird, reported as common by Olivares in the past; we recorded only one or two cases of this species inside a cage in the first years of count. On the other hand, there was also disruption of a nesting colony of *B. ibis* and *Nycticorax nycticorax* in 2000, apparently caused by "hunters" in one of the wetland localities (Vargas, personal communication).

However, the most important human actions during the study period were those that caused changes in bird habitats. There were three types of interventions: changes in several wetlands, urbanization and tree planting in several urban parks and avenues, and some wetland boundaries and reduction of open pastures. During several years Bogotá's water company (EAAB) performed major works in the city's wetlands, some beneficial and some harmful. New open water areas were opened and bulrush (*Schoenoplectus californicus*) areas increased in some wetlands, benefiting some aquatic species. However, the water supply of some other wetlands was reduced or eliminated by flood control measures, and the interruption of the constant and necessary removal of water hyacinth eliminated open water habitat in one of the most important wetlands, eradicating the largest population of the endemic and endangered subspecies of

TABLE 5 | Complex changes in abundance trends in seven resident species throughout the years of the Audubon's Christmas Bird Counts of the Sabana of Bogotá (1989–2014).

Species	Years	Slope	*p*	Interpretation	Possible cause and comments
Bubulcus ibis	1989–1999	3.237	0.043	Significant increase	Reproduction increase in circle
	2000–2014	−1.633	0.019	Significant decrease	Reduction in pasture areas because of urbanization, drainage of a wetland site
Zenaida auriculata	1989–2003	3.729	0.001	Significant increase	Climate change and adaptation to urban environment?
	2004–2014	−2.606	0.009	Significant decrease	Increase in mortality due to predation, diseases?
Elaenia frantzii	1989–1998	0.182	0.165	No change	
	1999–2014	−0.045	0.006	Significant decrease	Unknown
Thraupis episcopus	1989–2005	0.055	0.010	Significant increase	Climate change and adaptation to urban environment?
	2006–2014	−0.122	0.062	Slight decrease	Brood parasitism by *Molothrus bonariensis*?
Thraupis palmarum	1989–2006	0.092	0.001	Significant increase	Climate change and adaptation to urban environment?
	2007–2014	−0.080	0.062	Slight decrease	Brood parasitism by *Molothrus bonariensis*?
Zonotrichia capensis	1991–2009	−0.273	0.186	No change	Two atypical outliers removed (see text)
	2010–2009	−2.707	0.044	Significant decrease	Brood parasitism by *Molothrus bonariensis*? Changes in urbanization?
Molothrus bonariensis	1989–1998	1.086	0.014	Significant increase	Reproduction increase in circle
	2000–2014	0.168	0.102	No change	

P. melanops. Another population of this species was eliminated with the drainage of a rural pond between 2009 and 2011, which also affected other species such as *O. jamaicensis* and *Podilymbus podiceps*. Most of the remaining population of *P. melanops* in the circle survives in some artificial ponds and along the Bogotá River.

The effect of urbanization was noted mainly with the construction of commercial establishments, greenhouses for flower production and housing projects over areas formerly covered by pastures and crops, removing habitat for several species including *B. ibis, T. alba, Sturnella magna, Sicalis luteola,* and *Catamenia analis* and hence the decrease of their populations. Another effect of urbanization in the northern portion of the city was the replacement of single family houses with gardens and trees by apartment blocks with few green areas, possibly affecting some urban birds such as *Diglossa humeralis, Z. capensis* and possibly the urban population of *T. fuscater*, which has apparently declined since ca. 2010; however, *Z. auriculata* could apparently adapt to this change.

There have been two types of tree planting programs: the planting of exotic plants including some with fleshy fruits (especially *Syzygium paniculatum*) in parks and city avenues, which seem to have benefited several migrants and *T. fuscater* in particular: its population has increased in the study period in the city but not in natural areas with shrubs and forest. Instead, tree planting in the boundaries of some wetlands was done with a predominance of native species and favored the increase of the urban populations of several species such as *M. leucophrys, T. aedon,* and possibly *Arremon assimilis* and the wintering populations of some migrant species. The establishment or improvement of gardens in some parks could also have favored species such as *C. coruscans* and *Diglossa sittoides*; the latter seems to have expanded its local distribution during the counts.

"Natural" Changes in Some Habitats

Even though these types of changes may be the secondary effects of human actions, they were not produced deliberately. An example is the secondary succession of native scrub toward secondary forest in several sites after deforestation or fires in previous years. In one of the forest localities that was visited continuously for 24 years, scrub vegetation has grown from a height of 1–3 m in 1991 to 3–6 m at present with more trees in several places. To a greater or lesser degree a similar situation has occurred in other localities with long-term observations. The increase in observations of species such as *C. helianthea, Grallaria squamigera* and *ruficapilla, Atlapetes schistacea* and *H. superciliaris* are possibly related to these changes. Another change that has occurred in several wetlands is related to increase in water pollution: the filling of water bodies with macrophytes reducing open water areas and eliminating exposed mud on the edges with shallow waters. This is related to the increase in some species such as *G. galeata* and decrease in probers like *Gallinago* and *Tringa* spp. (Rosselli and Stiles, 2012a). The increase in the shrub vegetation around the edges of some wetlands has probably induced the increment in *S. subpudica* and perhaps also *T. aedon*. The disappearance of the endemic and endangered subspecies *Eremophila alpestris peregrina* is probably related to the coverage with introduced kikuyu grass in a newly established park at the southern end of the circle that had bunch grasses and areas of bare soil, an important habitat for this species, at the beginning of the study.

Interactions with Other Species

An interesting case is the increase in the abundance of *Buteo platypterus* as a wintering migrant, which is probably related to the significant increase of its favorite prey, the dove *Z. auriculata,* during most of the study period. The reduction of

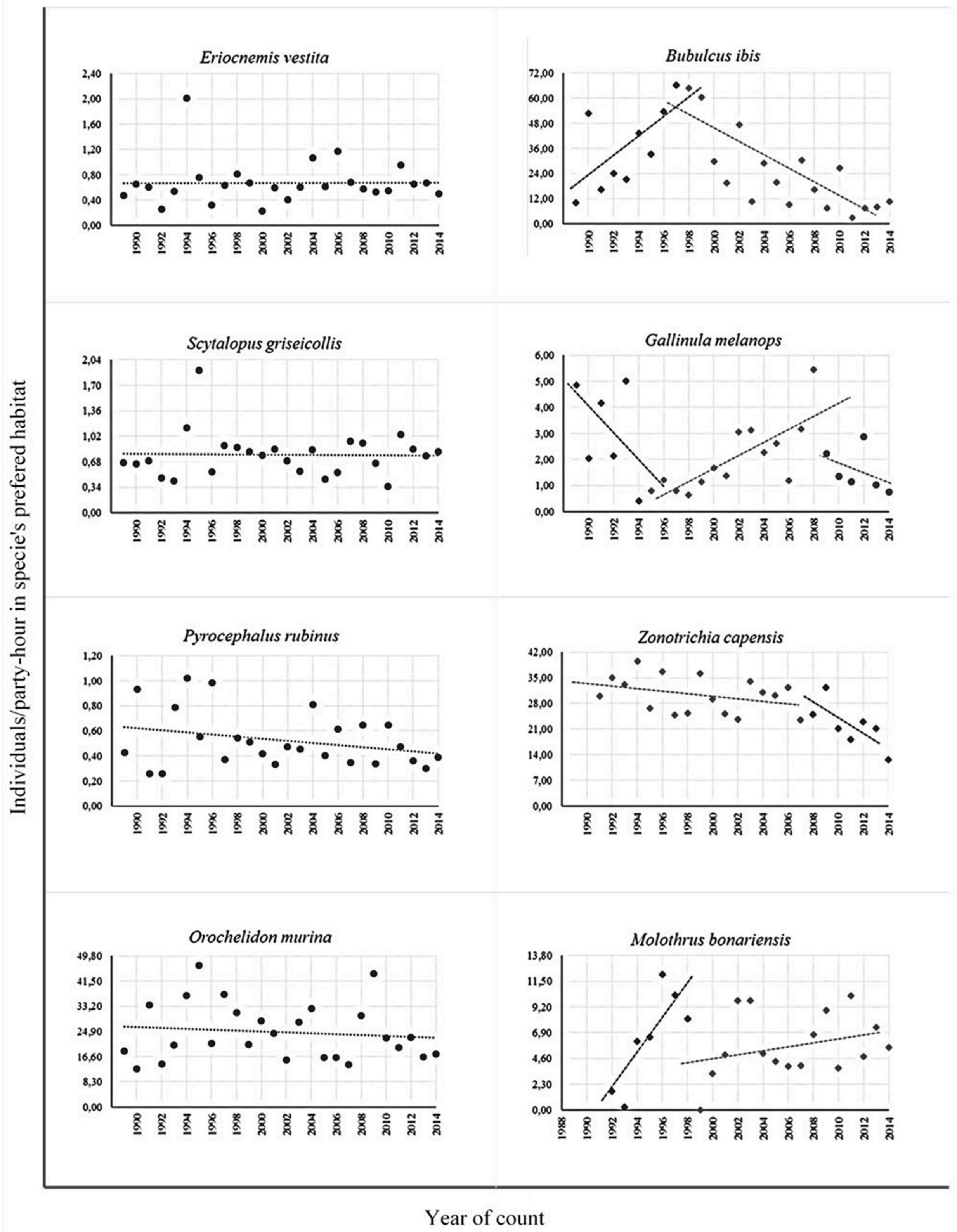

FIGURE 8 | Examples of bird species that were stable or had complex tendencies in which the direction of change varied along the period between 1989 and 2014 in the Sabana de Bogotá Audubon's Christmas Bird Count circle.

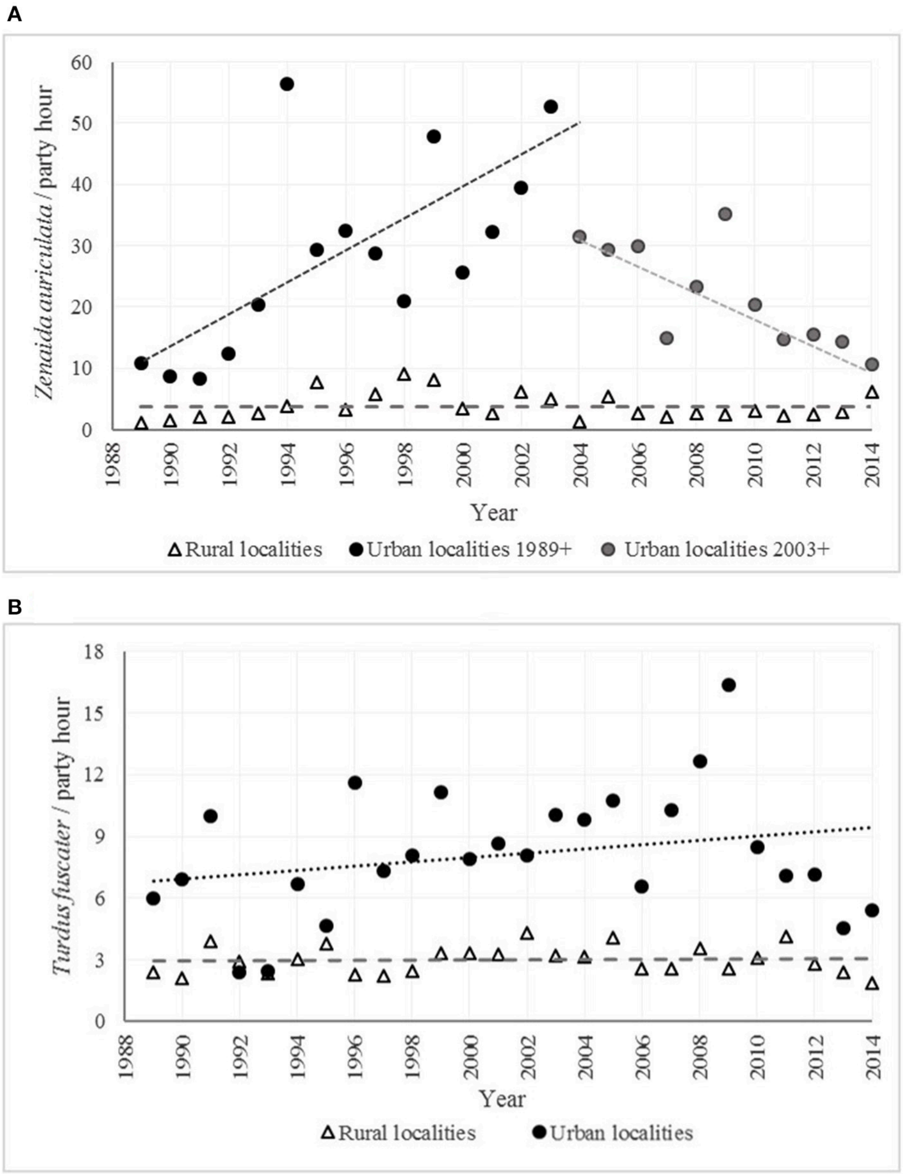

FIGURE 9 | Abundance tendencies in rural and urban localities for two of the most common species in the Sabana de Bogotá Audubon's Christmas Bird Counts. **(A)** *Zenaida auriculata* (Eared Dove). **(B)** *Turdus fuscater* (Great Thrush).

Z. auriculata in the last years (even though it is still the most common species in the circle) could be associated partially with this predation, although its high reproductive rate (nests are present all year long) would not be enough to explain its recent decline. Another possibility could be the increase in parasites or diseases in the population. Until recently we have observed dormitories of hundreds of birds in dense trees in city parks and such congregations are favorable for their transmission (Delgado-V and French, 2011). The recent occurrence of the snail specialist *Chondrohierax uncinatus* apparently reflected the appearance of the Giant African Snail (*Achatina fulica*) in the Sabana de Bogotá and nearby mountain localities; this species is a rapidly increasing agricultural pest and health threat (Linares et al., 2013).

