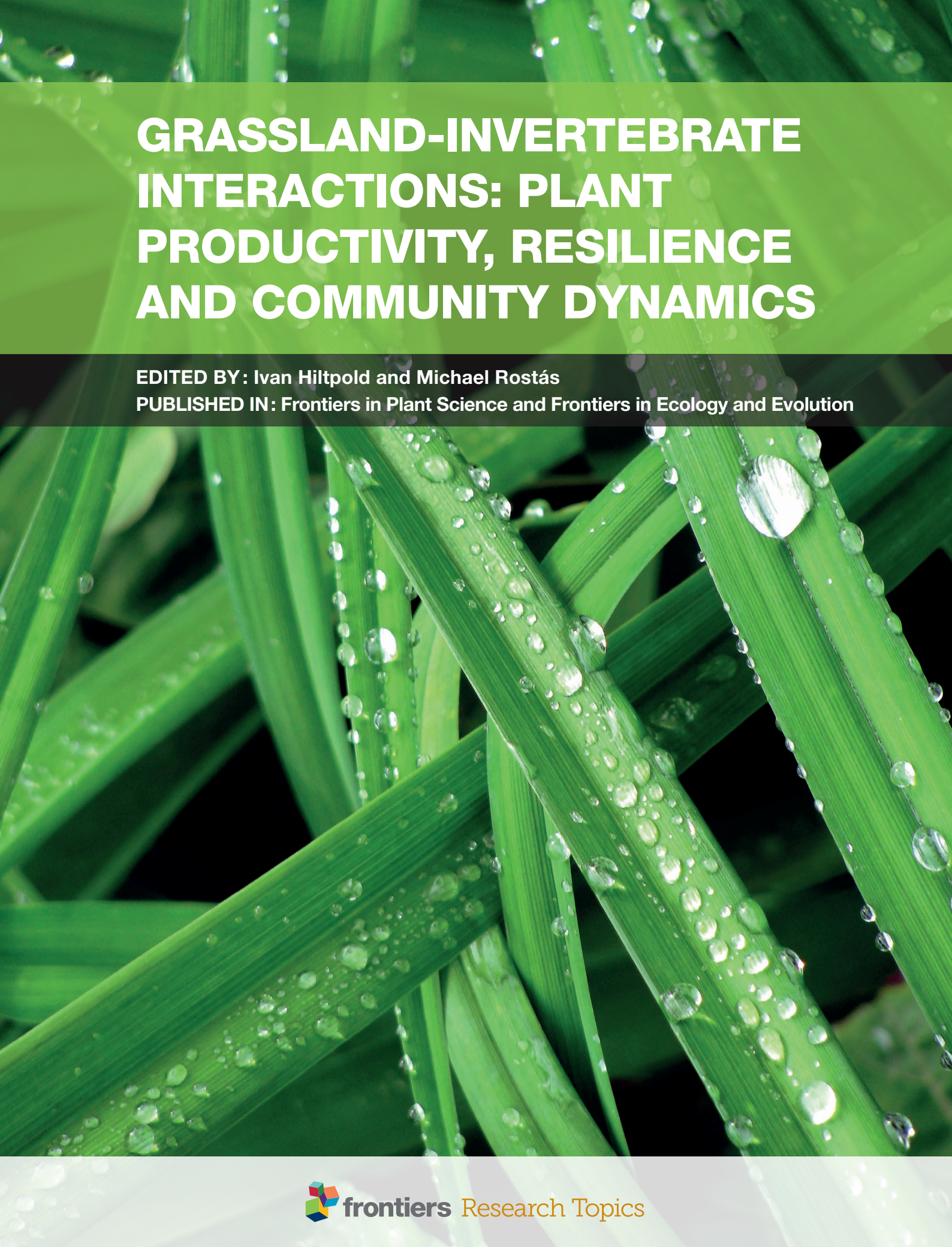


GRASSLAND-INVERTEBRATE INTERACTIONS: PLANT PRODUCTIVITY, RESILIENCE AND COMMUNITY DYNAMICS

EDITED BY: Ivan Hiltpold and Michael Rostás

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GRASSLAND-INVERTEBRATE INTERACTIONS: PLANT PRODUCTIVITY, RESILIENCE AND COMMUNITY DYNAMICS

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Ryegrass
Credit: Mohammad Mohammadi

Natural and anthropogenic grasslands such as prairies, meadows, rangelands, and pastures cover more than 40% of the planet's surface and provide a wealth of ecological services. Grasslands alone store one third of the global carbon stocks and grass roots, through their specific architectures, ensure water cycling and prevent the erosion of fertile topsoil. In addition, grasslands are of vital importance for human food production as vast areas of rangelands and pastures provide feed for livestock. Pastoral legumes mobilize atmospheric nitrogen and improve fertility of arable soils. Not least, grasslands are an essential genetic resource. The three major crop species that feed half of the global population have been bred from wild grasses. Ancestors of our contemporary turf cultivars, common components of urban landscapes and recreation spaces, originated from wild grasslands.

Although natural and managed grasslands represent pivotal ecosystems, many aspects of how they function are poorly understood. To date, most attention has focused on grassland primary producers (i.e. forage plants) and mammalian grazers but invertebrates are likely to play an equally, if not more important role in grassland ecosystem functioning. In Australian pastures, for example, the biomass of root-feeding scarab beetles can often exceed that of sheep and plant damage caused by invertebrates is sometimes equivalent to an average dairy cow's grass consumption. Indeed, grasslands are one of the most densely populated ecosystems with invertebrates being probably the most important engineers that shape both plant communities and the grassland as a whole. In a rapidly changing world with increasing anthropogenic pressure on grasslands, this Research Topic focuses on:

1. How grassland habitats shape invertebrate biodiversity
2. Impacts of climate change on grassland-invertebrate interactions

3. Plant and invertebrate pest monitoring and management
4. Plant-mediated multitrophic interactions and biological control in grasslands
5. Land use and grassland invertebrates
6. Plant resistance to invertebrate pests

Given the increasing demand for food and land for human habitation, unprecedented threats to grasslands are anticipated. Resilient to some extent, these key ecosystems need to be better comprehended to guarantee their sustainable management and ecosystem services.

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EDITORIAL
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Editorial: Grassland-Invertebrate Interactions: Plant Productivity, Resilience and Community Dynamics

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Keywords: biological control, biosecurity, climate change, community ecology, pests

Editorial on the Research Topic

Grassland-Invertebrate Interactions: Plant Productivity, Resilience and Community Dynamics

Grasslands are plant communities dominated by non-woody vegetation, in particular species of the Poaceae family. Such communities occur naturally from the tropics to the tundra in areas of low rainfall where soils do not hold enough moisture to support forest growth. Where climatic conditions fail to support natural grasslands, however, they exist due to more or less intensive farming practices and are known as unimproved (= semi-natural) or improved grasslands (Curry, 1994). Grassland types differ significantly in biodiversity and productivity (Kruess and Tscharntke, 2002); the latter being characterized by intensive management, including reseeding, fertilization, and irrigation to encourage the growth of only few plant species with particular value for grazing livestock. Natural and semi-natural grasslands, on the other hand, are often habitats to many rare plant and invertebrate species that depend on no or low-intensity farming, respectively. Natural and anthropogenic grasslands cover more than a quarter of the earth's surface and provide a wealth of ecological services. Grassland ecosystems store a third of the global carbon stocks, ensure water cycling, and are vital for human food production (Gibson, 2009). Invertebrates play major roles in such ecosystems as they contribute to soil fertility, plant growth, pollination, and biological control on the one hand but cause considerable economic loss through herbivory on the other.

This research topic reports new findings and concepts on grassland–invertebrate interactions in semi-natural and improved grasslands with emphasis on the effects of climate change, invasive species, and sustainable control methods of invasive pests. Five reviews, one opinion paper, two methods, and fourteen research articles explore the influence of biotic and environmental factors and management practices on the communities of invertebrates and their relationships with plants and natural enemies. The majority of contributions is dedicated to Australian and New Zealand grassland systems resulting from an invitation to the participants of the ninth Australasian Conference on Grassland Invertebrate Ecology held in Sydney in April 2016. Several studies on invertebrate communities in European grasslands complement our Topic.

What drives the diversity and distribution of grassland invertebrates and what role do agricultural management practices play? This question is explored in a number of papers such as the one by Kergunteuil et al. who investigate the nematode fauna in Swiss alpine meadows. Surprisingly, and in contrast to aboveground ecosystems, the study shows that the abundance and diversity of nematodes increases along an elevation gradient, which suggests a more important role for nematodes in the functioning of high-altitude alpine grasslands than previously anticipated. The distribution of soil invertebrates is also the focus of Benefer et al. albeit in low-land permanent pastures dominated by perennial ryegrass (*Lolium perenne* L.). Their work demonstrates that spatial

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scale is an important factor in describing species distribution. Focussing on the aboveground community of invertebrates, Fournier et al. analyse the rules that determine the co-occurrence network of orthopterans (grasshoppers and crickets) and plants in semi-natural grasslands of the French Jura Mountains. Such networks have a modular structure and the distribution of orthopterans into modules results from trophic and other interactions with plants. The presented models are valuable for biodiversity conservation and will allow for predictions on how such networks will be affected by changes in agricultural practices. Impacts of such practices on grassland invertebrate communities are the focus of a study by Liu et al. who show that herbicide spraying, plowing, and reseeding of permanent grassland can have opposing effects. While herbicide treatment tends to increase soil invertebrates, in particular decomposers due to enhanced food supply, a decrease in abundance can be found after plowing, which constitutes a significant disturbance. Interestingly, most populations were able to recover over a period as short as 1 year after reseeding.