T. fuscater regularly predates on nestlings and fledglings of *Z. capensis* but this is unlikely to have caused the decrease of the sparrow in recent years; the data show that the *Z. capensis* population was stable for many years during which the *T. fuscater* population increased. Another more worrying example of predation is the hunting of aquatic birds by dogs (domestic dogs let loose by neighboring owners or feral dogs that reproduce in the wetlands). This hunting almost certainly has caused the local extinction of the endemic subspecies of *I. exilis* and possibly the decreases of *B. striata* and the endemic and endangered *R.*

semiplumbeus in the circle. Although, cats are widely known to have significant effects on bird and other wildlife populations, especially in the United States and on some islands previously lacking mammalian predators (Medina et al., 2001; Nogales et al., 2013; Seress and Liker, 2015; Doherty et al., 2016), they do not appear to have had a significant impact on the birds of the Bogotá area. Contrary to the high number of feral dogs ranging in the wetlands, cats were rarely seen in the circle sites. Campos et al. (2007) compared the distribution and analyzed the diet of feral cats and dogs in Brazil and found that both were more abundant in suburban than in rural areas and their diet consisted mostly of invertebrates, followed by mammals and birds. We have found no other published studies of cat predation on birds in Latin America, but such studies are badly needed.

Nevertheless, the most worrisome interaction has been the increase in the brood parasitism by the cowbird *M. bonariensis* on various species. The recent decrease of *Z. capensis* may rather be the result of cowbird parasitism and in a lesser degree, to urbanization. An informal count on the Universidad Nacional campus in 2013–2014 (Stiles, personal observation) recorded more pairs of this species feeding fledgling cowbirds than those of their own species. This parasitism is the most probable cause of the dramatic decline of the endemic species *C. apolinari*, now at the brink of extinction in the city's wetlands despite the protection and improvement of its bulrush habitat (*S. californicus*) in several wetlands (Rosselli and Stiles, 2012b). Velásquez-Tibatá et al. (2000) first noted this parasitism and later it was observed in other localities; a nest collected in one wetland in the circle had more *M. bonariensis* eggs than those of its host (Castro et al., 2007). Villaneda and Rosselli (2011) found that *M. bonariensis* is more abundant in urban wetlands than in rural ones. The decline of *T. episcopus*, *T. palmarum,* and *Ramphocelus dimidiatus* in the urban areas could also be related to this parasitism; these species and others in their genera have been reported as hosts of *M. bonariensis* by Friedmann and Kiff (1985). However, another species also reported as a *M. bonariensis* host, *Chrysomus icterocephalus* (Naranjo, 1995), maintained a stable population during the time of the study. Villaneda and Rosselli (2011) noted aggressions from this species against the parasite, and it apparently may defend its nests more effectively.

Climate Change

During the CBC period the mean annual temperature in Bogotá's airport increased by 0.65°C and the mean minimal annual temperature has risen by almost 2°C (**Figure 4**, **Table 3**). Several changes recorded in the avifauna in the CBCs match the predictions of transformations made by some theoretical models developed to assess such phenomena (Velásquez-Tibatá et al., 2013; see also the revision by Herzog et al., 2011). The clearest indication of the effect of global warming on birds is the change in the lower or upper elevational limits of species. The counts have documented the displacement of the lower limits of *E. cupreoventris, O. fumicolor, Catamenia inornata,* and *D. lafresnayii* from ca. 2,600 m at the level of the flat area of the Sabana to at least 2,850 m in the surrounding mountains. Several species have become established as residents in the flat part of the Sabana at a higher elevation than that reported for reproduction according to Hilty and Brown (1986), including *P. infuscatus, R. magnirostris, A. clamator, Q. lugubris,* and *I. icterus* (although the origin of this population is probably from escaped cage birds, it has been reproducing and it seems to be increasing). Both *I. icterus* and *Q. lugubris* were exclusive to urban areas suggesting the influence of the city's "heat island"; otherwise they could have been registered in areas outside the city where exotic as well as native vegetation can be found and would provide resources to these species. Other species known mainly or exclusively from the southwestern drier and warmer parts of the Sabana have expanded their distributions as residents widely toward the north and east (*E. leucurus*, *V. chilensis, Mimus gilvus* and probably *M. bonariensis* and *B. ibis*). It is possible that the "heat island" in most of the city also has facilitated this expansion. The activity and abundance of heterothermic animals including insects may be related to temperature (Garcia et al., 2008; Coutinho-Silva et al., 2017); lower temperatures can cause longer biological cycles, reduced fertility and even total interruption of development (Hodkinson, 2005; Nava et al., 2007). Moreover, upslope displacements of various insect groups in response to climate change have been recorded (e.g., for butterflies see Forister et al., 2010 and Chen et al., 2011). Hence, the warming of the Sabana could have produced an increment in prey for insectivorous birds such as *T. melancholicus* in addition to the various insectivorous boreal migrants that increased their abundance and frequency during the study period. In fact, it appears that climate change and the heat island might have acted synergistically in such cases, particularly favoring the establishment of those species now found mainly in the flat parts of the Sabana. However, the upslope movements of some forest species in parts of the circle beyond the heat island are more likely to reflect climate change.

Climate change may be related to observed changes in the elevational movements of some species, hummingbirds in particular. During the first years of the study hummingbirds from higher elevations were fairly frequently recorded in the counts in scrub vegetation on lower mountain slopes around the Sabana; these included *Aglaeactis cupripennis, Pterophanes cyanoptera,* and *Lesbia victoriae.* Examining specimens from the ornithological collection of the Universidad Nacional de Colombia, the time of the counts seems to match the molting season for these species: effectively this period represented a "molt migration." All of them declined in frequency or ceased to appear during the CBC period. The contrary apparently happened with *C. coruscans*, which at the beginning became much scarcer between December and March–April, also in the molting period. However, presently many more individuals now remain in the Sabana during that season, possibly reflecting in part the planting of trees and increase in gardens, particularly in the urban areas.

Finally, we recorded changes in a few species that do not match easily with the main categories. The increase of *C. atratus* could be related simply to the increase of human population (and the consequent increase in dumps and waste); we have no explanation for the reduction of *Contopus fumigatus* and for others (e.g., *T. aedon*), we are not sure that the suggested

TABLE 6 | Number of species with changes in status or abundance recorded in the Audubon's Christmas Bird Counts of the Sabana of Bogotá over 26 years (1989–2014), for each of the five factors that could have caused these changes (positive or negative).

Factor	Number of species
1. Lack of continuity	12–15
a. Changes in localities and habitat representation	11+
b. Changes in observation time in some localities	1+
c. Changes in methodologies (using more vocalizations)	2–3
2. Direct human actions	18–21
a. Changes in certain habitats	
i. Wetlands intervention	5+
ii. Reforestation actions in some parks and other urban areas	4–6
iii. Urbanization: Building of housing projects, warehouses and commercial buildings on pastures and crop areas; replacement of houses that had front and backyard gardens with apartment buildings with little green areas	6–8
b. Hunting and other disturbances	2
3. Habitat changes with no human intervention	17–20
a. Secondary succession of native thickets to next stages (higher scrub, secondary forest) or bare soil changing to lawn	10+
b. Filling of water bodies with macrophytes reducing open water areas and eliminating narrow mudflats along the water edges	7
c. Increase in the shrub vegetation around some wetlands or from the edges inside the wetland	3–5
4. Interactions with other species	10–12
a. Predation	
i. Predation among bird species	1–2
ii. Predation by dogs in wetlands	3+
b. Parasitism	
i. Parasitism by other birds, mainly *Molothrus bonariensis*	4–5
ii. Parasitism by parasites, microbes, etc. and disease spreading	1?
5. Climate change because of local and global warming	37–42+
a. Changes in the lower or upper elevational limits of species	8+
b. Easier movement of some species inside the Sabana	7–9
c. Changes in the altitude movement patterns	4–5
d. Changes in status or abundance of species already registered in the circle	18–20

The number of species is greater than the registered changes number (see Table S1) because many changes reflect the effects of a combination of factors.

factors as causes may explain the magnitudes of the observed changes.

Summarizing the results (**Table 6**) we find that climate change, possibly abetted by the city's heat island, and their related effects seem to be the most important factor associated with the changes in abundance in permanent and seasonal residents in the Sabana. The pattern of the population changes is consistent with climate change for more than a third of the cases (ca. 38%). Next in importance are changes resulting from direct human actions (ca. 19%) and those resulting from "natural" changes (ca. 18%): combining these two results in the diverse changes in habitats in the circle having a similar magnitude to climate change. The lack of continuity could partially explain 12% of the observed changes, although possibly not reflecting real changes in populations in several cases; interactions between species appears to be related to ca. 11%, with a small residual of 1–2% without clear explanations.

The recent study by Rosselli et al. (in press) in high Andean forest and subpáramo between 2,800 and 3,300 m complements the results we present here. The work was done in high Andean forest, lower paramo and paramo areas between 2,800 and 3,300 m to replicate a previous study (Stiles and Rosselli, 1998) that comprised a period of time comparable to the counts although it was not continuous. Among the species that the counts were found moving toward those elevations were *R. magnirostris, E. frantzii,* and *T. melancholicus*. The increase of *E. frantzii* concurred with its decline in the Sabana, as did the increase of *O. fumicolor* in the paramo, so the abundance changes of these species could have well been the result of climate change.

A Look at the Past

Despite differences in methods and scope, the study by Olivares (1969) provides a basis for comparison with our results comprising the period up to the 1960's and for evaluating some changes that occurred during the two decades prior to the initiation of the CBC. Among the differences, the areas are not exactly the same: Olivares included larger areas both south and west from our CBC circle with habitats and species not found in it; on the other hand the CBC included high locations on the eastern mountains and a river valley not mentioned by Olivares, who apparently only studied places on the western base of the mountains. However, his comments on the abundances of a considerable number of are applicable to the CBC circle and will be discussed here.

Olivares (1969) recorded ca. 180 species in his more broadly defined Sabana de Bogotá. At least 15 of these species, including *V. chilensis* and *R. magnirostris,* were recorded only in the drier and warmer parts of the Sabana south and west of the CBC circle, within which they have since become established. In addition, several species that Olivares considered accidental in the Sabana had apparently become established in the two decades between 1969 and the initiation of the CBC, all originating from lower elevations in the Magdalena valley to the west, including *E. flavogaster, P. sulphuratus, T. episcopus, T. palmarum, R. dimidiatus, Sicalis flaveola,* and *Icterus nigrogularis.* The origins of these species could suggest that the effects of global warming were already being felt in the Sabana and the temperature records from the Eldorado airport since 1960 support this, although the rate of temperature increase and the intensity of the city's heat island have evidently augmented through the CBC period. On the other hand, two species recorded by Olivares as common (*E. alpestris* and *Asio flammeus*) had become very rare by 1969, and several other species of agricultural areas had decreased during this interval (e.g., *Colinus cristatus, S. luteola,* and *Sporophila luctuosa*), which coincides with the replacement of croplands by pastures over much of the Sabana. The CBC counts also recorded at least 20 species from the highest forests in the circle that apparently were not visited by Olivares or his collaborators. Very few of the species recorded in the counts as visitors from lower elevations were mentioned by Olivares, as well as none of those recorded as visitors in transit (TV) or introduced (IV), and the CBCs did not record several visitors from mountain forests (MV) (*Pharomachrus antisianus, Geotrygon montana, Claravis mondetoura*) mentioned by Olivares. Some transient migrants considered as common in the Sabana are at best rare now, including *Progne subis*; *Antrostomus carolinensis*, and *Charadrius vociferus*, which he considered to be common winter residents, were not recorded in the CBC although there are a few recent records from beyond the circle.

The situation of the aquatic birds is even more dramatic, and the information gathered by Fjeldså (1985) and van der Hammen et al. (2008) supplements the records of Olivares (1969) for the pre-CBC period, during which the most drastic reduction of the wetland areas occurred. Populations of several waterbird species were extirpated during this period, including *Podiceps andinus*, *Anas spinicauda niceforoi, A. cyanoptera borreroi, Circus cinereus, Botaurus pinnatus,* and *Netta erythrophthalmus*, the first three of which were endemic. Also, several waterbird migrants considered regular by Olivares were not recorded during the CBC counts, including *Anas acuta, A. americana, A. clypeata, Aythya affinis, Pluvialis squatarola* y *P. dominica, Charadius vociferus* y *Numenius phaeopus*. Moreover, ca. 10 species of large waterbirds mentioned as accidental by Olivares have not been recorded since.

Clearly the avifauna of the Sabana de Bogotá has been highly dynamic at least since the middle of the twentieth century. Prior to the CBC period, direct human actions including wetland drainage, expansion of urbanization, changes in rural land use appear to have been the most important factors affecting the avifauna, diminishing aquatic bird diversity in particular. The present study has documented the impacts of urbanization in rural areas, human intervention and succession in wetlands and forest succession on some mountain slopes as explanations for many avifaunal changes over the last 26 years. However, it has also added climate change, perhaps abetted by the city's heat island, as a possible driver of avifaunal changes.

A Glimpse into the Future

All indications are that the changes in Bogotá's avifauna during the CBC will continue; the effects of climate change and urbanization may well intensify in some of the sites, especially if the present administration's intentions to urbanize the largest remaining green area close to the northern city limits (Reserva Forestal Van der Hammen, CAR, 2011) materialize. We may therefore expect that the frequency and variety of boreal migrants remaining to winter in the Sabana will probably increase and that some native species now considered as visitors from lower elevations may become established. On the other hand, we could expect that some species whose lower elevation limits have moved upward might disappear from the count circle, as almost surely will the endemic and threatened wren species *C. apolinari* due to cowbird parasitism; it will be important to monitor the abundances of other species known or suspected to be suffering from this brood parasite. The plans of the district government to urbanize much of the rural area remaining in the circle could, if implemented, affect the populations of several open country species. Although, La Sorte et al. (2009) reported delays of up to 35 years in the latitudinal response to climate change of some North American species, data are still scarce for altitudinal changes by Andean species to climate change (Forero-Medina et al., 2011; Velásquez-Tibatá et al., 2013) and the Sabana de Bogotá could continue to contribute important data on this phenomenon. The most general conclusion is that the Sabana de Bogotá CBC has supplied much important information about its changing avifauna, and highlights the importance of continuing it for many more years.

The Present Study in the Context of Urban Ornithology in Latin America

When the Sabana de Bogotá CBC was begun, urban ornithology was still in its infancy in Neotropical countries although it was gaining recognition as a major field of study in North America and Europe (e.g., Chace and Walsh, 2006; Ortega-Álvarez and MacGregor-Fors, 2011; Marzluff, 2016). Because the Neotropics host the world's richest avifauna, biodiversity loss through urbanization was the main concern of most publications. Major reviews of this field in Latin America (Ortega-Álvarez and MacGregor-Fors, 2011) and Colombia (Delgado-V and Correa-H, 2013) found two main themes of most publications: compilation of species lists and comparisons of avian communities varying from natural vegetation through rural, periurban and urban areas, mostly around major cities (e.g., Fandiño et al., 2010; Biamonte et al., 2011; Lessi et al., 2016). In particular, many studies attempted to evaluate the importance of urban green islands like urban parks and university campuses with different proportions of native and exotic vegetation for conservation of native avian biodiversity (e.g., Muñoz et al., 2007; Vásquez-Muñoz and Castaño-Villa, 2008; Lessi et al., 2016). However, the temporal dynamics of avifaunal change due to urbanization over periods of decades were rarely documented because data for most studies were obtained over short periods, usually 2 years or less. That the importance of urban green islands cannot be appreciated over such short periods is demonstrated by the results for the Bogotá Botanical Garden: the mean number of species counted per year was ca. 32, but over the 26 years of the CBC, three times that number of species have been recorded. An exception was a qualitative description of avifaunal changes over a 20-year period as coffee plantations were converted to suburbs in Costa Rica (Stiles, 1990). The few studies that attempted to include a temporal component involved a comparison of historical data with current inventories 50 or more years later (e.g., Castaño-V and Castaño-V, 2000; Donegan et al., 2009).

The present study thus helps to fill a gap in Neotropical urban ecology and is the first to explicitly address the importance of climate change in the context of urban-periurban-rural-natural vegetation involving a range of elevations. However, several other CBCs have been conducted in Colombia since the 1990s in similar situations, notably in the cities of Medellín and Manizales (e.g., Cuadros, 1992), but the results of these have yet to be synthesized and analyzed. We hope that the present study will stimulate such analyses, particularly with respect to the possible impacts of climate change. Although, not a primary objective, our study has produced 26-year species lists for sites with different degrees of habitat alteration that could be analyzed from our data. However, the present study also is incomplete as regards the latter stages of urbanization in that it included no sites in completely urban or suburban situations without such green islands, which should be considered for the future.

Other aspects requiring more study, as recommended by Delgado-V and Correa-H (2013) deal with comparisons of the ecology of species successful vs. unsuccessful in occupying urban habitats, the importance of specific factors favoring (e.g., additional food resources, possible reductions of predation, especially of nests) vs. inhibiting (predation by cats, collisions with structures and windows, parasites and diseases, lack of specific food types such as insects in exotic vegetation) for the occupation of urban areas by native species. A further area needing study is the human-bird interaction itself, involving the attitudes of local residents toward birds and methods of environmental education to permit increased citizen involvement in biodiversity conservation. At least in Colombia, a number of such programs have been instituted but their results need to be evaluated and published. Finally, an aspect emphasized by Marzluff and Rodewald (2008) is the importance of involving city planners and managers in designing and improving green areas as urbanization proceeds, a critical need for the future of a rapidly expanding city like Bogotá.

AUTHOR CONTRIBUTIONS

FS coordinated the Bogotá CBC in 25 of the 26 years included in this article, collected and curated the observers' field data. FS, LR and SD initiated the CBC circle in Bogotá, participated in the counts, digitized and analyzed the data, and wrote and approved the manuscript.

FUNDING

The Universidad Nacional de Colombia and the Universidad de Ciencias Aplicadas y Ambientales U.D.C.A. granted time to FS and LR for the writing of the paper. The Science and International Alliances from National Audubon Society provided funding that made possible the publication of this article.

ACKNOWLEDGMENTS

The authors express their gratitude to landowners and institutions that have permitted the entrance of CBC participants to many localities for more than 20 years. The counts could not have been made without the hundreds of bird watchers and the locality compilers that with enthusiasm have risen early and have enjoyed the birds. This type of participation is the essence of citizen science. We thank Bogotá's Ornithological Association (ABO) for its continuous support and coordination of the CBC. J. Niño and J. Pinzón from the Universidad de Ciencias Aplicadas y Ambientales (U.D.C.A.) offered valuable help with data organization. J. Cifuentes and S. Velásquez-Licona greatly helped with data analysis and preparation of the figures, and J. A. Vargas-González helped prepare **Figure 1**. For many years the National Audubon Society waived registration fees for the Bogotá CBC count participants. We especially thank Matthew Jeffery, Gary Langham, and Geoffrey S. LeBaron from the National Audubon Society for their help in obtaining funds to assure the publication of this article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00058/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 19 October 2016
doi: 10.3389/fevo.2016.00122

Predicting Metapopulation Responses to Conservation in Human-Dominated Landscapes

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Loss of habitat to urbanization is a primary cause of population declines as human-dominated landscapes expand at increasing rates. Understanding how the relative effects of different conservation strategies is important to slow population declines for species in urban landscapes. We studied the wood thrush *Hylocichla mustelina*, a declining forest-breeding Neotropical migratory species, and umbrella species for forest-breeding songbirds, within the urbanized mid-Atlantic United States. We integrated 40 years of demographic data with contemporary metapopulation model simulations of breeding wood thrushes to predict population responses to differing conservation scenarios. We compared four conservation scenarios over a 30-year time period (2014–2044) representing (A) current observed state (Null), (B) replacing impervious surface with forest (Reforest), (C) reducing brown-headed cowbird *Molothrus ater* parasitism pressure (Cowbird removal), and (D) simultaneous reforesting and cowbird removal. Compared to the Null scenario, the Reforest scenario increased mean annual population trends by 54%, the Remove cowbirds scenario increased mean annual population trends by 38%, and the scenario combining reforestation and cowbird removal increased mean annual population trends by 98%. Mean annual growth rates (λ) per site were greater in the Reforest ($\lambda = 0.94$) and Remove cowbirds ($\lambda = 0.92$) compared to the Null ($\lambda = 0.88$) model scenarios. However, only by combining the positive effects of reforestation and cowbird removal did wood thrush populations stop declining ($\lambda = 1.00$). Our results suggest that independently replacing impervious surface with forest habitat around forest patches and removing cowbirds may slow current negative population trends. Furthermore, conservation efforts that combine reforestation and cowbird removal may potentially benefit populations of wood thrushes and other similarly forest-breeding songbird species within urbanized fragmented landscapes that typify the mid-Atlantic United States.

Keywords: brood parasite, brown-headed cowbird, *Hylocichla mustelina*, impervious surface, metapopulation, *Molothrus ater*, urbanization, wood thrush

Edited by:
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Lund University, Sweden

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Specialty section:
This article was submitted to
Urban Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *31 August 2016*
Accepted: *03 October 2016*
Published: *19 October 2016*

Citation:
Ladin ZS, D'Amico V, Baetens JM, Roth RR and Shriver WG (2016) Predicting Metapopulation Responses to Conservation in Human-Dominated Landscapes. Front. Ecol. Evol. 4:122. doi: 10.3389/fevo.2016.00122

INTRODUCTION

The conservation of species is faced with overcoming multiple anthropogenic factors that can negatively affect populations across multiple scales (Jarzyna et al., 2015). Understanding population responses to regional and local factors is essential for defining priorities and achieving conservation goals (Walsh et al., 2015). Using empirically-based integrated population models (IPM; Abadi et al., 2010), predictive site occupancy models (Meineri et al., 2015), and individual-based metapopulation models (Hanski and Gaggiotti, 2004) can improve our understanding and predictions of population responses to conservation efforts. Additionally, spatially-explicit metapopulation models and their extensions (e.g., source-sink models) have been successfully used for this problem, particularly in fragmented landscapes (Pulliam, 1988; Hanski, 1999). Metapopulation ecology has provided a powerful set of tools for applied conservation (Soulé et al., 1988), and is ideally suited for modeling population responses to anthropogenic stressors such as urbanization, habitat loss, and fragmentation (Kawecki, 2004). Particularly in the face of rapidly changing landscapes due to anthropogenic factors, these approaches can help increase the accuracy of population estimates across spatial scales, and help elucidate how multiple interacting drivers may be related to observed population dynamics (review in Walther et al., 2002; Faaborg, 2014).

Since 1966, 49% of Neotropical migratory songbird species have undergone significant population declines (Sauer et al., 2012). These populations are likely negatively affected by a synergy of habitat loss and fragmentation (Hoover et al., 1995; Burke and Nol, 2000; Rushing et al., 2016), which is exacerbated by urbanization (Suarez-Rubio et al., 2011). In previous studies, urbanization has been shown to reduce fecundity in forest-breeding Neotropical migrants (Rodewald et al., 2013), can influence probability of species occupancy (Goodwin and Shriver, 2011), and hence can influence community-level dynamics (Ladin et al., 2016b). Although, research has shown how urbanization can negatively influence populations and alter community dynamics, it remains unclear how populations might respond to particular conservation strategies.

We used long-term demographic data of wood thrushes *Hylocichla mustelina*, considered an umbrella species for declining forest-breeding migratory songbirds (Simons et al., 1999; Russell et al., 2004), to understand how these species may respond to conservation in human-dominated landscapes. Similar to other forest-obligate species, regional patterns in wood thrush occupancy and abundance are positively related to the amount of forest habitat on the landscape (Smith et al., 2011). As forest habitat is replaced *via* urbanization, the increasing proportion of impervious surface on the landscape can have profound effects on watersheds, habitat quality, and species occurrence (Kaplan and Ayers, 2000). Impervious surface has been documented as a leading stressor on landbird community structure (Donnelly and Marzluff, 2006; Schlesinger et al., 2008), abundance (Lussier et al., 2006), and dispersal (Whittaker and Marzluff, 2012).

Locally, wood thrush breeding density and fecundity are negatively related to human development (e.g., impervious surface; Phillips et al., 2005; Richmond et al., 2012), and nest parasitism by the brown-headed cowbird *Molothrus ater* (hereafter cowbird; Hoover et al., 1995; Ladin et al., 2015). Similar to findings from previous studies on the negative effects of Cowbird parasitism on other species (Mayfield, 1977; Brittingham and Temple, 1983; De Groot and Smith, 2001). Ladin et al. (2016a) have recently demonstrated how coincident long-term population trends in Wood Thrushes and Cowbirds has exacerbated negative effects of Cowbirds on Wood Thrush reproduction. Moreover, within our study area located within the mid-Atlantic United States, 97% of 29 forest-breeding species with similar life-history traits to the wood thrush (e.g., migratory, multiple-broods) are actively parasitized by cowbirds (Lowther, 2013). Previous studies of long-term localized wood thrush population demographics (Roth and Johnson, 1993; Holmes, 2011) and regional source-sink dynamics (Lloyd et al., 2005; Tittler et al., 2006) suggest that multiple-scale approaches can help determine linkages between population dynamics and conservation of forest ecosystems (Bonnot et al., 2011).

We constructed an individual-based model for breeding wood thrushes that assumed heterogeneity among site quality and differential inter-site transition probabilities, to evaluate how among- and within-site characteristics influence metapopulation dynamics (Ovaskainen and Hanski, 2004). We used 40 years of demographic information and contemporary count and population genetics data to evaluate population vital rates in relation to four conservation scenarios. We predicted that conservation scenarios would have a positive effect on population growth rates, however, we were unsure of the magnitudes of respective population responses. Here we present a predictive metapopulation model that evaluates alternative conservation strategies that can be used as a tool in guiding conservation and management decisions for wood thrushes and other similarly forest-breeding migratory species.

MATERIALS AND METHODS

Study Area and Species

Our study took place in the mid-Atlantic United States on the boundary of the Piedmont plateau and Atlantic Coastal Plain physiographic regions (Fenneman and Johnson, 1946), within the White Clay Creek and Christina River watersheds. We randomly located 21 sites within 18 distinct forest fragments in and near Newark, DE, USA (39°41′1N 75°44′58W; **Table 1**). Each site lay along an edge adjacent to non-forest land cover to control for edge effects. Sites ranged in size from 2.1 to 16.3 ha, and included Ecology Woods (Bray et al., 1965), where an ongoing 40-year demographic study on breeding wood thrushes occur. Wood thrushes breed in deciduous forest from southeastern Canada throughout the eastern United States and winter in Central America (Evans et al., 2011). Throughout their breeding range, wood thrushes are typically found nesting in under- and mid-story vegetation within forest patches ranging in size from 1 to >1000 ha (Keller and Yahner, 2007), often raise two

TABLE 1 | List of 21 study site names, IDs, locations, and site and patch areas (hectares) in and near Newark, Delaware, USA.

Site Name	ID	Latitude	Longitude	Site area (ha)	Patch area (ha)
Christina Creek 1	CC1	39°39′39N	75°46′14W	4.2	50.8
Christina Creek 2	CC2	39°40′30N	75°46′48W	8.2	12.7
Coverdale	CD	39°41′47N	75°45′5W	6.2	155.5
Chrysler Woods	CW	39°39′33N	75°45′21W	4.5	5.5
Dorothy Miller	DM	39°41′18N	75°44′7W	4.2	68.8
Ecology Woods	EW	39°39′44N	75°44′39W	16.2	16.6
Folk	FO	39°38′43N	75°45′26W	5.0	150.1
Glasgow 1	GG1	39°36′39N	75°43′29W	5.2	75.6
Glasgow 2	GG2	39°36′47N	75°43′54W	4.7	75.6
Iron Hill 1	IH1	39°38′19N	75°44′46W	5.2	150.1
Iron Hill 2	IH2	39°38′19N	75°45′33W	5.1	150.1
Laird	LA	39°41′31N	75°45′32W	4.5	10.1
Motor Pool	MP	39°40′33N	75°44′21W	5.1	5.9
Phillips	PH	39°40′10N	75°45′34W	3.6	4.6
Reservoir	RE	39°41′55N	75°44′36W	5.0	53.3
Rittenhouse	RH	39°39′14N	75°45′29W	11.0	50.8
Sunset Lake 1	SL1	39°37′35N	75°43′46W	4.5	157.9
Sunset Lake 2	SL2	39°37′4N	75°43′48W	4.6	157.9
White Clay Creek 1	WC1	39°42′32N	75°45′58W	5.2	163.6
White Clay Creek 2	WC2	39°42′45N	75°45′53W	5.4	163.6
Webb Farm	WF	39°40′17N	75°44′2W	8.7	11.1

broods per season (Evans et al., 2011), and exhibit a high degree of breeding site fidelity (Roth and Johnson, 1993).