What are the cascading effects of climate change on invertebrate communities in semi-natural and improved grasslands? In addition to agricultural intensification, grasslands, and their invertebrate communities need to cope with rising CO_2 levels, temperature, and changes in precipitation. How prognosed precipitation patterns affect grassland insects is reviewed for the first time by Barnett and Facey. The authors constitute that overall the effects on invertebrates, caused indirectly by changes in plant biomass and diversity, are highly idiosyncratic and dependent on the grassland ecosystem under scrutiny. This conclusion comes with a recommendation for further experiments in this under-studied area of research, which should consider multiple climate factors at the same time, thus reflecting the complex reality of climate change. This notion is reiterated in an Opinion paper by Johnson et al. which furthermore analyses the inherent problem of pseudoreplication in climate change experiments and offers advice on how this can be dealt with. Better results from climate change studies was also the motivation for designing an improved experimental platform to examine ecosystem responses to drought and root herbivory. The so-called DRI-Grass (**D**rought and **R**oot Herbivore **I**nteractions in a **Grass**land) system described by Powers et al. consists of rain-exclusion shelters with a sophisticated irrigation system that measures local rainfall and responds by delivering water in specific proportions of the actual amount with the effect of realistically mimicking natural precipitation patterns. Using the DRI-Grass platform, Torode et al. take a close look at plant and invertebrate community responses, above- and belowground and across a range of expected rainfall scenarios. Their findings suggest that summer drought, in particular, may favor outbreaks of sucking herbivores, probably followed by a density-dependent response in parasitoid abundance. Ryalls et al., in a similar setting, aim at teasing apart the complex interactions between above- and belowground herbivores in a grass-legume model system when exposed to altered precipitation patterns. Interestingly, in this case drought and root damage by weevils lead to decreases in aphid numbers, thus underpinning Barnett and Facey's conclusions about the idiosyncratic and system-specific nature of such interactions.

How do invasive invertebrates affect grassland diversity and functioning, and how can these invaders be sustainably managed to mitigate their impact on grasslands? This topic is covered by a third series of papers which focuses in particular on management strategies involving biotic resistance factors. To start with, Frew et al. give an overview of economically important pest species of the Scarab family in Australasia. Their timely review presents basic information on a group of beetles, whose larvae (grubs) cause significant damage by feeding on the roots of pasture plants. A range of abiotic and biotic soil factors as well as plant traits are explored that influence oviposition by adult beetles, larval behavior and survival, and ultimately population dynamics. Three further scarab papers discuss only a single species, the African black beetle *Heteronychus arator*, an invasive grub which is of particular concern to farmers in several countries. Mansfield et al. focus on the dispersal behavior of *H. arator* and address the question why control measures such as insecticide treatment have failed to reduce population levels. Their findings support the hypothesis that African black beetles re-colonize areas of low density by walking and pitfall traps are confirmed as a valuable monitoring tool. An improved method for rearing *H. arator* in the laboratory is presented by Hiltpold et al. which allows *H. arator* to be reared from egg to adult. The protocol may also be useful as a template for rearing other root herbivores, thus facilitating future studies in this important area as research of root–herbivore interactions is often hampered by the availability of the herbivore. Karpyn Esqueda et al. finally give us a broad historical account of improved grassland development in Australia, the establishment of *H. arator* as a major pest and discuss the use of endophytic fungi that produce insect-deterring toxins, as a control method.

Hennessy et al. focus on such grass endophytes (i.e., symbiotic fungi in the genus *Epichloë*) and the impact on insect herbivores. Their chemo-ecological study demonstrates the anti-feedant effect of fungally produced epoxy-janthitrems against caterpillars of *Wiseana* spp. and assesses the role of temperature on the production of these secondary metabolites. Studying several endophyte strains with different metabolic profiles, Popay and Cox confirm the beneficial effects of epoxy-janthitrem producing *Epichloë festucae* Tul. & Tul. against root aphids. Novel endophyte technology, which is based on using selected strains with specific metabolite profiles, is a sustainable control method against insect pests that is well-established in New Zealand and Australia. Bell et al. also highlight the importance of fungi as natural enemies of pasture pests. Using a combination of next-generation sequencing and bioassays, root nematode catching Orbiliomycetes fungi were identified as potential biocontrol agents from suppressive soils. Studies as the above are promising and will ultimately lead to better control of hard-to-tackle soil herbivores.

Adults and larvae of weevils (Curculionidae) are major pasture pests that can feed above- and belowground. In a multi-year study by McNeill et al., the question is raised whether abundance of the host plant *Trifolium repens* L. determines population density of the weevil *Sitona obsoletus* Gmelin. While

such a correlation is absent, mortality caused by introduced parasitoids seems to regulate weevil populations. In another weevil study Goldson and Tomasetto aim at elucidating the mechanism behind the observed decline in the initially successful biological control of *Listronotus bonariensis* Kuschel by its parasitoid *Microctonus hyperodae* Loan (Tomasetto et al., 2017). Their laboratory study suggests that the weevil has acquired host plant dependent resistance against its natural enemy, leading to the conclusion that low plant and enemy diversity in agriculture may facilitate the evolution of host resistance. Barratt et al., on the other hand, assess the potential impact of *L. bonariensis*, a pest of improved grasslands, on natural grassland ecosystems and are able to show that the weevil, although present, is not a significant threat to native grass species.

Apart from insects, invasive plants also pose a serious threat to natural and agricultural grasslands, and in many cases specialized herbivorous arthropods such as the thistle leaf beetle, *Cassida rubiginosa* Müller, have been introduced as weed control agents. Cripps et al. examine the evolution of host plant specialization in this beetle and present work that uses a quantitative measure of evolutionary separation between hosts to predict herbivore performance. Studies like these contribute to our understanding of contemporary evolution in novel environments and will aid in predicting non-target risks and host range expansion of biological control agents.

What makes grasslands resilient to invertebrate threats and community changes? Two review papers finally highlight the capacity and need for resilience in grassland ecosystems. Moore and Johnson present a thorough survey of the many physical and chemical resistance mechanisms that grasses have evolved against insects and draw our attention to the roots as little is known of belowground defenses in grasses. Strengthening these plant resistance mechanisms in order to deal with new invasive pests is also one of the main recommendations by Goldson et al. Their analysis on pasture biosecurity in New Zealand comes to this conclusion because pre-border controls are deemed less effective for pastures compared with other agricultural sectors due to a range of inherent constraints.

In this Research Topic, we have covered a broad range of themes with an emphasis on the invertebrate fauna of managed but also semi-natural grasslands, the abiotic and biotic factors that affect their dynamics, and some of the control measures that have the potential to provide ecologically and economically sustainable plant protection. Although grasslands are pivotal ecosystems, several aspects of their ecology are still elusive. With increasing anthropogenic pressure on fragile natural and semi-natural grasslands and the need for sustainable management of improved grassland systems, it is of paramount importance to better comprehend their complexity and functioning in order to conserve these resources. We are confident this compilation of papers will be a valuable resource for researchers and others interested in grassland ecology.

AUTHOR CONTRIBUTIONS

MR wrote the first draft with substantial contributions from IH. Both authors jointly edited successive versions and approved it for publication.

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ORIGINAL RESEARCH
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The Abundance, Diversity, and Metabolic Footprint of Soil Nematodes Is Highest in High Elevation Alpine Grasslands

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Nematodes are key components of soil biodiversity and represent valuable bio-indicators of soil food webs. Numerous community indices have been developed in order to track variations in nematode-mediate soil ecosystem processes, but their use is mainly restricted to anthropogenic stresses. In this study, we propose to expand the use of nematodes' derived ecological indices in order to shed light on variations of soil food webs in natural systems distributed along elevation gradients. For this purpose, we aimed at determining how elevation affects the community structure and the trophic diversity by studying the abundance, the composition and the functional diversity of nematode communities. Nematode communities were sampled every 200 m across five transects that span about 2000 m in elevation in the Alps. To understand the underlying ecological parameters driving these patterns we studied both abiotic factors (soil properties) and biotic factors (trophic links, relationships with plant diversity). We found that (1) nematode abundance increases with elevation of lowland forests and alpine meadows; (2) differences in nematodes communities rely on habitat-specific functional diversity (e.g., tolerance to harsh environments, "colonizer/persister" status) while most trophic groups are ubiquitous; and (3) the metabolic footprint of the complete nematode community increases with elevation. We thus conclude that the contribution of soil dwelling nematodes to belowground ecosystem processes, including carbon and energy flow, is stronger at high elevation. The resulting cascading effects on the soil food web structure are discussed from an ecosystem functioning perspective. Overall, this study highlights the importance of nematodes in soil ecosystems and brings insights on their functional role along ecological gradients.

Keywords: elevation gradient, entomopathogenic nematodes, nematophagous fungi, plant-herbivore interaction, soil ecosystem functioning

INTRODUCTION

It has been estimated that under the earth's surface, the myriad of soil habitats shelter about 25% of the worldwide described species, thus providing crucial reservoirs of biodiversity and subsequent ecosystem functioning (Fitter et al., 2005; Decaëns, 2010; Bardgett and van der Putten, 2014). While research on soil biota continues to bear inherent challenges, the combination of traditional research with genomic tools has accelerated the exploration of soil diversity and our understanding of ecosystem dynamics (Johnson et al., 2007). In addition, numerous studies have become increasingly focused on replacing soil diversity within trophic interactions for unraveling soil ecosystem processes (Bardgett and van der Putten, 2014). Indeed, soil fauna is essential for ecosystem functioning through different processes, such as primary production and nutrient cycling of carbon, phosphorous, or nitrogen (Brussaard, 1997). The role of soil functional diversity in the decomposition of organic matter and, more importantly, in the assimilation of carbon in food webs, governs energy flows worldwide (Hunt and Wall, 2002; Krumins et al., 2013).