Demographic Data Collection

We discovered and monitored active wood thrush nests every 2–3 days (Martin and Geupel, 1993). We recorded the numbers of wood thrush and cowbird eggs and nestlings present during each nest check. For each nest, we recorded the location (UTM coordinates), height (m), plant species, and subsequently the distance to the non-forest edge using ArcMap 10.1 (ESRI, 2011). We considered nests parasitized if at least one cowbird egg was present in the nest. Within our study area, cowbirds were detected at all sites, and we used logistic regression to test for effects of distance to edge (m), site, and month (i.e., May, June, July) on cowbird parasitism occurrence, and found no effects for distance to edge (z-value $= -0.895$, $P = 0.371$), or for site or month (all $P > 0.998$). We captured and sampled adult wood thrushes using mist nets (36 mm mesh size), and 6–10 day old nestlings by hand extraction from nests (Federal Bird Banding permit #: 23475) between 6 May and 15 August 2011–2013. We fitted all birds with aluminum US Geological Service bands, and adults were given unique color-band combinations to allow future identification by sight.

Demographic Parameters

We estimated wood thrush demographic parameters (i.e., adult survival, recruitment, fecundity, and immigration) using an integrated population model (IPM; Kéry and Schaub, 2012; Schaub et al., 2013). We used 40 years (1973–2013) of mark-recapture, population census, and fecundity data containing information from 2,592 marked individuals and 1,692 nests collected annually within Ecology Woods (EW), a 16-ha forest patch within our study area (Roth and Johnson, 1993), to run a female-based, age-structured population model (Ladin et al., 2016a) using the R package "R2WinBUGS" (Sturtz et al., 2005) and WinBUGS (ver. 1.4.3; Lunn et al., 2000). We used data from 1974 to 2013 on cowbird parasitism of wood thrush nests, which has increased by over 400% over the past four decades, to build regression models correlating cowbird parasitism pressure (i.e., mean proportion of parasitized nests and mean number of cowbird eggs per nest) with IPM-estimated wood thrush survival, fecundity, and recruitment (see Ladin et al., 2016a for details). We then computed contemporary demographic parameters (mean ± SD), by averaging 10 years of IPM annual estimates from 2002 to 2012 for adult survival, fecundity, recruitment, and immigration (**Table 2**).

Transition Matrix

To determine transition probabilities among sites, we genotyped individuals that were sampled over 3 years during the 2011–2013 breeding seasons at five microsatellite loci and estimated relatedness between all site pairs. In order to genotype individuals, we collected blood samples via brachial artery puncture using 27 gauge, 1.27 cm syringes in heparinized capillary tubes (75 µL) according to IACUC regulations, under permit #:1129-2012-2. We sealed capillary tubes containing blood with crito-seal clay and stored immediately on ice in the field. We then transferred blood samples to 1.5 mL cryo-vials and stored frozen at −80°C within 2–3 h of collection. We extracted deoxyribonucleic acid (DNA) from blood samples using Qiagen

TABLE 2 | Parameter estimate means (SD) used in four metapopulation model scenarios for wood thrushes breeding in small urban forest fragments in and near Newark, Delaware, USA.

Model parameter	MODELS Null	Reforest	Cowbird removal	Reforest and Cowbird removal
Adult survival	0.49 (0.03)	0.49 (0.03)	0.51 (0.01)	0.51 (0.01)
Recruitment	0.06 (0.003)	0.06 (0.003)	0.085 (0.01)	0.085 (0.01)
Fecundity	0.97 (0.29)	0.97 (0.29)	1.19 (0.04)	1.19 (0.04)
Immigration	0.43 (0.01)	0.43 (0.01)	0.43 (0.01)	0.43 (0.01)

DNeasy kit (Qiagen, Venlo, Netherlands), and used polymerase chain reaction (PCR) in Bio Rad T100 thermal cyclers (Bio Rad, Hercules, CA, USA) to amplify five neutral polymorphic microsatellite loci, that have been previously used in genetic studies of wood thrush (Dawson et al., 1997; Lisle Gibbs et al., 1999; Evans et al., 2008). We analyzed florescent dye-labeled PCR products at the Delaware Biotechnology Institute using fragment analysis on a ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). We used the program BayesAss (Rannala, 2013) to estimate genetic distances and compute transition probabilities of wood thrushes among sites (Wilson and Rannala, 2003). Pair-wise site transition probabilities were then averaged between all site pairings and used to construct a transition matrix (see Appendix S2).

Wood Thrush Occupancy and Abundance Estimates

We conducted fixed-radius point count surveys (Buckland, 1987; Ralph et al., 1995) between 30 min before and 5 h after dawn from 1 May to 15 August 2011–2012 at one central point in each of the 21 sites. We visited each site 5 times per year and conducted a 10-min point count recording all birds detected within 0–50 m. We analyzed point count data using the "unmarked" package (Fiske and Chandler, 2011) in R (R Development Core Team, 2014) to estimate site occupancy (MacKenzie et al., 2002) and abundance (Royle, 2004). We used Akaike's information criterion (AIC; Akaike, 1974) with ΔAIC values ≤ 2 as a criterion to compare models predicting wood thrush site occupancy and abundance. In order to test for potential drivers of wood thrush site occupancy based on findings from previous studies (James et al., 1984; Donovan and Flather, 2002), we included the detection covariate (ordinal day) and the following site-level covariates: year, forest patch size (ha), proportion of impervious surface within 500 m buffer, proportion of agricultural land cover within 500 m buffer, mean forest site age, mean number of woody stems, and mean exchangeable soil calcium (Ca) (meq/100 g) in models. We computed the following site-level covariates [i.e., forest patch size (ha), proportion impervious surface within 500 m buffer, and proportion of agricultural land cover within 500 m buffer] in ArcMap (ver. 10.1) using the National Land Cover Data (NLCD) 2006 raster data. We compared 11 models to capture potential effects of patch-level covariates on wood thrush site occupancy. We extrapolated a population estimate to establish the initial conditions for model simulations by multiplying the estimated wood thrush density (i.e., individuals per ha) by the total area (ha) of forest habitat within our study area. Given documented even sex-ratios of males and females from previous studies (Roth and Johnson, 1993), we halved this number to estimate the number of females on the landscape.

Metapopulation Model Structure

We modeled wood thrush metapopulation dynamics among 21 discrete sites within our study area as a discrete-time Markov process in Mathematica 10.1 (Wolfram Research, 2014) and created an additional (22nd) site to represent emigration (i.e., removal from the study area). By drawing from distributions based on empirically-derived demographic parameters, model simulations mimicked a stochastic process (containing temporal variation from long-term data) where the probability of future states of the population were dependent on the current state at a given time step t (Caswell and Cohen, 1991; Verboom et al., 1991; Hanski and Ovaskainen, 2003).

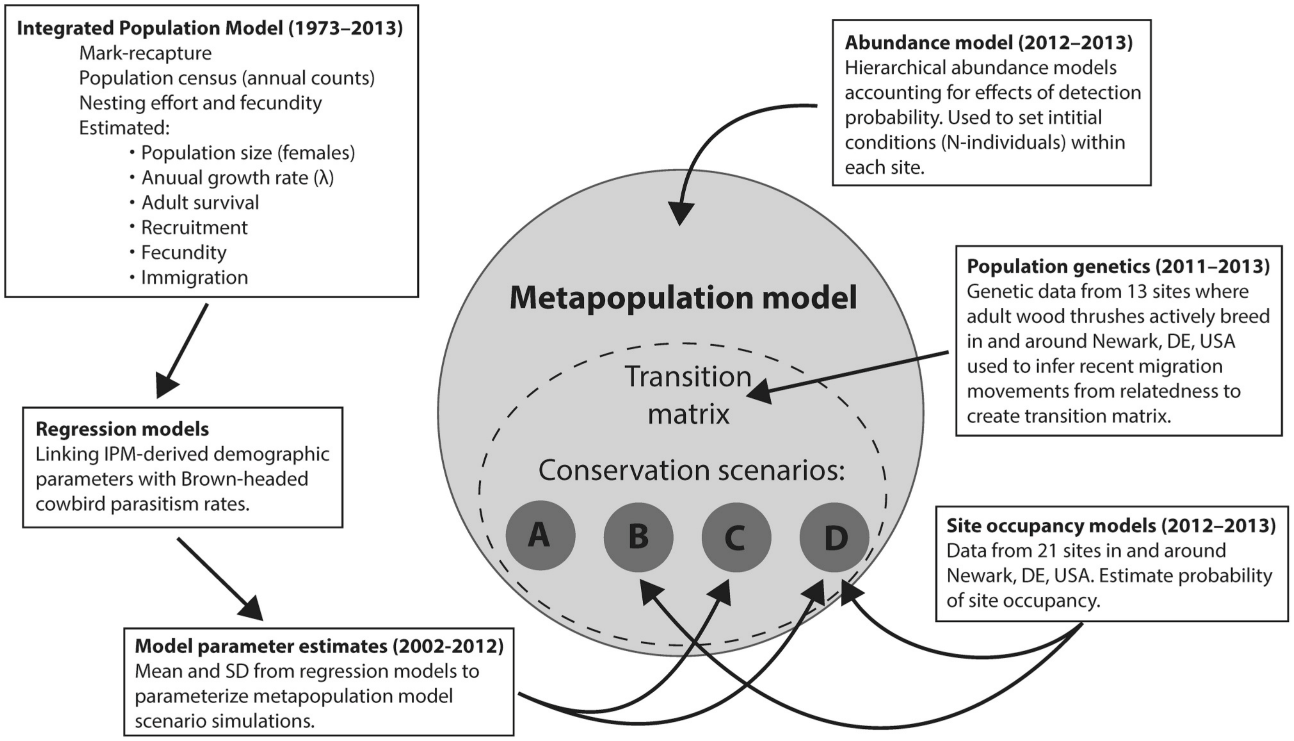
We simulated four conservation scenarios by varying metapopulation model parameters based on regression functions that represented Scenario (A) the Null scenario, akin to no conservation efforts under current observed conditions (hereafter "Null"), Scenario (B) replacing impervious surface with forest (hereafter "Reforest"), Scenario (C) reducing cowbird parasitism pressure (hereafter "Cowbird removal"), and Scenario (D) coincident replacing impervious surface with forest and reducing cowbird parasitism (hereafter "Reforest and Cowbird removal"; see **Figure 1** for conceptual diagram). All model simulations consisted of 10,000 iterations, and were run for 30 years into the future with $P\left(\text{emigration}_i^t\right) = 0.03$ as a calibration term that we adjusted so Null scenario trends mimicked that of current BBS-estimated trends within Delaware. We chose a 30-year timeframe based on the Null scenario performance, where after 30 years, the local population reached near-extinction levels. All simulations started from an initial distribution of birds across sites following the current observed distribution of birds (2011–2013) among 13 known occupied sites and 9 empty sites (including the idealized 22nd site representing emigration).

We initialized the model by distributing 400 birds (based on abundance estimates) by observed relative proportions among the 21 sites. At each time step (years), the number of birds per site (φ_i^t) for all 22 sites, was computed by multiplying the number of birds per site i at time step t (which we rounded to the nearest integer) by the transition probabilities of individuals moving between site i and any of the other sites, and then subsequently multiplying φ_i^t independently by adult survival, recruitment, and immigration rates to compute the number of returning adults, recruited individuals, and immigrants, respectively, with the equations:

$$Returns_i^{t+1} = \varphi_i^t \times P\left(\text{adult survival}_i^t\right) \quad (1)$$

$$Recruits_i^{t+1} = \varphi_i^t \times P\left(\text{recruitment}_i^t\right) \quad (2)$$

$$Immigrants_i^{t+1} = \varphi_i^t \times \text{proportion of immigrants}_i^t \quad (3)$$



Integrated Population Model (1973–2013)
Mark-recapture
Population census (annual counts)
Nesting effort and fecundity
Estimated:
- Population size (females)
- Anuual growth rate (λ)
- Adult survival
- Recruitment
- Fecundity
- Immigration

Abundance model (2012–2013)
Hierarchical abundance models accounting for effects of detection probability. Used to set intitial conditions (N-individuals) within each site.

Population genetics (2011–2013)
Genetic data from 13 sites where adult wood thrushes actively breed in and around Newark, DE, USA used to infer recent migration movements from relatedness to create transition matrix.

Regression models
Linking IPM-derived demographic parameters with Brown-headed cowbird parasitism rates.

Site occupancy models (2012–2013)
Data from 21 sites in and around Newark, DE, USA. Estimate probability of site occupancy.

Model parameter estimates (2002-2012)
Mean and SD from regression models to parameterize metapopulation model scenario simulations.