Several groups of soil-dwelling organisms (e.g., bacteria, fungi, protists, collembolan, enchytraeid worms or earthworms) can partition their task in order to optimize trophic interactions and energy flow. In addition, among soil inhabitants, the group of roundworms (i.e., nematodes; phylum Nematoda) is a key component of the belowground living mosaic. Indeed, nematodes, with more than 14,000 described species, are distributed in almost every habitat on Earth, and represent more than 80% of metazoan taxonomic and functional diversity in soils (Bongers and Bongers, 1998; Hodda et al., 2009). Nematodes can be assigned to basically all functional trophic guilds, and span the whole gamut of ecological adaptations, ranging from "colonizer" (r strategists) to "persister" (K strategists) along a colonizer-persister ("cp") scale (Bongers, 1990). Besides the diversity in life history traits, nematodes sustain a large range of trophic groups and eight feeding types have been described: herbivore, fungivore, bacterivore, substrate ingester, predator of animals, unicellular eukaryote feeder, parasites, and omnivore (Yeates et al., 1993). The combination of both cp groups and feeding habits provides a wide diversity of functional guilds. In addition, nematodes occupy a central position in soil food-webs by linking microbial communities with macrofauna. Hence, nematodes are widely used as appropriate bioindicators to track changes in the environment and the resulting cascading effects on soil food-web structure (Sochová et al., 2006; Wilson and Kakouli-Duarte, 2009). Several community and metabolic footprints indices have been developed in order to assess how nematode communities affect (or are affected by) soil quality (Bongers and Ferris, 1999; Ferris et al., 2001; Ferris, 2010), although such studies remain mostly restricted to anthropogenic systems (Salamún et al., 2014; Zhao et al., 2015). Here, we propose to expand the use of nematodes' derived ecological indices for increasing our understanding soil-driven ecosystem functioning along natural ecological gradients.

Studying the causes and consequences of species abundance and distribution along environmental clines remains crucial for providing insights into community assembly and ecosystem functioning (Gaston, 2000; Doherty et al., 2011; Oliver et al., 2015). In this context, ecological gradients act as potent environmental filters and thus provide powerful tools for dissecting biotic and abiotic factors driving species diversity and ecosystem dynamics. For instance, elevation gradients have been classically used to develop key ecological concepts such as the niche theory or the species-energy hypothesis (Grinell, 1917; Brown, 1971; Lomolino, 2001). More recently, various authors have considered elevation gradients as promising "natural experiments" to test evolutionary hypotheses in species niche-breadth or predict plant adaptation to changing environment (Körner, 2007; Alexander et al., 2015; Rasmann and Pellissier, 2015). Indeed, mountain slopes present strong variation in both biotic and abiotic factors that can to alter ecological niches, abundance in species population, or community assemblage (Hodkinson, 2005). In addition, biotic variations occur over short distances, thereby limiting the confounding effect of phylogeography when studying inter-specific interactions from a comparative ecology approach (Rasmann et al., 2014). While, numerous studies have demonstrated the ability of nematodes to colonize the harshest environments, such as the polar regions (Loof, 1971; Yeates, 2010), only few studies have been interested in studying their distribution along elevation, and to our knowledge, none of them have assessed changes in nematode communities along continuous elevation gradients (Hoschitz and Kaufmann, 2004).

With the present work, we aimed at unraveling the community ecology of soil-dwelling nematodes along steep elevation gradients, from the colline regions up to the Alpine grasslands. Specifically, we hypothesized: (1) a decrease in nematode abundance at high elevation following classic views on biodiversity gradients (McCain and Grytnes, 2010); (2) changes in the nematode communities' structures according to variations of ecological niches along the gradient; and (3) changes in the nematode-mediated metabolic footprint indices along the elevation gradient of the Alps.

MATERIALS AND METHODS

Study Site

To dissect nematode food web structure along elevation gradients, between July and August 2013, we sampled soils ranging from 700 m above sea level (asl) up to 2700 m asl across five transects in the Swiss Alps (Figure S1). The five transects were collected over 130 km in order to assess variations in nematode communities over a large scale in Alpine systems. Along each elevational transect, sampling sites of 2 × 2 m were chosen approximately separated from each other by an elevation of 200 m asl ($n = 48$ sites, Table S1). As we aimed to measure soil diversity in the most pristine conditions, we sampled within the climacic vegetation at each site (Delarze et al., 2015). In lowlands, soil samples were predominantly collected within *Fagus sylvatica*, *Quercus* spp., or *Castanea sativa* dominated forests. Sites in the mountain and the subalpine belts were mainly collected in *F. sylvatica*, *Pinus sylvestris*, *Abies alba,* or *Picea abies* dominated

forests, while sampling in the alpine zone was done in Alpine grasslands found above the timberline (Figure S1, Table S1). All along the elevation gradient we avoided cultivated, urban, or heavily grazed areas and selected sites with a similar exposition and slope for a same transect.

Sampling sites of three transects out of the five (i.e., 28 sites along the Mont d'Or, Salgesch, and Vallon de Nant transects, Table S1) were selected according to the study conducted by Pellissier et al. (2010), who described plant communities (abundance of plant taxa based on Braun-Blanquet categories) within a 40 m^2 (grasslands) or 250 m^2 (forests) quadrat at each site (Vittoz and Guisan, 2007). Hence, over three transects, each site was also described with the corresponding plant species list and plant cover estimation for each species. This allowed testing for potential spatial correlations between nematode species and the local flora (see below).

Soil and Nematode Sampling

At each site, we randomly collected 10–30 soil cores of 5 cm diameter within a 2 × 2 m area and with a maximal depth of 30 cm till we reached a total of 1.5 Kg of soil after the removal of all rock particles bigger than 2 cm in diameter. The bulk soil was homogenized before dividing it into several subsamples.

Initially, 300 g of the bulk soil were used for measuring soil traits (soil humidity, pH, conductivity and root fraction, i.e., percent root biomass). For soil humidity, we calculated the difference between soil fresh weight and soil dry weight after 7 days at 70°C. Both pH and conductivity were measured using a 914 pH/Conductometer (Metrohn, Herisau, Switzerland) after mixing 50 g of this subsample with 100 ml of deionized water. Finally, under the microscope, we visually separated all discernible root fragments from the other soil components and weighed them to obtain the proportion in percent of root biomass, calculated as the ratio of root biomass on total soil mass.

Next, out of the initial soil bulk, a sub-sample of 200 g of fresh soil was used for extracting soil nematodes: bacterivores, fungivores, herbivores, predators, and omnivores. For this purpose, we used the sieving and Baermann funnel method (Barker, 1985). All free-living nematodes in each sample were then counted under the dissecting microscope and mounted into a slide. At least 100 nematodes in each sample were then identified under a dissecting microscope to family or genus level, and assigned to a functional guild based on their trophic group and life-histories (Yeates et al., 1993).

Finally, in order to improve the description of nematode communities, we also performed targeted genomic approach on 200 additional grams of fresh soil to identify entomopathogenic nematodes (EPNs; i.e., nematodes parasites of invertebrates that are only free-living in the soil as the third instar infective juvenile state), and nematophagous fungi (NF) which are important in shaping nematode communities but remain virtually impossible to distinguish under a dissecting microscope. Using species-specific primers/probe and quantitative real time PCR procedures, we screened 13 EPNs species and 6 NF according to well-established methods (Atkins et al., 2005; Zhang et al., 2006; Torr et al., 2007; Campos-Herrera et al., 2011a,b, 2012, 2015; Pathak et al., 2012; Table S2). The procedures for the establishment of the pure cultures of the organisms used as positive controls, and the protocols followed for the DNA extraction and the standard curves design were performed following Campos-Herrera et al. (2015). Briefly, EPNs and NF were collected using the sucrose extraction method (Jenkins, 1964), recovering the nematodes in a sieve of 25 μm mesh. The DNA from both the known quantities of the target organisms and the field samples were extracted by Power Soil® DNA Isolation Kit (MoBio laboratories, Inc., protocol for maximum yield, see Campos-Herrera et al., 2015). Details of the concentration and protocols for all the target species were described by Campos-Herrera et al. (2015). We employed a 10-fold dilution for the enumeration of nematodes in the qPCR reactions, whereas, for NF used the total DNA with no dilution. All the organisms quantified by qPCR were expressed as per 100 g of dry soil. In addition, we estimated the NF relative biomass rate by dividing the NF DNA quantity of each species by the total amount of DNA (de Rooij-van der Goes et al., 1995; Campos-Herrera et al., 2012, 2015; Duncan et al., 2013).

Statistical Analyses

All statistical analyses were performed with R software, version 3.2.2 (R Core Team, 2015).

Soil Traits

The correlations between the four soil traits recorded (soil humidity, pH, conductivity, and root fraction) and the elevation were individually tested through Pearson's coefficient. Those relationships were plotted in Figure S2 using either non-linear regressions (package "nls2": Grothendieck, 2013) or mixed linear regressions (package "lme4": Bates et al., 2015) with "elevation" as fixed factor and "transects" as random factor.

Abundance and Species Diversity of Nematodes along Elevation Gradients

The total number of nematodes was expressed as number of individuals per 100 g of dry soil and the Simpson diversity index (D) was calculated as a measure of nematode diversity using the package "vegan" (Oksanen et al., 2015). Mixed linear regressions (package "lme4": Bates et al., 2015) were used to analyze the total number of nematodes, the diversity of nematode communities and the infestation rate of nematophagous fungi along elevation gradients, using "elevation" as fixed factor and "transects" as random factor.

Nematode Community Structure along Elevation Gradients

In order to describe the structure of nematode communities (composition and abundance of nematode taxa) along elevation gradients, we performed a partial least square discriminant analysis, PLS-DA (package "mixOmics"; le Cao et al., 2015). The PLS-DA is well suited for dealing with a large number of variables (47 taxa of nematodes) across a limited number of samples (43 sites). Before the analysis, a logarithmic transformation was applied to the data to improve the symmetric distribution of the variables and one outlier was removed from the original dataset based on the initial score plot. In order to plot a multivariate

analysis appropriately interpretable, the final model retained was computed with the four soils traits measured and the 19 taxa capturing the most of the variations between nematode communities' structures across elevation zones (i.e., the 19 taxa with a variable importance in the projection, VIP, superior to 1).

Association between Nematode Communities and Plant Communities

First, we constructed plant community structure along the three transects where floristic inventories were conducted by performing a second PLS-DA on the 100 plants with a VIP superior to 1 (results not shown; LV1 = 40%, LV2 = 38% of intergroup variance).