FIGURE 1 | Conceptual diagram depicting data inputs (years shown in parentheses), analyses, and various modeling approaches used in parameterizing metapopulation model framework for comparison among conservation scenarios: (A) Null, (B) Reforest, (C) Cowbird removal, and (D) Reforest and Cowbird removal.

where φ_i^t is the number of birds in site i and $P\left(\text{adult survival}_i^t\right)$, $P\left(\text{recruitment}_i^t\right)$, and proportion of immigrants$_i^t$ were computed for each site i at each time step t, by randomly drawing values from uniform distributions with means and 95% CIs taken from IPM-estimated means for adult survival, recruitment, and immigration rates from 2002 to 2012. We defined recruited individuals as birds that were born and subsequently returned to the study area as sexually mature ($\geq$1 year old). For each of 22 sites, the total number of birds at time $(t+1)$ was computed with the equation:

$$N_i^{t+1} = \sum \left(\begin{matrix} Returns_i^{t+1} + Recruits_i^{t+1} + \\ Immigrants_i^{t+1} \end{matrix} \right) \tag{4}$$

We computed the annual growth rate lambda (λ_i) within each site i with the equation:

$$\lambda_i = \frac{N_i^{t+1}}{N_i^t} \tag{5}$$

where N_i^{t+1} *and* N_i^t are the total number of birds per site i (from Equation 4) at time step $(t+1)$ and t, respectively. To estimate metapopulation annual growth rate (λ) for each model scenario, we summed the number of birds across 21 sites at each time step (Equation 4), and divided the total number of birds in our study area at time step $t+1$ (N^{t+1}) by the total number of birds from the previous time step t (N^t). We calculated mean annual growth rates (λ_i) per year at each site i before averaging across 21 sites (excluding emigrants in site 22) for each ensemble of 10,000 model iterations. We calculated the probability of lambda ($\lambda_i \geq$ 1) for each scenario by tallying the number of sites where the local annual growth rate was equal to or >1 at each time step and dividing by the total number of sites. We estimated population trends (mean % annual change) for each model scenario using the equation:

$$r = log\left(\frac{Nf}{No}\right) \times \frac{100}{T} \tag{6}$$

where N_f is the number of birds at the final time step, N_o is the number of birds at the initial time step (t_o), and T is the total number of years the model is run for. We estimated mean annual site occupancy by dividing the number of occupied sites per year (defined as having at least two individuals) by the number of years.

After testing simulation-derived demographic data for departures from normality with a Shapiro–Wilk test, we used one-way analysis of variance (ANOVA) with Tukey's *post-hoc* tests to test for differences in population trend, mean annual growth rate (λ), and a multiple pair-wise comparison test (Marascuilo, 1966) for differences in site occupancy among the four model scenarios.

MODEL SCENARIO DESCRIPTIONS

Scenario (A) Null

Null scenario parameters at each time step were derived independently for each site by drawing randomly from uniform distributions constrained by upper and lower 95% CI around the means of IPM annual estimates from 2002 to 2012 in EW for adult survival, recruitment, and fecundity (i.e., number of fledglings per female). Similarly, we estimated the proportion of immigrants for the metapopulation at each time step by randomly drawing from a uniform distribution with a mean and SD taken from IPM estimates. To model current observed conditions within the study area, we limited breeding (i.e., set fecundity = 0), and constrained immigration rates (i.e., 1% of total birds per site) to occupy currently unoccupied sites at (t_o), while allowing birds to immigrate at estimated rates into currently occupied sites and to reproduce at currently estimated levels of fecundity.

Scenario (B) Reforest (Replace Impervious Surface)

We modeled the effect of replacing the proportion of impervious surface with forest habitat on wood thrush metapopulation dynamics (based on results from predictive site occupancy models) by allowing 90% of total individuals at each time step to enter into and effectively breed (i.e., no reduction of fecundity) in previously unoccupied sites. These adjustments in the likelihood of site occupancy follow from concomitant reductions in impervious surface from ca. 0.8 (Null scenario) to around 0.05 using a predictive site occupancy model fit using the R package "unmarked" (Fiske and Chandler, 2011). Adult survival, recruitment, and fecundity were all estimated using the methods described above for the Null scenario.

Scenario (C) Cowbird Removal

We based our modeled reduction in cowbird parasitism pressure on beta regression models by increasing the probability of adult survival corresponding to a reduction in the proportion of cowbird nests parasitized from 0.65 (current observed parasitism rate) to range between (0.25 and 0.05). We increased estimated mean fecundity (i.e., number of fledglings per female) from 0.97 (current observed fecundity) to 1.19 using a linear model representing a predictive relationship between fecundity and the mean number of cowbird eggs laid per nest. We additionally increased mean recruitment based on the linear model relationship between the proportion of cowbird parasitized nests and annual number of recruited individuals. In this model scenario, immigration probabilities and fecundity in initially unoccupied sites were the same as in the Null scenario.

Scenario (D) Reforest and Cowbird Removal

We modeled the simultaneous effects of reducing impervious surface and cowbird parasitism pressure by using regression-based models to estimate according increases in immigration probabilities to all sites, and allowed breeding in all sites (as described in the Reforest scenario above). Under this model scenario, we used parameters estimated for adult survival, fecundity, and recruitment following the methods described above for the Cowbird removal scenario, and immigration probabilities were estimated as a function of reduced proportion of impervious surface (as stated above).

RESULTS

Occupancy Model Results

The impervious surface model had the most support (Akaike weight = 0.62) as indicated by AIC model comparison (Appendix S1). Site occupancy was negatively related to the proportion of impervious surface within a 500 m buffer around forest sites (**Figure 2A**), and differed from the null model according to the likelihood ratio test (Kent, 1982) ($\chi^2 = 11.6$, df = 1, $P < 0.01$). From significant correlation between impervious surface within a 500 m buffer around sites and wood thrush site occupancy, we derived the equation:

$$logit\ (y) = -1.31x + 1.01 \tag{7}$$

representing the predicted probability of patch occupancy (y) given the proportion of impervious surface (x) within a 500 m buffer around a forest patch (**Figure 2A**).

Regression Model Results

Adult wood thrush survival from 1974 to 2012 was negatively related to the proportion of parasitized nests ($\chi^2 = 80.1$, df = 3, $P < 0.01$; **Figure 2B**). We inferred the following equation from the beta regression model of the negative relationship between adult wood thrush survival and the proportion of cowbird parasitized nests:

$$logit\ (y) = -0.24x + 0.067 \tag{8}$$

where y is annual adult survival and x is the proportion of cowbird parasitized wood thrush nests (**Figure 2B**), to estimate a mean adult survival (0.51 ± 0.01) corresponding to a simulated reduction in the proportion of parasitized nests by 62% (**Table 2**).

Wood thrush annual fecundity was negatively related to mean cowbird eggs laid per nest ($F = 9.72$, df = 37, $P < 0.01$; **Figure 2C**). We used linear regression in the "stats" R package and found that wood thrush annual fecundity (i.e., number of offspring produced per female) was negatively related to mean cowbird eggs laid per nest ($F = 9.72$, df = 37, $P < 0.01$). With the governing linear regression equation:

$$y = -0.25x + 1.25 \tag{9}$$

where y is predicted wood thrush fecundity and x is the mean cowbird eggs laid per nest (**Figure 2C**), we estimated a mean fecundity (1.19 ± 0.04) given the simulated reduction in mean number of cowbird eggs per nest from 1.18 to 0.5 (**Table 2**).

The mean number of annually recruited wood thrushes was also negatively related to the proportion of parasitized nests ($F =$

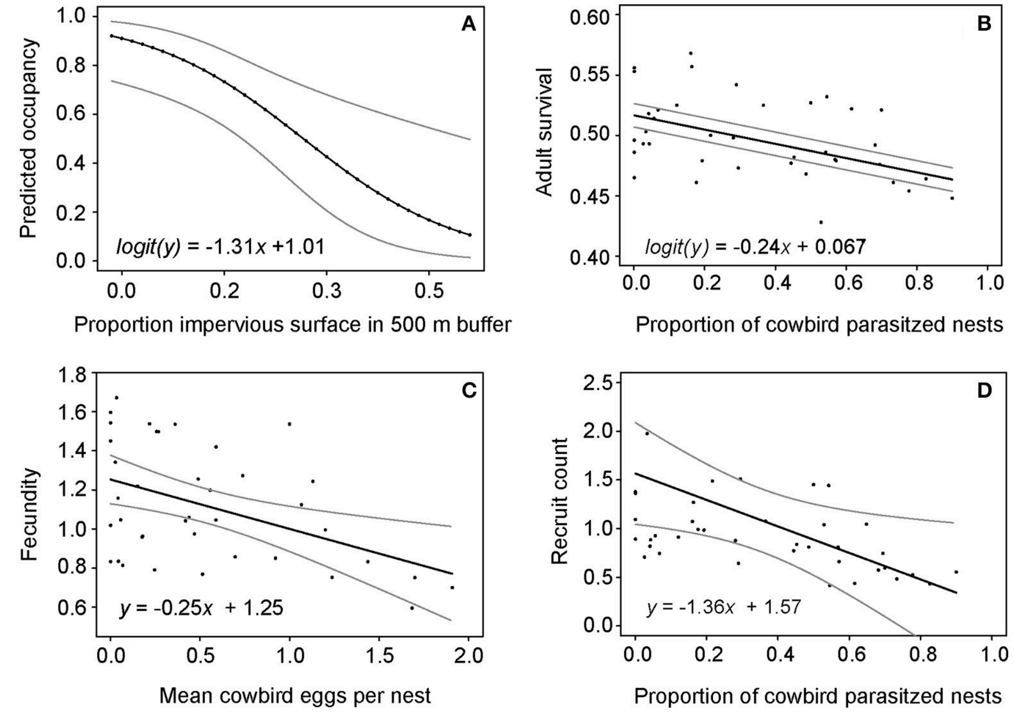


FIGURE 2 | Regression models showing relationships between (A) proportion of impervious surface (within 500 m buffer around sites) and site occupancy, (B) proportion of cowbird-parasitized nests and adult wood thrush survival, (C) mean cowbird eggs laid per nest and wood thrush fecundity (mean fledglings per female), and (D) proportion of cowbird parasitized nests and mean number of recruited individuals per year.

5.61, df = 37, $P < 0.05$), resulting in the following linear model equation:

$$y = -1.37x + 1.57 \tag{10}$$

where y is the number of recruited birds and x is proportion of parasitized nests (**Figure 2D**). We simulated increases in annual wood thrush recruitment rates by randomly drawing values from a uniform distribution with mean and SD of 0.085 ± 0.01 (**Table 2**).

Transition Matrix

We estimated transition probabilities among sites inferred from mean relatedness of birds among sites using microsatellite data from 177 genotyped adult wood thrushes sampled from 13 discrete sites (see Appendix S2). The final normalized transition matrix contained estimated transition probabilities from the 13 sampled sites, transition probabilities of 0.008 and 0.85 for leaving and remaining in a site, respectively, for the 8 initially unoccupied sites, and a 22nd site representing emigration out of the study area (see example transition matrix in **Table 3**).

Metapopulation Simulation Results

Metapopulation-wide annual growth rates (λ) (mean ± SD) differed among all model scenarios ($F = 567.1$, df = 3, $P < 0.001$; **Table 4**), and were lowest for the Null scenario (0.88 ± 0.01) after 30 years (**Table 4**, **Figure 3**). The annual growth rate for the Cowbird removal scenario (0.92 ± 0.02) was lower than in the Reforest scenario (0.94 ± 0.01), and both were lower than the combined Reforest and Cowbird removal scenario (1.0 ± 0.01; **Table 4**, **Figure 3**).

TABLE 3 | Example transition matrix containing probabilities (Θ) of dispersal movement among 21 discrete forest sites, and functional emigration (ε) from the study area.

Site	1	2	3	...	21	ε
1	$\Theta_{1,1}$	$\Theta_{2,1}$	$\Theta_{3,1}$	...	$\Theta_{21,1}$	ε_1
2	$\Theta_{1,2}$	$\Theta_{2,2}$	$\Theta_{3,2}$	...	$\Theta_{21,2}$	ε_2
3	$\Theta_{1,3}$	$\Theta_{2,3}$	$\Theta_{3,3}$	...	$\Theta_{21,3}$	ε_3
⋮	⋮	⋮	⋮	⋱	⋮	⋮
21	$\Theta_{1,21}$	$\Theta_{2,21}$	$\Theta_{3,21}$	...	$\Theta_{21,21}$	ε_{21}
ε	ε_1	ε_2	ε_3	...	ε_{21}	ε_{22}

Annual growth rates per site (λ_i) (mean ± SD) differed among model scenarios ($F = 46.3$, df = 3, $P < 0.001$). Mean growth rates per site for the Null scenario (0.92 ± 0.03) were lower than all other scenarios, and mean growth rate per site for the Cowbird removal scenario (0.99 ± 0.04) was lower than both the Reforest scenario (1.02 ± 0.06), and the combined Reforest and Cowbird removal scenario (1.05 ± 0.08; **Table 4**). Additionally, we found that the probability that site-level mean annual growth rates (λ_i) were >1 over the 30-year simulation period was low under the Null scenario (0.06 ± 0.05), and higher probabilities were observed for Cowbird removal, Reforest, and combined

TABLE 4 | Model-estimated means (SD) for metapopulation lambda (λ), lambda per site (λ_i), probability $\lambda \geq 1$, annual population trend (%), and the proportion of occupied sites for four model scenarios.