Second, we assessed the correlations between nematode distribution (presence and abundance) and local flora (presence and cover percentage based on Braun-Blanquet categories) across sites using two hierarchical clusterings (package "pvclust": Suzuki and Shimodaira, 2015; the 19 taxa of nematodes and the 100 plants with a VIP superior to 1 were retained). For both clustering, a dendrogram was created using the Ward agglomerative method applied on a distance matrix computed from correlation method. Bootstrap replications were set to 10,000 and stable clusters with a significant approximatively-unbiased (AU) *p*-value were highlighted (significance level 0.05). Significant correlations between nematode and plant taxa across these two dendrograms were indicated by different segments (Spearman's rank correlation test, $\alpha = 0.05$; *p*-values adjusted by the Benjamini and Hochberg method: Benjamini and Hochberg, 1995).

Variation in Nematode Trophic Function along Elevation Gradients

In order to assess the functional role of nematode-based soil food webs along elevation gradients, we calculated several indices, which are based on the abundance of functional guilds of nematodes (Bongers and Bongers, 1998; Ferris et al., 2001; Table S3).

For this purpose, first, all identified nematodes were classified into the main five trophic habits (bacterial-feeders, fungal-feeders, plant-feeders, omnivores, and predators; Yeates et al., 1993), and along the colonizer-persister (cp) scale (Bongers, 1990). The colonizer-persister (cp) scale classifies nematode families into five groups (from 1 to 5) reflecting the life-history characteristics similarly to the r/K scale (Bongers and Bongers, 1998). Nematodes belonging to the cp 1 group are fast-growing, bacterivore enrichment-opportunistic nematodes, which increase their population fast after soil enrichment processes; nematodes belonging to cp 2, cp 3, and cp 4 groups present progressively longer life cycles and are more sensitive to environmental perturbation. Nematodes in groups 4 and 5 are in general predators and omnivores, K-strategists, very sensitive to soil perturbation.

Next, we calculated five nematode-based ecological indicators: (1) the sigma-maturity index (ΣMI; Bongers, 1990), (2) the maturity index (MI; Bongers, 1990), and (3) and the plant-parasitic index (PPI; Bongers, 1990). These first three indices represent the proportions of the different cp groups for the whole nematode community, the free-living nematodes, and the plant-parasitic nematodes, respectively. For all three, a higher value indicates that nematodes harboring "persiter" life history traits are predominant within each of those different nematode categories. (4) The enrichment index (EI; Ferris et al., 2001) is based on the biomass of opportunistic nematodes that respond rapidly to the increase of bacterial and fungal populations that arise from organic matter decomposition. High values indicate high soil enrichment and high fertility. Finally, (5) the channel index (CI; Ferris et al., 2001) is the ratio between the biomass of fungivore to bacterivore nematodes, and greater values indicate that fungal decomposition (the fungal "channel") predominates over bacterial decomposition for a given site. For specific calculation of each index see equations provided in Supplementary Materials (Equations 1–3).

In addition to the five nematode community indices described above, we also calculated the metabolic footprints (MF) according to the equation developed by Ferris (2010), and using the Nematode Joint Indicator Analysis tool (Sieriebriennikov et al., 2014; https://sieriebriennikov.shinyapps.io/ninja/; see the Equation 4 provided in Supplementary Materials). The MF balances the mass of carbon used by nematodes for both production (growth and egg production) and respiration (metabolism activities) components. MF can be computed either for specific functional trophic guilds or for the whole nematode community. In this later case, the so-called "composite MF" represents an indicator of the energy flow channeled by nematodes in general within soil food webs. High composite MF suggests that nematode assemblage store high amount of soil carbon (Ferris, 2010).

The effect of elevation on all nematode community indices and MF was tested using mixed linear models (package "lme4": Bates et al., 2015) with "elevation" as fixed factor and "transect" as random factor.

RESULTS

Soil Traits

Elevation was correlated with soil moisture (Figure S2A; $r = 0.38$; $t = 2.75$, $df = 46$, $P = 0.008$), conductivity (Figure S2B; $r = -0.44$; $t = -3.37$, $df = 46$, $P = 0.002$), root fraction (Figure S2D; $r = 0.66$; $t = 5.99$, $df = 46$, $P < 0.001$), while no correlation was observed for the pH (Figure S2C; $r = -0.09$; $t = -0.64$, $df = 46$, $P = 0.526$). Both the root fraction and the soil humidity increased along the altitudinal gradient: the root fraction was multiplied by 4 between locations sampled under 900 m and those sampled over 2000 m while the humidity ranged between 27 ± 3 and $35 \pm 2\%$ over these two elevation levels. On the contrary, soil conductivity sharply decreased with elevation from 1772 ± 78 mS.m^{-1} under 900 m to 1478 ± 35 mS.m^{-1} over 2000 m.

Abundance and Species Diversity of Nematodes along Elevation Gradients

Overall, we extracted 34.752 nematodes from the 48 soil samples collected along the five transects. Nematodes were assigned to 44 genera or three additional families when the identification

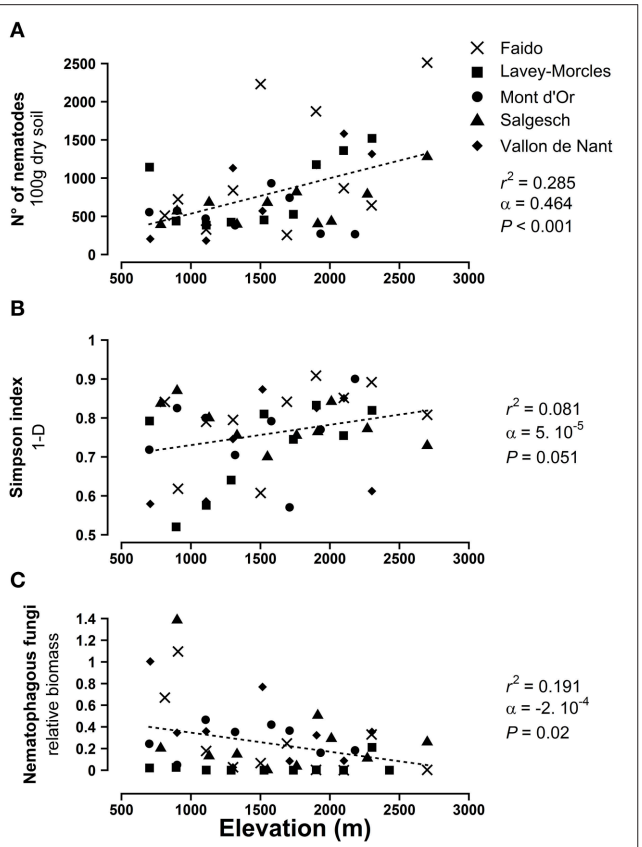


FIGURE 1 | Effect of elevation on (A) total number of nematodes, (B) nematode species diversity, and (C) infestation rate of nematophagous fungi. Shown are the sampling sites beloning to five mountain transects as shown in Figure S1. The dashed lines represent the predictions of the mixed linear models. r^2, coefficient of determination; α, regression slope; *P*, significance of the regression slope.

of the genus was uncertain. The total number of nematodes increased along elevation gradient (**Figure 1A**). Although the specific richness was not affected by elevation (LMM, $\chi^2 = 0.61$, $df = 1$, $P = 0.44$), the simpson index measured on nematode communities and taking into account taxa abundance was positively correlated with elevation (**Figure 1B**). Finally, the increase in both nematode numbers and biodiversity up along the mountain slope was coupled with an important drop in the relative biomass of nematophagous fungi (**Figure 1C**).

Beyond the general increase in nematode population along mountain clines, all the trophic groups of nematodes also increased, except predators and parasites, i.e., entomopathogenic nematodes (**Figure 2**).

Nematode Community Structure along Elevation Gradients

The composition of nematode communities also differed along elevation gradients (**Figure 3**). The two axes of the PLS-DA retained for the projection explained 57 and 21% of the inter-group variance (**Figure 3A**). Over the two axes, we were able to discriminate clusters of nematode communities based on elevation zones. Particularly, communities observed between 1500 and 2000 m and those collected above 2000 m were clearly separated from each other (**Figure 3A**). As shown in **Figure 3B**, nematode communities at low elevations, i.e., under 1500 m, were mainly characterized by the presence of *Tripyla*, *Alaimus*, *Wilsonema* and *Cervidellus*. *Tripyla,* and *Alaimus* genera were scarcely present between 1500 and 2000 m and completely absent at higher elevations while *Wilsonema* and *Cervidellus* remained at high elevation but in a much lower abundances. The genera *Aphelenchoides*, *Plectus*, *Prodorylaimus,* and *Mesodorylaimus* mostly dominated the nematode communities between 1500 and 2000 m. *Prodorylaimus* and *Mesodorylaimus* were not present in soil originating from other elevational levels. Among soil traits, soil humidity was driving intermediate elevation communities. Over 2000 m, four nematode genera were found in high abundance; three of them (*Eudorylaimus*, *Paratylenchus*, *Teratocephalus*) were also recorded at lower elevations, although in lower abundance, while *Pratylenchus* was only collected over 2000 m. These high elevation communities were strongly associated with high root fractions in soils.

Association between Nematode Communities and Plant Communities

We next assessed the spatial correlations between nematode and plant communities along elevation gradients. First, the dendrogram based on the distance matrix analysis of plant communities inventoried across three transects indicated a strong clustering of plants according to three elevational levels (**Figure 4**). Plants characterizing low (i.e., under 1500 m) and intermediate elevations (i.e., between 1500 and 2000 m) were grouped in two single clusters, while plant communities sampled over 2000 m were split in four stable clusters. Second, the dendrogram based on nematodes communities showed a broader, less structured distribution in relation to elevation. Nevertheless, the eight taxa characterizing high and low nematode communities were separated in two single clusters of the dendrogram, while the four nematode's taxa specific to intermediate altitudes were more largely spread across the classification tree.