	Models			
Model derived estimates	**Null**	**Reforest**	**Cowbird removal**	**Reforest and Cowbird removal**
Metapopulation lambda (λ)	0.88 (0.01)	0.94 (0.01)	0.92 (0.02)	1.00 (0.01)
Lambda per site (λ_i)	0.92 (0.03)	1.02 (0.06)	0.99 (0.04)	1.05 (0.08)
Prob($\lambda \geq 1$)	0.06 (0.05)	0.52 (0.08)	0.33 (0.26)	0.89 (0.2)
Metapopulation trend	−12.8 (1.15)	−5.93 (0.30)	−7.92 (0.68)	−0.32 (0.21)
Population trend per site	−11.7 (1.84)	−3.79 (3.98)	−5.68 (2.96)	1.92 (3.90)
Proportion of occupied sites	0.42 (0.36)	0.98 (0.17)	0.67 (0.36)	0.98 (0.17)

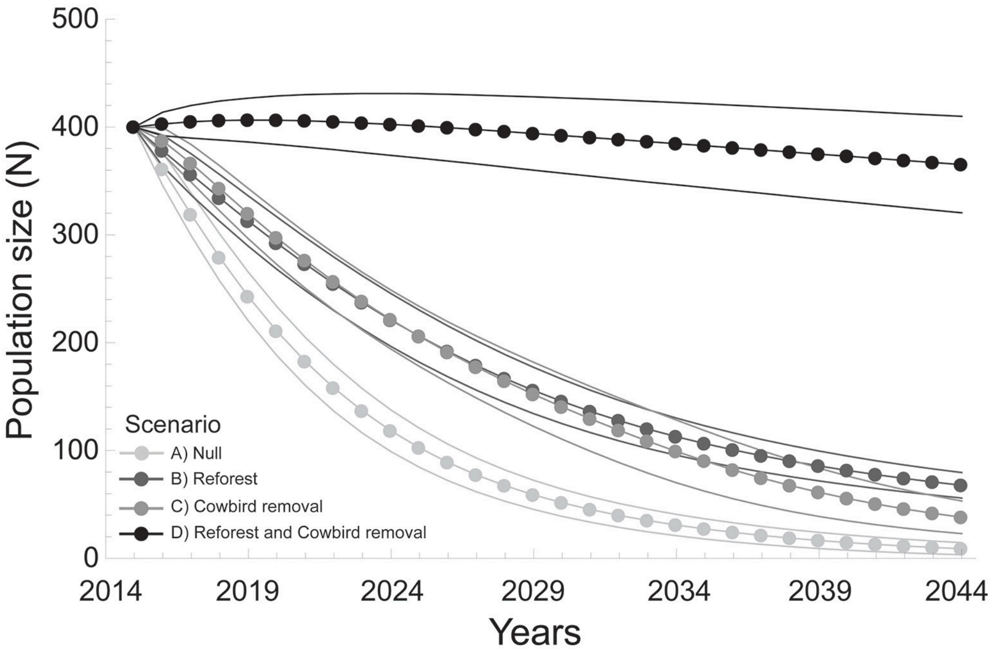


FIGURE 3 | Wood thrush population size (N) estimates (mean and 95% CI) over a 30-year period under four model scenarios: (A) Null (light gray), (B) Reforest (dark gray), (C) Cowbird removal (gray), and (D) Reforest and Cowbird removal (black).

Reforest and Cowbird removal scenarios (0.33 ± 0.26, 0.52 ± 0.08, and 0.89 ± 0.2, respectively; **Table 4**, **Figure 3**).

Annual population trend estimates (mean ± SE) differed among the four model scenarios ($F = 61.1$, df $= 3$, $P < 0.001$; see Appendix S3). Trend estimates were lower for the Null scenario (−11.7 ± 0.40) compared to all other scenarios, and both Cowbird removal (−5.68 ± 0.65) and Reforest (−3.79 ± 0.87) scenarios were lower than the combined Reforest and Cowbird removal scenario (1.92 ± 0.85; **Table 4**).

The proportion of occupied sites were similar under the Reforest scenario and the combined Reforest and Cowbird removal scenario (0.98 ± 0.17), and both were greater than the Null scenario (0.42 ± 0.36) and the Cowbird removal scenario (0.67 ± 0.36; χ^2, df $= 3, 7.815$), which did not differ (**Table 4**).

The relationship of combinations of proportions of impervious surface (ranging from 0 to 0.35) and cowbird index (ranging between 0 and 0.5) to mean annual growth rate per site (λ_i) is shown in **Figure 4**. Mean annual growth rate (λ_i) was lowest (<0.94) when both impervious surface and cowbird index were 0.35 and 0.5, respectively (**Figure 4**). Annual growth rate was stable ($\lambda = 1$) between 0.05 and 0.15 impervious surface over the range of cowbird index values, and mean annual growth rate was positive ($\lambda > 1$) between 0.02 and 0.10 impervious surface over the range of cowbird index values (**Figure 4**).

DISCUSSION

Simulated conservation scenarios had a positive effect on wood thrush population growth rates. Our results suggest that coincident replacement of impervious surface with forest habitat and reducing cowbird parasitism rates would have the greatest benefit for wood thrush populations. Although both conservation measures (Reforest scenario and Cowbird removal scenario) had positive effects on wood thrush population growth, they were

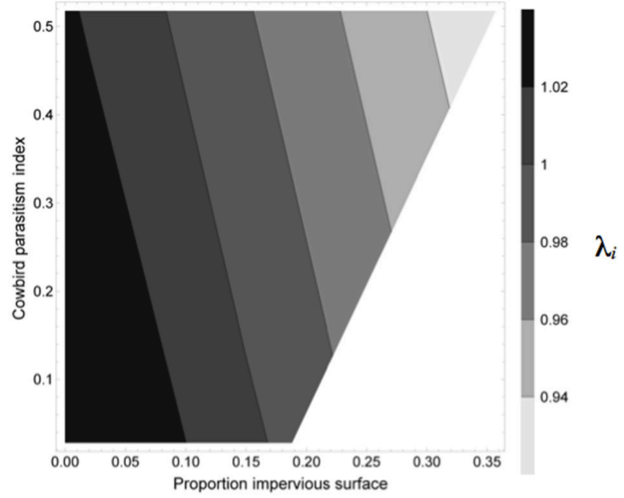


FIGURE 4 | Contour plot showing the relationship of combinations of proportions of impervious surface and index of cowbird parasitism (i.e., proportion of cowbird parasitized nests × number of cowbird eggs per nest) to mean lambda per site (λ_i).

insufficient to stabilize population growth when implemented individually. We think wood thrush conservation efforts in the New England/mid-Atlantic region could combine local and regional strategies to reduce current rates of regional population declines and stabilize the population within the urbanized mid-Atlantic USA.

The implementation plan for the New England/mid-Atlantic Coast Bird Conservation Region 30 (BCR 30; characterized by a highly developed landscape), has identified habitat loss, degradation, and fragmentation as the greatest threat to bird species, and has designated wood thrushes as a species of "Highest Priority" with a regional population estimate 1/3 lower than objective goals (Atlantic Coast Joint Venture, 2012). We estimated that 23% of all forest habitat within BCR 30 (590,794 ha), is contained in forest fragments $\leq$20 ha that can provide valuable breeding and habitat for wood thrushes and other forest-breeding birds. Given observed differences in wood thrush occupancy across a range of patch sizes (Keller and Yahner, 2007; MacIntosh et al., 2011), we think that solely focusing conservation efforts on larger forest patches would be a mistake in attempting to achieve established population goals for wood thrushes in BCR 30.

We compared population trends among model scenarios and 40 years of empirical data from EW (−2.79), which were similar to Breeding Bird Survey (BBS) route regression trend estimates (1966–2012) for the New England/mid-Atlantic Coast (−2.77) and the state of Delaware (−2.1) (Sauer et al., 2012). However, more recent trends (from 2002 to 2012) have decreased to −8.42 in EW, and to −3.73 and −10.52 for the New England/mid-Atlantic Coast and Delaware, respectively (Sauer et al., 2012). *In silico* population trends for 30 years into the future were similar for Null in EW (−11.5), and for the overall study area (−12.8). This confirmed continuity among regional BBS, locally-observed, and model-predicted population trends which supports our comparison of population-trend estimates under modeled conservation scenarios. Given differences among growth rates throughout the breeding range (Sauer et al., 2012), conservation efforts for wood thrushes should address both regional and local factors that likely drive source-sink dynamics (Keagy et al., 2005).

Negative effects of urbanization and impervious surface on wood thrushes and likely other forest-breeding birds may be a key driver of site occupancy (Longcore and Jones, 1969; Roth and Johnson, 1993, this study). Our findings indicate that reducing the proportion of impervious surface from 0.60 (in Null) to 0.05 (Scenarios B and D), would increase site occupancy by 57% and the probability of ($\lambda \geq 1$) by 88.5%. We found an upper threshold of 0.17 for the proportion of impervious surface where mean growth rate per site was positive ($\lambda_i \geq 1$).

Despite known challenges regarding habitat loss and increasing rates of impervious surface in urban areas (Nowak and Greenfield, 2012), reforestation and "greening" of urbanized landscapes provide many plausible and beneficial opportunities for helping populations of forest-breeding migratory species (De Sousa, 2014; Haase et al., 2014). Existing strategies to mitigate current rates of habitat loss include tools and methods for guiding future sustainable design and development of urban areas (Le Roux et al., 2014; Scott and Lennon, 2016).

However, we should not be limited in our creativity or by our ability to work within existing urban areas (Madre et al., 2014). Several examples of successful reforestation within urban areas exist including long-term (20-year) benefits of reforestation within New York City parks where reforestation efforts led to increases in tree species diversity, forest structure complexity, and native tree regeneration (Simmons et al, 2016). Additionally, initiatives for maintaining urban green spaces and keeping pace with rates of human development have become important priorities within existing city plans (Tan et al., 2013; Norton et al., 2015).

Cowbird parasitism can also have negative effects on songbird species by reducing fecundity and can contribute to population-level declines (Mayfield, 1977; Kilpatrick, 2002). In addition, cowbird parasitism can have less obvious relationships to wood thrush population dynamics such as adult survival, perhaps due to adults incurring increased energetic costs related to provisioning cowbird nestlings (Ladin et al., 2016a). Simulations of cowbird removal showed that reducing the proportion of parasitized nests from 0.65 (Null) to between 0.25 and 0.0 (Cowbird removal), resulted in a 4.3% increase in metapopulation-wide annual growth rate (λ), a 7.1% increase in mean annual growth rates per site (λ_i), and an 82% increase in the probability that $\lambda \geq 1$. Additionally, we observed a 37% increase in site occupancy under the Cowbird removal scenario. Given the positive trend for cowbirds in the New England/mid-Atlantic (1.2; Sauer et al., 2012) and demonstrated efficacy of cowbird removal for benefiting host species (e.g., Kirtland's warblers *Dendroica kirtlandii*; Siegle and Ahlers, 2004; McLeod and Koronkiewicz, 2014), we propose that cowbird removal be tested in the field to evaluate model predictions.

While empirically-based studies on how populations will respond to potential conservation strategies are critical starting points aiding in the design and implementation of conservation efforts, there are several other factors that can influence (potentially in unpredictable ways) intended outcomes from the application of conservation strategies that should be considered. For instance, how populations are likely to respond to climate change both on breeding and wintering grounds within spatially-explicit areas must be considered in concert with other factors that are examined (Giannini et al., 2015; Regos et al., 2015). One broadly-applicable consideration would be how species distribution shifts, and variation within species among populations to climate change might influence responses to specific conservation actions within a given area (Hällfors et al., 2016). Given the centrality of latitude for the location of our study area within the breeding range of wood thrushes in the eastern United States, species distribution shifts are likely to have little to minor effects on the performance of our model scenarios. However, for situations where conservation actions are targeting populations located at the margins of species distribution ranges, this should be directly accounted for (Hampe and Petit, 2005). Also, due to weak migratory connectivity between breeding and wintering areas found for wood thrushes, it will be hard to predict how climate- and landscape-change on wintering grounds may influence (*via* carry-over effects) population-limiting responses during breeding (Rushing et al., 2016). We are aware of additional and difficult-to-predict population responses that our model does not account for that include likely increases in human population growth rates, as well as, synergistic effects among climate change, increased human population densities in urban areas, and continued resultant land cover change due to increased rates of urbanization. However, our model does indirectly capture some of the inherent variation in wood thrush population responses to these factors through our inclusion of "noise" from long-term empirical relationships that end up being propagated within model predictions of the conservation scenarios we compared.

Our predicted population responses of wood thrushes to conservation scenarios could be used to estimate growth rates in other mid-Atlantic locations. However, estimated transition probabilities (which we held constant over time) do not account for other potentially contributing factors to wood thrush population dynamics, and ignore variation in habitat quality over time. Future models could improve these shortcomings by modifying transition probabilities and emigration rates through time according to predicted changes in habitat quality. Predicting bird population responses to future land use change (Martinuzzi et al., 2015), and mitigating negative effects of urbanization using sustainable landscape planning tools (Mason et al., 2007; Lerman et al., 2014; Gagné et al., 2015) will aid the conservation of wood thrushes and other forest-obligates. In general, we advocate for future studies using integrated population models and other empirically-based model simulations to evaluate potential population responses to conservation efforts.

ETHICS STATEMENT

We followed recommended guidelines for ethical scientific research and publishing conduct provided by the University of Delaware's Center for Science, Ethics and Public Policy.

AUTHOR CONTRIBUTIONS

ZL was lead author on the study, and lead data collection and analyses. VD was involved in study design, study area establishment, and review of manuscript drafts. JB helped develop modeling framework for metapopulation models. RR was responsible for long-term data collection and review of manuscript drafts. WS was responsible for data management, budgetary management, study design, and review of manuscript drafts.

ACKNOWLEDGMENTS

We thank the Northern Research Station of the US Forest Service, McIntire-Stennis Forestry Research Program, and the University of Delaware for funding. We thank cooperators from the University of Delaware, City of Newark Municipal parks, and White Clay Creek State Park for study site accessibility. We thank S. Adalsteinnsson, J. Buler, A. Colavecchio, D. Delaney, D. Ecker, N. Hengst, S. Mkheidze, K. Pastirik, C. Rega, J. Richardson, K. Serno, and M. Walker for help with field work and data collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00122

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 11 May 2017
doi: 10.3389/fevo.2017.00041

The Degree of Urbanization of a Species Affects How Intensively It Is Studied: A Global Perspective

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The expansion of urban areas is currently one of the most important worldwide landscape changes. This process, termed urbanization, has important ecological effects and is known to alter many aspects of the biology of organisms (including birds). However, human-nature interactions can also be affected by this process. We hypothesized that urbanization can particularly affect how intensively we investigate birds. We predict that species living in close proximity to humans will be more easily or preferably studied, thus promoting a bias in research effort toward urban birds. In order to test this hypothesis we have collected a detailed database of urban and non-urban avian communities including information from five biogeographic realms and more than 750 bird species. We obtained four different indicators of research effort (two previously considered and two new ones) as well as information on different confounding factors that are known to affect research effort such as conservation status, body mass, distribution range and phylogeny, in addition to the previously unconsidered historical factor of year of description of the species. We found a positive and significant association between the degree of urbanization of a species and how frequently it is investigated. We also found the expected effect for biogeographic realm, body mass and distribution range, and year of description, but not for conservation status. In addition, we found a strong correlation among all research effort variables which support the use of Google Scholar as a reliable source for these kind of studies. Our findings suggest that urbanization is not only affecting the biology of organisms but also how we study them. These results might have important implications if this research bias is maintained in the long term. Future investigation should aim at exploring the ultimate reasons for this research bias toward urban birds and whether it is also happening for other groups of organisms.