A total of 44 significant correlations between nematode and plant taxa were observed (**Figure 4**). These correlations were homogenously distributed across the different trophic groups of nematodes even if 3 herbivore genera, mainly due to *Pratylenchus*, accounted for almost half of these correlations. Indeed, this genus characterizing alpine communities was related to 15 plant species, most of them typical to high elevations. *Prodorylaimus*, an omnivore genera particularly abundant in soils collected at intermediate elevations was correlated to six rather uncommon plant species in our dataset belonging to mesotrophic to eutrophic plant communities. In the same elevation zone, *Mesodorylaimus* was surprisingly correlated with *Cuscuta epithymum*, a parasite plant species. Finally, two nematode taxa associated to low elevation communities, *Tripyla* and *Cervidellus*, were related with only one plant.

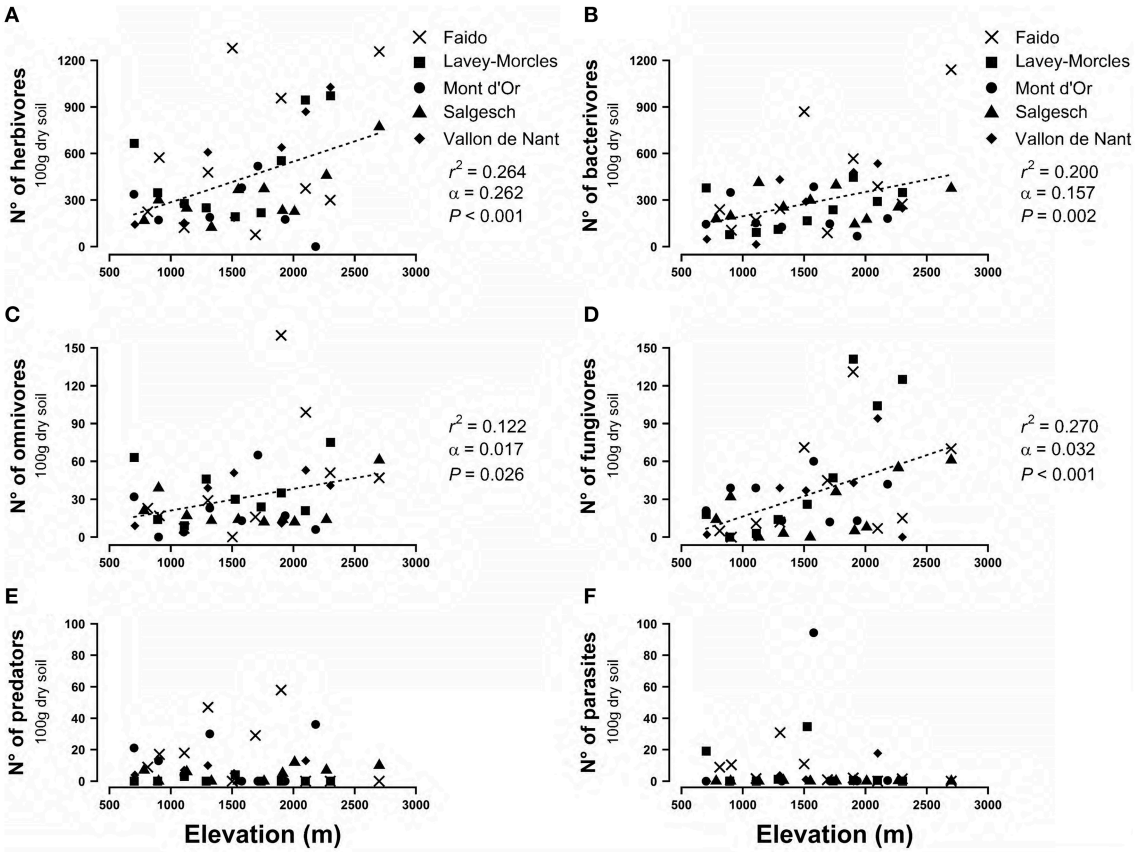


FIGURE 2 | Effect of elevation on the abundance of nematode's trophic group, (A) herbivores, (B) bacterivores, (C) omnivores, (D) fungivores, (E) predators, (F) parasites (i.e., entomopathogenic nematodes). Shown are the sampling sites beloning to five mountain transects as shown in Figure S1. When the mixed linear model is significant, the dashed lines represent the predictions of the models. r^2, coefficient of determination; α, regression slope; *P*, significance of the regression slope.

Variation in Nematode Trophic Function along Elevation Gradients

We recorded an increase in the relative abundance of colonizer to persistent nematodes up along the transects, as showed by an increase of the sigma maturity index (ΣMI) with elevation ranging from 2.21 ± 0.06 under 900 m, up to 2.43 ± 0.07 for soils collected above 2000 m (Figure S3A). This elevation pattern relied mainly on an increase of the PPI at high elevation (Figure S3C), while elevation did not affect the MI (Figure S3B). These results indicate that more persistent nematodes are found at high elevation, mainly due to the high abundance of plant-parasitic nematodes.

As shown in **Figures 5A,B** the EI informing about the relative abundance of opportunistic nematodes was stable with elevation but the CI increased. This indicates greater relative biomass of fungivore nematodes at high elevation. The CI was multiplied by three to reach 38.11 ± 11.06 in soils located over 2000 m compared to soils under 900 m. Finally, the composite MF clearly increased with elevation, indicating that the amount of carbon entering the soil food webs from nematode food sources is progressively higher at higher elevations (**Figure 5C**).

DISCUSSION

The decrease in species diversity along elevation gradient is a common feature for aboveground ecosystems. On the contrary, our results indicate that nematode communities become more abundant and richer at high elevation within the range included in this study (700–2700 m asl). In addition, a steady increase in the composite metabolic footprint (MF) of nematode communities along elevation gradient indicates that nematodes sustain greater part of the soil's energy flow at high elevation. Hence, this study brings novel insights on the role of soil fauna driving soil ecosystem functioning along elevation gradients.

Abundance and Species Diversity of Nematodes along Elevation Gradients

Species richness generally decreases with elevation for most of the organisms studied across a broad range of taxonomic groups, including soil fauna like mites (Chapin and Körner, 1995; Nagy et al., 2003; Hodkinson, 2005; McCain and Grytnes, 2010; Vittoz et al., 2010; Mumladze et al., 2015). Therefore, opposite to general predictions, we observed that both nematode abundance and

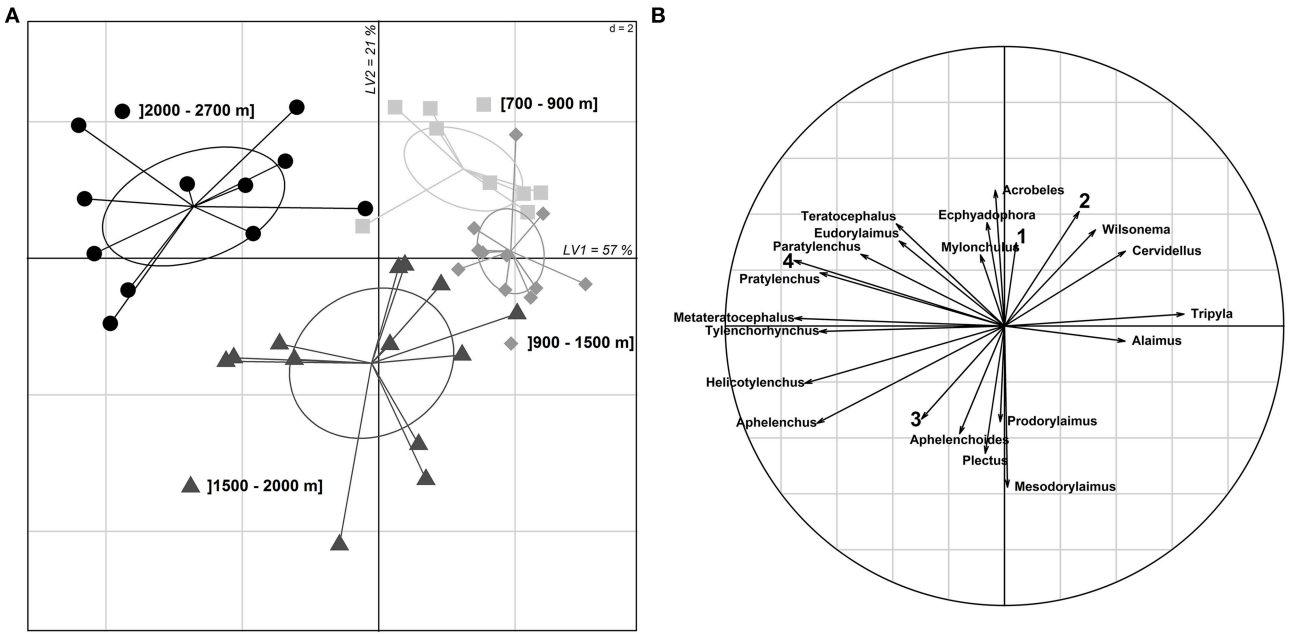


FIGURE 3 | Nematode distribution along elevation gradients. (A) PLS-DA scores plot of the 43 sampling sites where nematodes were collected. Only the 19 nematode taxa with a VIP>1 are retained in the model. The first and the second axis explain 57 and 21% of the inter-group variance respectively. **(B)** Correlation disk representing the correlations of the two axis of the PLS-DA with each nematode taxon and the four soil traits: (1) pH, (2) conductivity, (3) humidity, and (4) root fraction.

nematode diversity increases at high elevation (**Figures 1A,B**). Because our linear models showed no sign of attenuation, it even suggests that the altitudinal threshold after which nematode abundance should decline might be located above 2700 m. Nematodes occur in every ecosystem, often providing available organic carbon sources, and this study confirms their previously reported ability to colonize harsh environments such as Antarctic or high elevation biotopes (Yeates, 2010). Nonetheless, in the Alps, above 3000 m (the alpine and nival stages), vegetation and organic soil layers becomes extremely rare, very inducing a reduction of nematode diversity.

Where vegetation is still relatively abundant (i.e., below 3000 m), different ecological factors can be proposed for understanding this elevation pattern in nematode distribution. Free water in the soil matrix is certainly one of the most important parameter controlling nematode activity and several authors have shown that water availability promotes nematode populations (e.g., Todd et al., 1999; Landesman et al., 2011). Hence, higher nematode abundance at mid- to high-elevation could be linked with the observed increase in soil moisture at high elevation (**Figure 3** and Figure S2), which is related higher rainfall frequency and amount at high elevation in the Alps (Körner, 2003). High elevation nematodes are also clearly associated with denser root systems, probably shaping soil micro-habitats that offer shelters against abiotic stresses and increase water retention for free-living nematodes. Additional biotic factors like top-down pathogen pressures over the nematode community are also probably involved, since high elevation soils bare lower amounts of nematophagous fungi (**Figure 1C**), thereby providing enemy-free zones for nematodes to thrive.

The elevation pattern observed for nematode distribution is in line with Hoschitz and Kaufmann (2004) who recorded relatively high densities of nematodes and diversity within nematode communities collected above 1950 m in the Austrian Alps and is in line with the hypothesis that nematodes might be predominant within high elevation mesofauna because they harbor better adaptations to extreme habitats than most of the other soil–dwelling invertebrates (Procter, 1990). In this context, the ecology of nematode taxa and the functional diversity within nematode communities is expected to vary along mountain clines (see Discussion below).

Nematode and Plant Communities along Elevation Gradients

As shown in **Figure 3A**, three different nematode communities can be distinguished across four elevational zones. In the alpine zone, i.e., over 2000 m, the composition of nematode communities is characterized by four taxa: *Eudorylaimus*, *Teratocephalus, Pratylenchus,* and *Paratylenchus* (**Figure 3B**). Numerous taxa of nematode have a worldwide distribution although some of them are more frequently found in specific habitats. *Eudorylaimus* and *Teratocephalus* have been previously found able to colonize arctic or high elevation soils due to their ability to cope with extreme cold temperature (Loof, 1971; Ruess et al., 1999; Hoschitz and Kaufmann, 2004). *Pratylenchus*, an endoparasitic herbivore, might survive harsh conditions due to its life style within root tissues, which confers appropriate protection against unfavorable environmental conditions (Jones and Fosu-Nyarko, 2014).

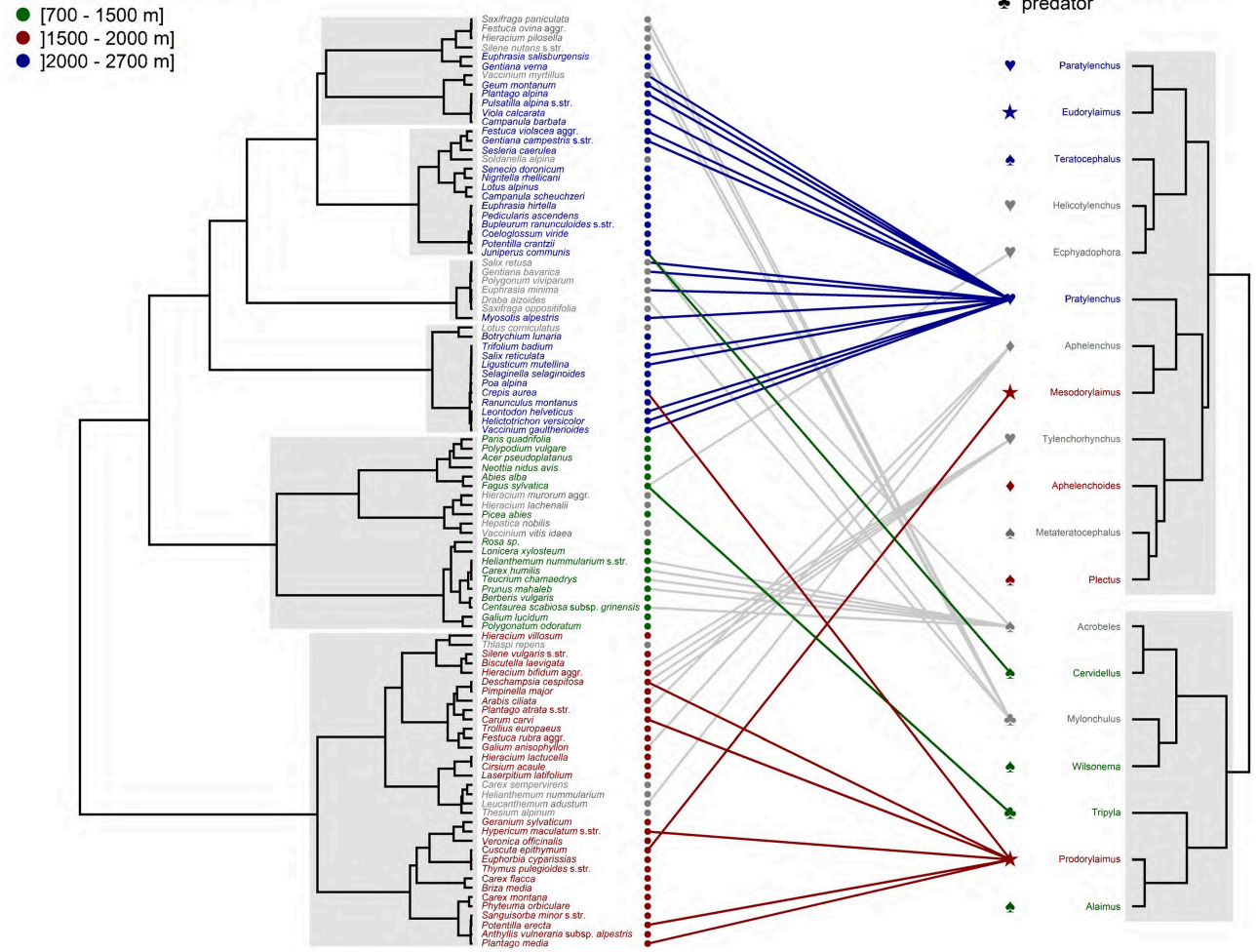


FIGURE 4 | Hierarchical clustering and correlations between plant and nematode distribution. Clustering are performed with the 100 plants and the 19 taxa of nematodes with a VIP > 1 in the previous PLS-DAs (distances based on correlation method; agglomeration based on Ward's method). Stable clusters are highlighted (bootstrap replication: 10,000; approximatively-unbiased $p < 0.05$). Nematodes and plants characterizing elevation communities are colored according to the identification of their respective communities based on the previous PLS-DAs. Nematodes and plants not characteristic of elevation communities appear in gray.

Within the *Paratylenchidae* family, many infective juveniles form resistant stages to survive harsh conditions (Bongers, 1990). Consequently, the establishment and winter survival of *Paratylenchus* at high elevation could be promoted by the highly resistant juvenile larvae, but this needs to be further studied.

Variation in the local flora might also explain variation in nematode community clustering along elevation gradients. Indeed, the hierarchical clustering based on nematode diversity shows that nematode taxa specific to high and low elevation are distributed within two single clusters whereas nematode genera characterizing intermediate communities are spread across the whole dendrogram (**Figure 4**). While the hierarchical clustering indicates that practically all trophic groups are ubiquitous across the different nematode communities at all elevations, the ecology of nematodes and the functional traits conferring adaptations to elevation niches might be consequently predominant in driving patterns of community spatial variation.

Along the same lines, we could highlight a strong clustering of plant diversity along elevation gradients. At low elevation, quite pristine environments are dominated by beech forests, an habitat where 75 nematode species have been previously inventoried in Denmark (Yeates, 1972). Among nematode taxa characterizing the lowland communities, *Tripyla* is the most characteristic genus, mainly correlated with the dominant tree species, *F. sylvatica*. *Cervidellus* is counterintuitively correlated with the subalpine species *Juniperus communis*. This might be

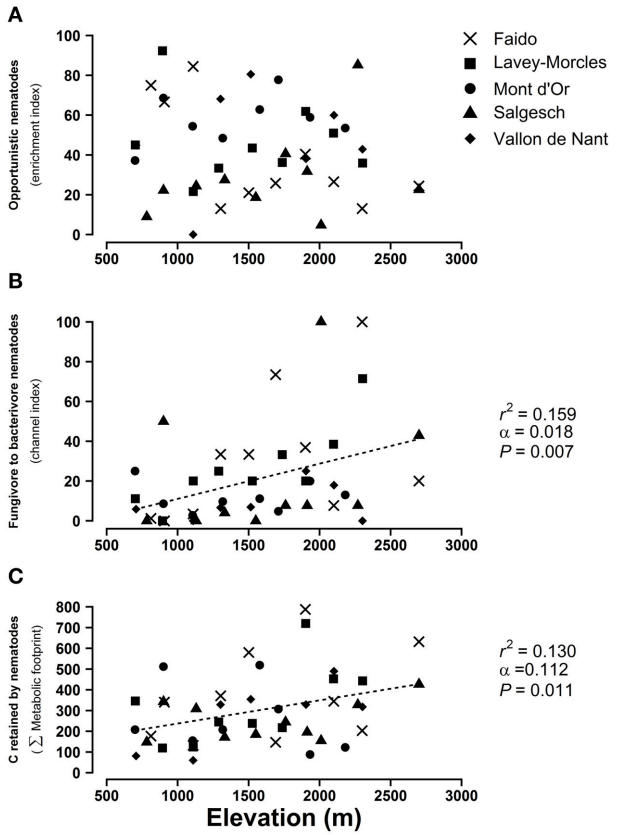


FIGURE 5 | Effect of elevation on (A) opportunistic nematodes (enrichment index), (B) fungivore to bacterivore nematodes (channel index), and (C) carbon retained by nematodes in the soil food web (Σmetabolic footprint). Shown are the sampling sites beloning to five mountain transects as shown in Figure S1. When the mixed linear model is significant, the dashed lines represent the predictions of the model. r^2, coefficient of determination; α, regression slope; *P*, significance of the regression slope.

driven by the fact that *Cervidellus* is also present in the subalpine and alpine zones, although in a much lower amount compared to low elevation.