Keywords: birds, human-nature interactions, research effort, urbanization

Edited by:
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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *10 February 2017*
Accepted: *20 April 2017*
Published: *11 May 2017*

Citation:
Ibáñez-Álamo JD, Rubio E and Bitrus Zira K (2017) The Degree of Urbanization of a Species Affects How Intensively It Is Studied: A Global Perspective. Front. Ecol. Evol. 5:41. doi: 10.3389/fevo.2017.00041

INTRODUCTION

The process of urbanization is dramatically changing the environment, modifying not only abiotic elements such as habitat structure or connectivity, but also biotic elements (Grimm et al., 2008; Gaston, 2010; Forman, 2014). There is mounting evidence suggesting that this anthropogenic landscape change modifies different components of biodiversity, including taxonomic, functional and evolutionary diversity (Devictor et al., 2008; McKinney, 2008;

Newbold et al., 2015; Ibáñez-Álamo et al., 2016; Knop, 2016; Morelli et al., 2016) and other aspects of the biology of organisms like animal behavior or life-history traits (Ibáñez-Álamo and Soler, 2010; Møller and Ibáñez-Álamo, 2012; Díaz et al., 2013; Gil and Brumm, 2014; Møller et al., 2015). This intensive alteration of the environment has attracted increasing attention by the scientific community (Marzluff, 2016; McDonnell and MacGregor-Fors, 2016) and has ultimately lead to recognize urbanization as a major global challenge (United Nations, 2016).

Humans are intrinsically associated with the urbanization process (Forman, 2014). The urban habitat is created by us to meet our species-specific requirements and now the majority of the World's human population is living in cities (United Nations, 2012). It is thus normal that many papers in the field of Urban Ecology have focused on investigating the interaction between organisms and humans. The majority of them focused on how humans can affect organisms, for example by altering animal's escape behavior (Díaz et al., 2013, 2015; Samia et al., 2015), while others explored the opposite direction of the interaction, how urban nature can affect humans (Fuller and Irvine, 2010; Soga and Gaston, 2016). In relation to the latter, it is logical to think that scientists are not immune to this effect and the focus of their investigations might also be influenced by urban nature. For instance, researchers might be biased to study species that live in urban habitats more often, due to different reasons such as ease to study or preferences toward those species encountered more often by researchers or with scientifically attractive traits. These reasons have already been proposed to affect research effort (how intensely we study a topic or subject) and are used to explain for example why we tend to investigate more often larger species or those with broader distribution ranges (Brooke et al., 2014; Ducatez and Lefebvre, 2014; McKenzie and Robertson, 2015). Despite previous studies, it has been suggested that there is a need to explore alternative factors in order to explain research effort (Murray et al., 2015) and that urbanization might be one of these factors. The knowledge about how we carry out our research is very valuable as it can be used to re-orientate our effort toward those less studied topics or areas, it can help detect potential biases in our conclusions, or even better justify our management efforts (De Lima et al., 2011; Ibáñez-Álamo et al., 2012; McKenzie and Robertson, 2015).

Another important and related issue regarding the study of research effort is methodological. Even though there are alternative ways to measure research effort (Murray et al., 2015), the most commonly used variable is the number of published papers (De Lima et al., 2011; Brooke et al., 2014; Ducatez and Lefebvre, 2014; McKenzie and Robertson, 2015). Previous studies on the topic have used different research databases to look for published papers, from Web of Science to Zoological Records, including those obtained from different research organizations (e.g., Birdlife International; De Lima et al., 2011; Ducatez and Lefebvre, 2014; McKenzie and Robertson, 2015). However, even if it has been highly recommended for this type of study (Pautasso, 2016), to our knowledge there has been only one attempt to investigate whether different databases might be offering similar conclusions in relation to research effort in birds (De Lima et al., 2011). In this case the authors found a positive correlation between the number of papers obtained from Web of Knowledge and those of the Birdlife International library catalog. It is also worth to mention that no previous study has used Google Scholar as a source to test research effort predictions. All previously used databases are, to some extent, difficult to get access to because they are not free or easily accessible. Thus, exploring the feasibility of this open-access scientific database to investigate research effort and its relationship with other commonly used databases is important.

The aims of our study were: (i) to investigate whether the degree to which a species is urbanized (number of cities in which it is found) is related to the research effort it receives; (ii) to find other potential factors that can affect research effort in birds (including the previously unconsidered effect of year of description of a species); and (iii) to test whether there are correlations between research effort variables obtained from different sources (including Google Scholar and more traditional databases), in order to explore whether they can offer similar information. In order to do so, we used a global database of bird species found in urban and non-urban habitats and collected the number of papers published for each species in four different databases (Google Scholar, Web of Science, Zoological Records and the Handbook of the Birds of the World Alive). We decided to use birds as a model group because they are very well known in relation to urbanization (Marzluff et al., 2001; Lepczyk and Warren, 2012; Gil and Brumm, 2014) allowing us to compile a geographically wide database and extract general conclusions. This global coverage is important given the worldwide expansion of urban areas and will also allow us to identify differences in research effort allocation among regions.

MATERIALS AND METHODS

Bird Assemblages

We used a global database of studies presenting information for 17 countries and four continents on urban and non-urban bird communities published recently (Ibáñez-Álamo et al., 2016). Basically, the database was created using an exhaustive literature search in different websites (i.e., Web of Science, Google Scholar, and SmartCat) and a careful selection of papers including complete bird assemblages from urban and non-urban habitats (defined according to Marzluff et al., 2001; e.g., urban areas characterized with >50% of the surface built and >10 buildings per ha). These assemblages were collected following the same field method, during the same period and by the same field observer, thus offering standardized information that avoids many potential confounding factors in these kind of comparative studies. From each study, we obtained: (i) urban bird assemblage, (ii) non-urban bird assemblage, and (iii) site. There is a more detailed description of data collection in Ibáñez-Álamo et al. (2016).

Research Effort Data, Urbanization and Species' Traits

Using the database described above, we created a new dataset including all the 767 species from the 28 paired study sites (Supplementary Material 1). To quantify research effort for each species, we collected information on the number of papers published in different databases. This variable has been commonly used in previous studies and has been suggested to reflect the research effort invested in a species better than alternative ones (McKenzie and Robertson, 2015). First, we used Web of Science, as it has been previously used in these kinds of studies (McKenzie and Robertson, 2015; Murray et al., 2015). Using quotes, we searched for each scientific name in all available databases within the search engine and without time restriction. A recent study showed that this is an effective method that is not affected by changes in scientific names with time (Ducatez and Lefebvre, 2014). Second, we extracted the number of papers in Zoological Records from a previously published compilation (Ducatez and Lefebvre, 2014). Third, we also looked for the number of references per species obtained with Google Scholar, using the scientific name in quotes and without the patents option activated, again without time limits. The literature search in Google Scholar and Web of Science were both done in January 2017. Finally, we extracted the number of papers included in the reference section of the Handbook of the Birds of the World Alive during August 2016 (Del Hoyo et al., 2016). This compilation of birds is known for its quality and up-to-date information among ornithologists and thus, the number of references used for each species should indicate the overall knowledge for that particular species.

In order to investigate whether species that are found in urban areas are more studied, we calculated the number of cities for each species in which it was found in our database (max. 28 cities). This variable matches the definition of urbanization used by Croci et al. (2008), which considers a species to be urbanized if it is found in urban centers, but also accounts for the intensity of such effect by adding the number of cities in which the species can be found. Croci's definition of urbanization is strongly correlated (sensu Cohen, 1988) to all other indexes of urbanization previously used in Urban Ecology, and thus can be considered a good proxy for the extent of urbanization of a species (Møller, 2014).

Given that research effort in birds is multifaceted and can be affected by several different factors (Ducatez and Lefebvre, 2014; McKenzie and Robertson, 2015; Murray et al., 2015), we also collected information on different factors that might affect research effort according to previous studies, but we tried to avoid co-linearity of predictors (i.e., between distribution range and population size or body mass and clutch size Saether, 1987; Jetz et al., 2008). We obtained the following information for each species: (i) range of breeding distribution (square kilometers) according to Birdlife International (www.birdlife.org) given that species with large distribution are more likely to be studied than those occupying small areas (i.e., endemic) (Ducatez and Lefebvre, 2014; McKenzie and Robertson, 2015); (ii) Body mass collected from the Handbook of the Birds of the World Alive (Del Hoyo et al., 2016) as a proxy for size because large-sized species are more likely to be detected and manipulated and hence studied (Ducatez and Lefebvre, 2014; McKenzie and Robertson, 2015); (iii) Conservation status obtained from the International Union for Conservation of Nature (www.iucn.org) as threatened species might attract a greater attention by scientists (De Lima et al., 2011; Murray et al., 2015); (iv) the biogeographic realm for that species as the research effort can vary depending on the development of the region (i.e., highly developed countries investing more in research and consequently having more probabilities to investigate their species) (De Lima et al., 2011; Ducatez and Lefebvre, 2014). In addition to these factors capturing information on geographic (biogeographic realms), biotic (distribution range and body mass) and human effects (conservation status and urbanization), we wanted to control also for historical factors. Therefore, we also collected (v) the year of description of the species obtained from the Handbook of the Birds of the World Alive (Del Hoyo et al., 2016), as those birds described more recently may have received a lower research effort.

Statistical Analyses

We first calculated a correlation matrix of the four research effort variables in order to detect whether they offer similar information. All research effort variables in addition to body mass and breeding distribution range were log-transformed to achieve normality. We chose a single research effort variable based on the results of these correlations to run the subsequent analyses (see Results). Given that a previous study (Ducatez and Lefebvre, 2014) found differences among avian taxa in research effort, species cannot be considered independent units in our context. Therefore, we estimated the phylogenetic relationships of the species in our database using the Mesquite environment (Maddison and Maddison, 2015) and calculating the consensus (i.e., majority rules consensus) tree of 1000 phylogenetic trees downloaded from birdtree.org (Jetz et al., 2012; Supplementary Material 2). Then, we used a stepwise backward model selection running phylogenetic generalized least square models (PGLS; i.e., Díaz et al., 2013, 2015). The best models were selected based on corrected Akaike Information Criteria (AIC_c), using a threshold AIC_c value of 2. The full model included all single effects of the variables described above (**Table 2**) in addition to the interaction between biogeographic realm and the degree of urbanization as urban development vary geographically (Seto et al., 2012). We performed our analyses in R 3.3.2 (R Core Team, 2016) using the R libraries "ape" (Paradis et al., 2004), "MASS" (Venables and Ripley, 2002) and "mvtnorm" (Genz et al., 2016) as well as the function pglm3.3.r created by R. Freckleton which allows to run PGLS using an orthogonal (type III) fit of models. As a first step, we calculated the phylogenetic scaling parameter lambda (λ), which varies from 0 (phylogenetic independence) to 1 (variables completely covary according to their shared evolutionary history; Freckleton et al., 2002) and provides information on the variation explained by phylogeny. Secondly, we run the models correcting for the estimated λ and applied the backward procedure. Once the best model was found, we assessed the importance of each predictor regarding our (research effort) dependent variable based on their effect sizes calculated from P-values and t-tests (Díaz et al., 2015). We used Cohen's criteria (Cohen, 1988) to quantify their importance explaining our dataset as small ($r \leq 0.10$, explaining less than 1% of the variance), intermediate ($r = 0.11–0.49$, explaining between 2 and 24% of the variance), and large ($r \geq 0.50$, explaining more than 25% of the variance).

TABLE 1 | Correlations between all four variables of research effort.

R^2	Web of science	Handbook alive	Zoological records
Google scholar	0.94***	0.66***	0.92***
Web of science		0.62***	0.94***
Handbook alive			0.64***

****P < 0.001.*

RESULTS

Our dataset contained information on 767 bird species, of which 49.0% were found in an urban area. The three most urbanized species in our database were the Rock pigeon (*Columba livia*), the House sparrow (*Passer domesticus*) and the Common starling (*Sturnus vulgaris*) (Supplementary Material 1). Given the extreme degree of urbanization of these three species, we decided to run a sensitivity analysis in order to check the importance of those species in our findings (Supplementary Material 3).

The search in Google Scholar provided a higher number of references in comparison with the other databases (Supplementary Material 1), probably due to the use of different search engines and size of the database. Despite this, the results for our correlation analyses among research effort variables indicated that all of them are highly correlated (**Table 1**). The *r*-values indicated a strong and significant correlation among all four research effort variables, with Google Scholar showing the highest values with all others. Thus, we carried out our model selection procedure using Google Scholar as it is more easily accessible and correctly represents research effort.

The minimum adequate model of Google Scholar research effort retained six variables, including urbanization index, and explained 73% of the variance of our database [PGLS; $\lambda = 0.33$, Adjusted $R^2 = 0.73$, $F_{(1, 754)} = 477.87$, $P < 0.0001$; **Table 2**]. The phylogenetic signal of this model was relatively small. All variables retained in the final model were statistically significant and had intermediate effect sizes indicating that they are important for explaining research effort. The only exception was the interaction between the biogeographic realm and urbanization index, which involved a small effect size (**Table 2**), and in fact was not retained in the best model if we exclude the three most urbanized species (Supplementary Material 3). This interaction indicated that the relationship between research effort and urbanization was stronger in the Palearctic. We found a positive association between the level of species urbanization and the attention received by researchers (**Table 2**, **Figure 1**). In addition, we found a positive effect of body mass and breeding distribution suggesting that larger and more widely distributed species have been studied more (estimate ± standard error of 0.11 ± 0.03 and 0.44 ± 0.03, respectively; **Table 2**). But a negative influence of year of description showing that fewer papers have been published for those species described more recently (**Table 2**; **Figure 2**). The biogeographic realm also had a significant and intermediate effect *per se* to explain research effort as those species from Neotropical and Oriental realms have been less studied than those of the other three realms represented in our database (**Figure 3**). We also run the analyses using Croci's definition of urbanization more strictly, thus using a binary variable instead of the urbanization index described above. Our results were the same as those obtained with the urbanization index except for the interaction between biogeographic realm and urbanization that was not retained in the best model (Supplementary Material 4), similarly to what happened with our sensitivity analysis. This additional analysis provides support for the robustness of our results and suggest caution regarding the mentioned interaction.

DISCUSSION

As hypothesized, we found that the level of urbanization of a species is an important predictor of the attention it receives from scientists (**Figure 1**). This is a clear example that urbanization is not only affecting the biology of organisms but also how intensively we research them, and consequently altering human-nature interactions. Our results seem to point out that the urbanization effect on research effort is global and does not depend on the biogeographic realm given that the small effect size of the interaction between the degree of urbanization and the biogeographic realm disappeared in our sensitivity analysis (without the three most urbanized species) and in our analysis using a binary variable (Supplementary Materials 3, 4). Our results markedly contrast with those obtained in a recent study done with a group of mammals (Brooke et al., 2014). In that investigation, they found a significant negative relationship between the mean human population density of the distribution range of a species and its research effort. This effect disappeared when other factors where included in the model. The differences between the two studies could be due to group-specific effects regarding research effort or different methodological approximations (e.g., mean human population density being influenced by other factors in addition to urbanization). Future studies on avian research effort should investigate the effect of human population density in order to distinguish between these two options.

Our findings highlight the importance of biases in research effort like the one described here, even though it does not necessarily involve re-orientating our scientific aims. It is possible that urban birds are significantly more studied because they show a particular set of traits (Kark et al., 2007; Croci et al., 2008; Møller, 2014; Sol et al., 2014) that might be of special scientific relevance (i.e., more complex social breeding; Kark et al., 2007) or because they are involved in particularly important economic or health issues (i.e., spread of some diseases; Kilpatrick, 2011). Alternatively, other reasons of more concern (i.e., logistic/monetary constrains or personal preferences) may be behind our results. The ease of study has been raised as an important determinant to explain research effort in birds (Ducatez and Lefebvre, 2014; McKenzie and Robertson, 2015; Murray et al., 2015) and mammals (e.g., Brooke et al., 2014). Some of the traits of urban birds can facilitate their investigation. Nesting in holes, for example, is a common trait of birds living in cities (Croci et al., 2008; Sol et al., 2014) which allows the use of nest-boxes by scientists, making their study easier.

TABLE 2 | Full and minimum adequate models explaining avian research effort.

Predictor	df	SS	*F*	*p*	Effect size
FULL MODEL (AIC$_C$ = 1764.94)					
Number of cities	1	3.551	477.99	**<0.001**	0.371
Body mass (log)	1	0.088	11.87	**<0.001**	0.138
Conservation status	2	0.037	2.52	0.081	0.063
Breeding distribution (log)	1	4.507	603.74	**<0.001**	0.371
Year	1	0.772	103.96	**<0.001**	0.371
Biogeographic Realm	4	6.232	209.69	**<0.001**	0.371
Biogeog. Realm*N of cities	4	0.043	1.24	**0.040**	0.074
Error	752	5.587			
MINIMUM MODEL (AIC$_C$ = 1760.84)					
Number of cities	1	3.565	477.87	**<0.001**	0.371
Body mass (log)	1	0.089	11.88	**<0.001**	0.124
Breeding distribution (log)	1	4.523	606.40	**<0.001**	0.371
Year	1	0.797	106.84	**<0.001**	0.371
Biogeographic Realm	4	6.250	209.46	**<0.001**	0.371
Biogeog. Realm*N of cities	4	0.076	2.55	**0.038**	0.075
Error	754	5.624			

Effect sizes are partial correlation coefficients. P-values in bold indicate statistical significance (P < 0.05).

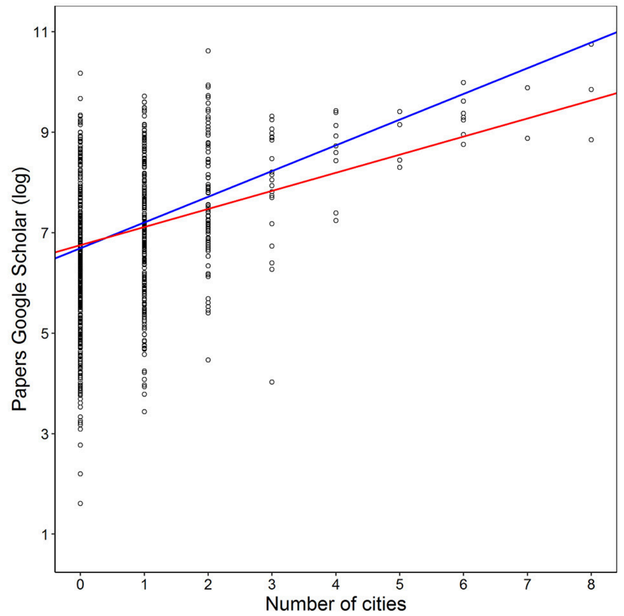

FIGURE 1 | Relationship between research effort (number of papers in Google Scholar) and the number of cities in which the species are found. The blue line represents the correlation between the two variables according to our model while the red line corresponds to the sensitivity analysis. The three most urbanized species (Rock pigeon, House sparrow and Common starling) are not represented to increase the clarity of the figure.

Furthermore, urban birds are present in a larger number of habitats than non-urban birds (Sol et al., 2014) and the number of habitats in which a species can be found (habitat breadth) is known to affect research effort in birds (Ducatez and Lefebvre, 2014). This could also be the reason why urban species are more easily accessed by a larger number of researchers and, consequently, more often studied. In addition, this bias could be due to human demographic patterns. Considering that scientists follow the same demographic trend as humans in general and are concentrated in urban areas (Nations, 2014), populations that are found closer to the residence place of scientists may be more frequently studied. But despite these potential explanations for the ease of study of urban birds, we cannot discard other (worrying) sociological effects unrelated to it. The extinction of experience is known to affect human-nature interactions in different contexts (Soga and Gaston, 2016). What if the scientists are more often studying those species encountered during childhood? The extinction of experience in researchers might also explain the bias toward urban birds and should be explored in future studies. Additionally, frequent contact with urban species and popularization of science programs can make them more charismatic (Duckworth, 2014), and this factor is known to influence research effort (Murray et al., 2015). If the ease of study or extinction of experience are the causes for the observed bias found in our study instead of their scientific relevance, maybe we should think about how to facilitate the study of non-urban birds as well.

Our model explained a high proportion of variance of our data, which supports the relevance of the factors selected for our analyses. The phylogenetic signal in our model explained 33% of the variation in research effort. This value was not as high as in Ducatez and Lefebvre (2014) study, in which it explained 74% of the variation, but it is still present and important to correct for. The reason for this difference between studies could be our use of a more restricted database in comparison to theirs presenting a phylogenetically biased subset. Similarly to other studies on avian research effort we found an intermediate and significant

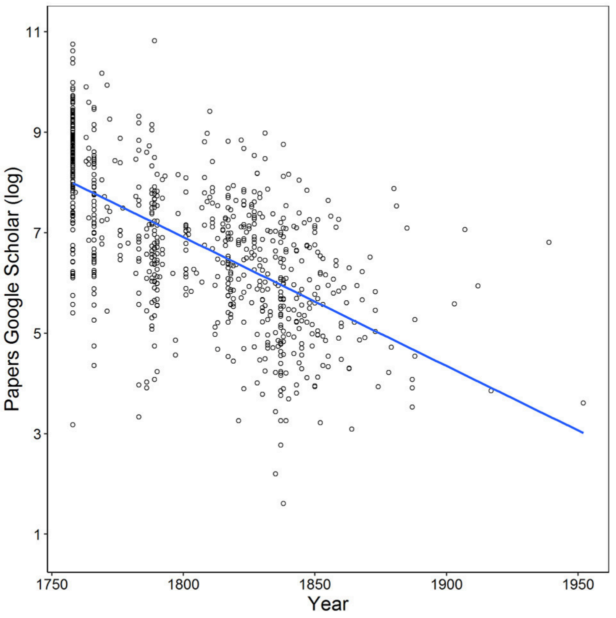

FIGURE 2 | Relationship between research effort (number of papers in Google Scholar) and year of description of the species. The blue line represents the correlation between the two variables.

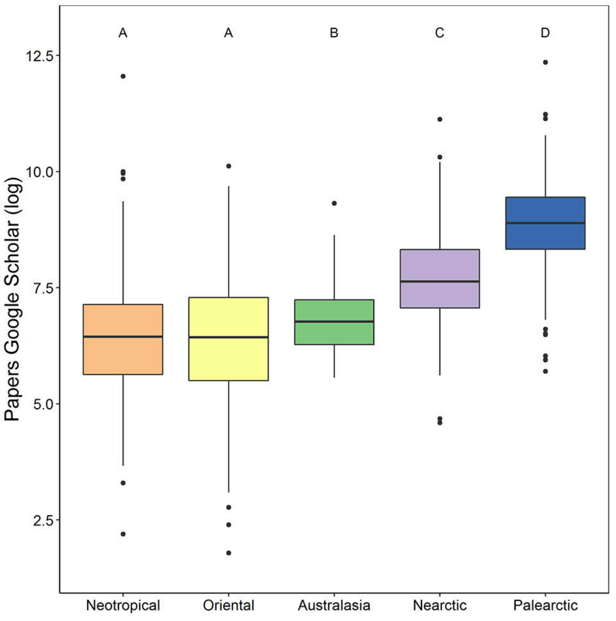

FIGURE 3 | The research effort (number of papers in Google Scholar) for each biogeographic region. Box-plots show median, quartiles, 5- and 95- percentiles and extreme values. Different letters indicate significant differences ($P < 0.02$) between regions according to Bonferroni *post-hoc* tests using the package "phytools" (Revell, 2012).

effect of body mass, distribution range and biogeographic region (De Lima et al., 2011; Ducatez and Lefebvre, 2014; McKenzie and Robertson, 2015; Murray et al., 2015). As stated in these papers, larger birds with wider distributions are more studied, probably because they are more easily accessible by scientists (endemic species can only be studied by a limited number of scientists), more easily manipulated and their large size allows to do more studies (i.e., allowing to extract more blood, attaching tracking devices; Bridge et al., 2011). Nevertheless, given that many bird species are studied outside their breeding range, future investigations should explore the effect of non-breeding distribution range in this context. We also found a geographical pattern showing that those birds from certain areas (i.e., Palearctic) are more often studied (**Figure 3**). Previous studies already suggested that this geographic bias was of concern as there is no match between the biodiversity levels of a region and the research effort to study their species (Brito and Oprea, 2009). In contrast, we did not find an effect of conservation status. Two recent studies using regional databases also failed to detect such an effect (McKenzie and Robertson, 2015; Murray et al., 2015), although other papers have found that more threatened species are investigated more often (Brooks et al., 2008; De Lima et al., 2011; Ducatez and Lefebvre, 2014). This difference in results can be due again to the subset of species in our database, which does not includes many threatened species (99% of them are considered of least concern). Interestingly, we found an intermediate effect for the year of description of the species indicating that those species described more recently have fewer papers published (**Figure 2**). Recently, it was proposed for mammals that such an effect could be due to larger and more widely distributed species being described first (Brooke et al., 2014). This however does not seem to be the case as the intermediate effect of year of description is present even after controlling for those two traits, which suggest an effect of time-restriction (species described long ago have had more time to be investigated). Further studies are needed to differentiate whether mammals or other groups follow the same pattern shown here for birds. To our knowledge, it is the first time that this factor is tested explicitly and, together with our other results, suggests that research effort is complex and multifactorial, influenced by many different factors including geographic, phylogenetic, biotic and human causes.

Another important result from our study is the positive and strong correlations among all research effort variables obtained from four different databases. These correlations exceed 0.60, accounting thus for strong effects in consistency, and being higher than those found by the only single study that has tested for such a relationship (De Lima et al., 2011). This consistency is particularly strong between the number of papers obtained from Web of Science, Zoological Records and Google Scholar, with values higher than 0.90, which suggest that any of these variables can be used for studies on research effort. Therefore, considering the facility of access to Google Scholar, we recommend its use for future studies on the topic. On the contrary, the lower correlation with the number of papers included in the Handbook of the Birds of the World Alive as well as the difficulty to extract this information (much more time consuming than for the other databases as it is not completely digitalized) recommend against its use for research effort studies.

To sum up, our findings complement previous studies on avian research effort adding two new variables (urbanization and year of description) that significantly explain how bird species are studied, but also provide an additional perspective to those papers more focused on investigating temporal and geographic trends in avian urban ecology research (Marzluff, 2001, 2016). The effect of urbanization in human-nature interactions can be very subtle, like in our case, and deserves more attention in the future (i.e., exploring if our findings apply to other organisms too). It could be particularly interesting to study the ultimate reasons of the bias toward urban birds in order to detect whether we should re-orientate the allocation of scientific resources. Finally, we confirmed the utility of Google Scholar as a good database to carry out these kind of studies and recommend its use in future investigations. We hope that our study is useful to better understand how we study urban birds and more broadly the impacts of urbanization on organisms, including humans.

AUTHOR CONTRIBUTIONS

Conceived the study: JDI. Collected data: All authors. Analyzed data: JDI and ER. Wrote the first draft of the manuscript: JDI. All authors edited the manuscript.

FUNDING

JDI was funded by a postdoctoral contract (TAHUB-104) from the program "Andalucía Talent Hub" (co-funded by the European's Union Seventh Framework Program Marie Skłodowska-Curie actions -COFUND- and the regional Government of Andalucía).

ACKNOWLEDGMENTS

We thank Maaike Versteegh and Juan J. Soler for their help with statistical analyses. Mario Díaz for his help with the Mesquite software. John C. O'Connor reviewed the English. Two reviewers provided very useful comments that considerably improved our manuscript. Caroline Isaksson kindly invited us to contribute to this special issue. Special thanks also to Olivia Sanllorente and Beatriz Rapado Tamarit for their help and constant support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00041/full#supplementary-material

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