At intermediate elevation, two characteristic taxa of nematodes are correlated to several plant species that encompass a range of diverse habitats. First, *Mesodorylaimus* is surprisingly correlated with *C. epithymum*, a parasite plant species developing haustoria on the host stem. Both *Mesodorylaimus* and *C. epithymum* were collected in only one sample and we cannot exclude a biased correlation, probably indirectly driven by other, more suitable plants. Second, in the same elevation zone, *Prodorylaimus* is significantly correlated to six plant species that occur mainly in mesotrophic to eutrophic pastures: *Plantago media*, *Crepis aurea*, *Potentilla erecta*, *Carum carvi*, *Hypericum maculatum,* and *Deschampsia cespitosa*.

Finally, among the nematode taxa characterizing high elevation communities, the herbivore genus *Pratylenchus*, is correlated with 15 plant species mainly characterizing two plant communities: the high alpine calcareous grasslands with long snow cover, and the subalpine-alpine acidic grasslands or heathlands (transition between the upper subalpine forests and lower alpine grasslands). These numerous correlations confirm that *Pratylenchus* has developed a wide host range including high diversity of plant habitats (Jones and Fosu-Nyarko, 2014). At high elevation, the relaxation in plant defenses against herbivores could explain this wide host range (Pellissier et al., 2012; Rasmann et al., 2014), but this needs to be confirmed. Thus, considering the number of significant correlations between local flora and the abundance of nematode taxa across elevation zones, this study suggests that nematodes with broader ecological niches including more diversified vegetation might be advantaged at high elevation, i.e., in more fragmented landscapes with higher variability of the vegetation (Rasmann et al., 2014). That said, our methodological approach has the intrinsic limitation of being purely correlative, and future research should address the specificity of plant-nematode interaction across different habitats.

Variation in Nematode Trophic Function along Elevation Gradients

In recent years, numerous studies have advocated the importance of replacing taxonomical biodiversity with functional diversity for uncovering mechanisms of ecosystem functioning (Thébaud and Loreau, 2006; Reiss et al., 2009; Thompson et al., 2012; Montoya et al., 2015). Hence, a shift from studying the composition of nematode communities from a taxonomical perspective to analyzing the assemblage of these communities based on trophic functional guilds is appropriate for better understanding changes in soil ecosystem functioning along elevation gradients. Here, we analyzed several major classes of community indices in order to estimate the contribution of nematodes to soil food web structure along elevations.

First, our results show an increase of the sigma-maturity index (ΣMI) along mountain slopes (Figure S3A), and, consequently, indicate that nematodes sensitive to environmental perturbations and harboring longer life cycles are more abundant at high elevation. This increase in the ΣMI relies on both free-living and plant-parasitic nematodes. However, our study suggests that higher ΣMI values on mountaintops are mainly driven by an increase of herbivorous nematodes with slow growing rates and longer life cycle at high elevation (Figure S3C). This increase in "persisters" within plant-feeding nematodes at high elevation could be due to low annual soil temperature and slow turnover and nutrient cycling, which might be more suitable for nematodes with longer life cycles and low reproduction rates. Furthermore, tolerance of "persister" nematodes toward stress conditions, like those occurring at high elevation, should be more suitable for plant-feeding than for free-living nematodes (Bongers, 1990).

Second, the increase of the CI with elevation (**Figure 5B**), i.e., the ratio of fungivore to bacterivore nematodes, reveals that

the fungal decomposition pathways support greater nematode biomass than bacterial decomposition at high elevation. This pattern might be explained by the variation of the productivity of soils as argued by Wardle et al. (2004). At high elevation, plant traits such as slow growing and long leaf life span result in slow mineralization rates, and thus in less fertile soils (see also our results of decrease in conductivity at higher elevation, Figure S2B). These high elevation conditions enhance fungal-based energy flows within ecosystems, consequently accompanied by slower decomposition rates compared to lowland soils with more bacterial-based pathways (Wardle and Yeates, 1993; Zhao and Neher, 2014).

The fifth community index retained in our study, the enrichment index (EI), is not affected by elevation, even if we recorded strong variability between soil locations (**Figure 5A**). Hence, the ratio of nematodes indicating enrichment and basal characteristics of the food web is likely to rely on local soil conditions, independently of elevation.

In addition to the above-mentioned indices, the metabolic footprints (MF) of nematode communities can inform on how carbon assimilation in soil food web from autotrophic organisms varies with elevation (Ferris, 2010). Overall, the increase of the composite MF (i.e., the MF for the whole nematode community) along mountain slopes is similarly quite surprising. Indeed, while abundance and species diversity of most of soil invertebrates decrease with elevation (Hodkinson, 2005; Rasmann et al., unpublished), we here show that the energy flow canalized through nematodes increases with elevation. These results are in line with a previous study performed in grasslands and demonstrating the stronger role of soil mesofauna in incorporating carbon within soil food-webs compared to macrofauna (Ostle et al., 2007). However, changes in habitats, like those occurring along elevation clines, trigger variations in soil biota, and might impact the resulting nutrient fluxes differently. For instance, the abundance and the biodiversity of nematodes and mites along grassland successional stages evolve in opposite directions, and this triggers variation in interactions between vegetation and soil biota and therefore variation in nutrient fluxes (Swift et al., 1998). In this context, our results along elevation pattern deserve further studies for better understanding to what extent nematodes replace other soil invertebrates (earthworms, collembolans, enchytraeidae, mites) for carbon cycling in Alpine soils.

CONCLUSIONS

Our results show that harsh elevation environments drive modifications in the composition of nematode communities based on ecological traits conferring local adaptations. In addition, the correlations between local flora and nematode distribution suggest that nematodes colonizing various high elevation habitats could cope with more fragmented and spatially variable vegetation on mountain tops. Further studies with higher taxonomic resolution are required to validate this hypothesis. Indeed, while genera of nematodes found at high elevation seem to harbor wider host-range, we cannot exclude that different species within these genera are specialized on different habitats and/or plant species. Finally, this study highlights the potential of nematodes' derived ecological indices in understanding ecosystem processes along ecological gradients. We could observe that the role played by nematodes in nutrient cycling increases with elevation, as they partially take over carbon assimilation in soil food web. Future accurate sampling strategies of nematodes across more specific habitats are nevertheless required to dissect how ecosystems types and ecological factors affect nematode-driven soil ecosystem processes along elevation gradient.

AUTHOR CONTRIBUTIONS

SR, RC, SS planned the experiment and collected the data. PV provided plant cover data. AK analyzed the data and wrote the manuscript. All authors reviewed and commented previous versions of the manuscript.

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ORIGINAL RESEARCH
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The Distribution of Soil Insects across Three Spatial Scales in Agricultural Grassland

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The effects of specific environmental factors on abundance and distribution of some individual soil insect taxa is known, but how scale influences spatial distribution is less well evaluated, particularly at the community level. However, given that many soil insects are pests or beneficial natural enemies, and that collectively they play a role in soil processes, this information is of potential value for predictive modeling and in furthering our understanding of soil ecology and management. The objectives of this study were to characterize the spatial distribution, relative population sizes, effect of sampling scale and taxa co-occurrence on a range of soil insects at the family level over 2 years. Soil cores were taken from agricultural grassland soils across three different sampling scales (farm, field, and core) using a systematic sampling approach. Spatial distribution was assessed using the variance-to-mean (VMR) ratio and taxa distribution plots and the contribution of scale, spatial (geographical location), and biotic (presence-absence of other species) factors determined using deviance partitioning. Tipulid larvae (leatherjackets) were the most abundant taxa in both years, but the composition of other Dipteran and Coleopteran taxa varied between years. The VMRs revealed differences in spatial distribution between taxa across scales and years, showing a range of underlying distributional patterns. Scale was the most important factor influencing species distributions, but a large proportion of deviance remained unexplained and there was much variation between taxa, suggesting biological and scale-specific factors are driving distributions, in agreement with a previous study.

Keywords: deviance partitioning, variance-to-mean ratio, Tipulidae, pest, belowground, soil communities

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INTRODUCTION

Whilst there is increasing research interest and focus on the roles of soil organisms in above and belowground processes and their interactions (e.g., Johnson et al., 2013), there remains a considerable lack of knowledge on the abundance and distribution of soil insects at an individual and a community level. Grassland soils support a diverse range of insects, many of which are direct pests of grass (e.g., Tipulidae) or nearby arable crops (e.g., wireworms) when present in sufficient numbers. Conversely, the stable habitat that grassland provides is also of value to beneficial natural enemies such as Carabids, adults, and larvae of which can help to suppress pest populations to non-damaging levels (Kromp, 1999). This is likely to be important in mixed farming systems that form

a mosaic of both cropped and grass fields, since grasslands can provide a reservoir of generalist natural enemies (Gravesen and Toft, 1987). Therefore, an understanding of the basic biology and ecology of these organisms is important for implementing sustainable management strategies. In order to do this, knowledge of the spatial structuring of populations, and the underlying mechanisms leading to this, is needed. It is known that there is natural stochasticity in insect abundance over short timescales, linked to their high reproductive rate and environmental factors (Schowalter, 2011), such as temperature, moisture, food availability and soil texture (King, 1939) and that abundance and distributions can vary even between even closely related species (Benefer et al., 2012). While factors affecting the biology of some notable pest taxa (e.g., wireworms) have been particularly well-studied in the laboratory and in the field at small scales (e.g., Campbell, 1937; Lees, 1943; Furlan, 1996), this has rarely been replicated at larger scales. Benefer et al. (2010) investigated the effects of spatial, biotic and scale variables on observed soil insect distributions and associations in a grassland soil, focusing on the soil core, site (within field), and field scale. It was found that the observed distributions and associations of most taxa changed according to scale, with this being the most important variable influencing distributions.

In this study, we used the North Wyke Farm Platform, which is a highly instrumented and monitored grassland farm to:

1. Characterize the spatial distribution of subterranean grassland insects over three spatial and time scales: soil core, field and farm, and 2 years.
2. To assess the relative population sizes and associations of soil insects at the family level over these spatial scales.
3. To determine the relative importance of scale, spatial, and biotic factors on the observed distribution and abundance of soil insects.

With limitations on the chemical controls that can now be implemented to manage grassland pests, understanding pest (and natural enemy) distribution and using this information in predictive modeling has increasing importance for sustainable management. Assessing these distributions and associations in the same location over time aids in strengthening our understanding of the variation in ecological processes occurring in the soil; this is again important for making predictions based on observational data. Use of the same data analysis approach as Benefer et al. (2010) has allowed a direct comparison of the use of these techniques for assessing soil insect distributions across sites, and tests whether the conclusions of the earlier study are robust or site-specific.

METHODS

Study Site and Soil Sampling

The sampling was conducted on the North Wyke Farm Platform (NWFP), in south west England, UK (Geographic Location: Lat: 50.73237; Long: −3.99635) (Griffith et al., 2013). During the study, the NWFP was managed as a conventional Beef and Sheep enterprise based on perennial ryegrass (*Lolium perenne*) dominant permanent pastures receiving 200 kg N ha^{-1} per annum. The soil types common on the NWFP are typical of soils under grassland management in England. The soil is predominantly of 2 similar soil types, Harrod and Hogan (2008), which are of a slightly stony clay loam topsoil (∼36% clay) overlying mottled stoney clay (∼60% clay), derived from the carboniferous Culm measures. Below 30 cm the soil is highly impermeable to water and is seasonally waterlogged. The mean annual rainfall (1982–2011) at the North Wyke site was 1042 mm with a mean temperature ranging from 6.6-13.4°C. North Wyke has relatively high and consistent summer rainfall which is characteristic of the major agricultural grassland areas in the UK. It has been calculated (Wilkins, 1982) that the environmental conditions are sufficient to support 280 days of grass growth, but the grazing season is restricted to ∼180 days due to soil wetness.

Soil samples were taken from 19 permanent grassland fields (>5 years old) split over 3 farms, ranging from 1.3 to 7.9 ha, with a total study area of 67 ha. Sampling points were plotted on a 25 m sampling grid using ESRI ArcMap (V.10) GIS software and determined in the field using a GPS system (a Trimble R8 base station with an R6 rover and TSC3 controller). Samples were taken between April and May 2012 and repeated between April 2013 and May 2013. Soil cores were taken using a standard soil corer with a 6.5 cm plastic pipe which was inserted to a depth of 10 cm. A total of 2260 soil samples were collected (1130 in each year, representing a total sampled area of ∼9 m^2). The individual samples were placed in labeled plastic bags and held at 4°C until processed, within 14 days of sampling.

Extraction and Identification of Soil Insects

A modified heat extraction method based on that of Blasdale (1974) was used to recover soil insects from the cores over a 24 h period. The modified apparatus consists of two layers, each layer holding 25 soil samples of 6.5 cm diameter. The insects collected were stored in 70% ethanol before being individually identified to family level using morphological keys (Freeman, 1983; Stubbs and Drake, 2001; Luff, 2007). Some specimens which could not be identified to Family with confidence were sent to the Natural History Museum, London, including those in the families Scatopsidae, Anthomyiidae and two types of Dolichopodidae larvae: Dolichopodidae "type A" was subsequently further identified as belonging to the Genus *Campsicnemus*, while Dolichopodidae "type B" was classified as an undescribed larva. In addition, there were larval individuals of an unknown beetle family which were labeled as "unknown Coleoptera."

Data Analysis

To visualize the distribution of each taxa across the sampling area in each year, the British National Grid coordinates (Easting and Northing) of each soil core sample were used to create a spatial distribution map for each taxa using ArcGIS 10.2. The distribution pattern (aggregated, random, or uniform) of each taxon was determined using variance-to-mean ratios (VMR) as an index of dispersion and indication of underlying patterns in abundance data (Benefer et al., 2010), using the mean and variance of the abundance data calculated separately for each scale (farm: 3 data points, field: 19 data points, and core: 1130

data points). In both sampling years there were a large number of cores that did not have any insects so we used a Generalized Linear Model (GLM) with a binomial distribution and logistic link function to determine the relative importance (measured by deviance explained) of space (geographical coordinates), biotic influences (presence/absence data for all other taxa), and scale (a nominal variable, divided into "farm" and "field" for each taxon in each year). Core scale data were not included due to the high number of zero counts (Legendre and Legendre, 1998; Lobo et al., 2002; Benefer et al., 2010).

RESULTS

Taxa Abundance and Distribution across the Sampling Site

A total of 512 adult and larval insects were recovered (233 in 2012 and 279 in 2013), representing a total of 13 families of Coleoptera, Diptera, and Lepidoptera (**Tables 1**, **2**). The larvae of Tipulidae (leatherjackets) were the most abundant taxa recovered in both years (45.5 and 41.9% in 2012 and 2013 respectively). While the abundance and proportion of the samples collected remained similar across years for some taxa (e.g., Muscidae), for others there was a substantial difference. For example, Sciaridae were the second most abundant insect recovered in 2012 (11.6% of all samples), but no individuals of this family were found in 2013, and several new families were recovered in 2013 that were not present in samples in 2012 and vice versa; only 6 taxa were found in both years (**Tables 1**, **2**). Leatherjackets were found across the NWFP, showing a similar distribution in 2012 and 2013 (**Figures 1**, **2**). Several taxa showed a tendency to be distributed on or close to the field margins, some across both years, including adult and larval Carabidae, Muscidae, Bibionidae, and Dolichopodidae. For some taxa found in both years there was also a change in distribution from the northern-most to central/southern fields, e.g., Stratiomyidae and Carabid adults and larvae (**Figure 1**).

Spatial Distribution

The underlying spatial distribution, as indicated by the variance/mean ratio (VMR) as an index of dispersion, varied between taxa, and generally between years for those taxa found in both 2012 and 2013 (**Tables 3**, **4**). Tipulidae were aggregated at the farm and field scale in both years (VMR >1), but had a more random distribution in 2013 at the core scale (VMR $= 1$), compared to a more uniform one (VMR <1) in 2012. Generally, taxa were more aggregated or randomly distributed at the field and farm scale and uniformly (in 2012) or randomly (in 2013) distributed at the core scale. Notable exceptions were Sciaridae, which was highly aggregated at all scales in 2012, and Psychodidae, highly aggregated at all scales in 2013.

Deviance Partitioning

The percentage of total deviance in taxa presence/absence explained by scale, space and biotic variables was generally low for both years, ranging from 10% for Tipulidae (2012 and 2013) to 56% for Sciaridae (in 2013; **Tables 3**, **4**). Individually, scale explained the most variation in this data, accounting for between 8 and 50% of deviance (for Tipulidae and Chironomidae in 2013, respectively) whereas spatial and biotic factors had less of an influence on most taxa. At the core scale (excluded from deviance partitioning due to high zero counts), 6 out of 172 cores containing taxa in 2012, and 12 out of 201 cores containing taxa in 2013, contained at least two different taxa. The observed and predicted co-occurrence was not significantly different in a chi-squared test ($\chi^2 =$

TABLE 1 | The number of individuals of each taxa, their percentage of the total 233 insects obtained from 1130 cores collected in 2012 over 19 fields in 3 farms, the population density based on an area of approximately 9 m^2 (the total area of soil cores taken from the study) and the variance-to-mean ratio for the taxa collected at the farm, field, and core scale in 2012.

Taxa	No. of individuals	%	Population density (m^{-2})	Variance-to-mean ratio		
				Farm	Field	Core
Tipulidae	106	45.5	11.94	13.2	4.5	0.6
Sciaridae	27	11.6	3.04	20.3	23.7	12.5
Stratiomyidae	18	7.7	2.03	3.6	2.8	0.7
Scatopsidae	18	7.7	2.03	3.6	5.1	0.8
Carabidae (adults)	13	5.6	1.46	4.80	5.9	1.7
Carabidae (larvae)	13	5.6	1.46	4.2	1.6	0.7
Dolichopodidae B	13	5.6	1.46	2.4	3.3	0.7
Muscidae	9	3.9	1.01	1.4	2.1	0.6
Dolichopodidae A	9	3.9	1.01	1.0	0.8	0.5
Unknown Coleoptera	5	2.1	0.56	2.2	0.8	0.5
Chironomidae	1	0.4	0.11	0.8	1.0	0.5
Anthomyiidae	1	0.4	0.11	0.8	1.0	0.5

TABLE 2 | The number of individuals of each taxa, their percentage of the total 279 insects obtained from 1130 cores collected in 2013 over 19 fields in 3 farms, the population density based on an area of approximately 9 m^2 (the total area of soil cores taken from the study) and the variance-to-mean ratio for the taxa collected at the farm, field, and core scale in 2013.

Taxa	No. of individuals	%	Population density (m^{-2})	Variance-to-mean ratio		
				Farm	Field	Core
Tipulidae	117	41.9	13.18	10.6	3.3	1.1
Chironomidae	38	13.6	4.28	24.7	19.2	2.3
Psychodidae	29	10.4	3.27	21.8	27.6	29.0
Dolichopodidae B	27	9.7	3.04	5.1	2.4	1.5
Bibionidae	17	6.1	1.91	5.2	7.8	3.0
Chrysomelidae	13	4.7	1.46	1.1	2.5	1.6
Stratiomyidae	13	4.7	1.46	2.5	2.7	1.6
Muscidae	11	3.9	1.24	6.4	4.8	1.2
Carabidae (adults)	4	1.4	0.45	3.0	1.4	1.0
Carabidae (larvae)	6	2.2	0.68	1.5	1.7	1.3
Cantharidae	2	0.7	0.23	0.5	0.9	1.0
Elateridae	1	0.4	0.11	0.8	1.0	1.0
Noctuidae	1	0.4	0.11	0.8	1.0	1.0

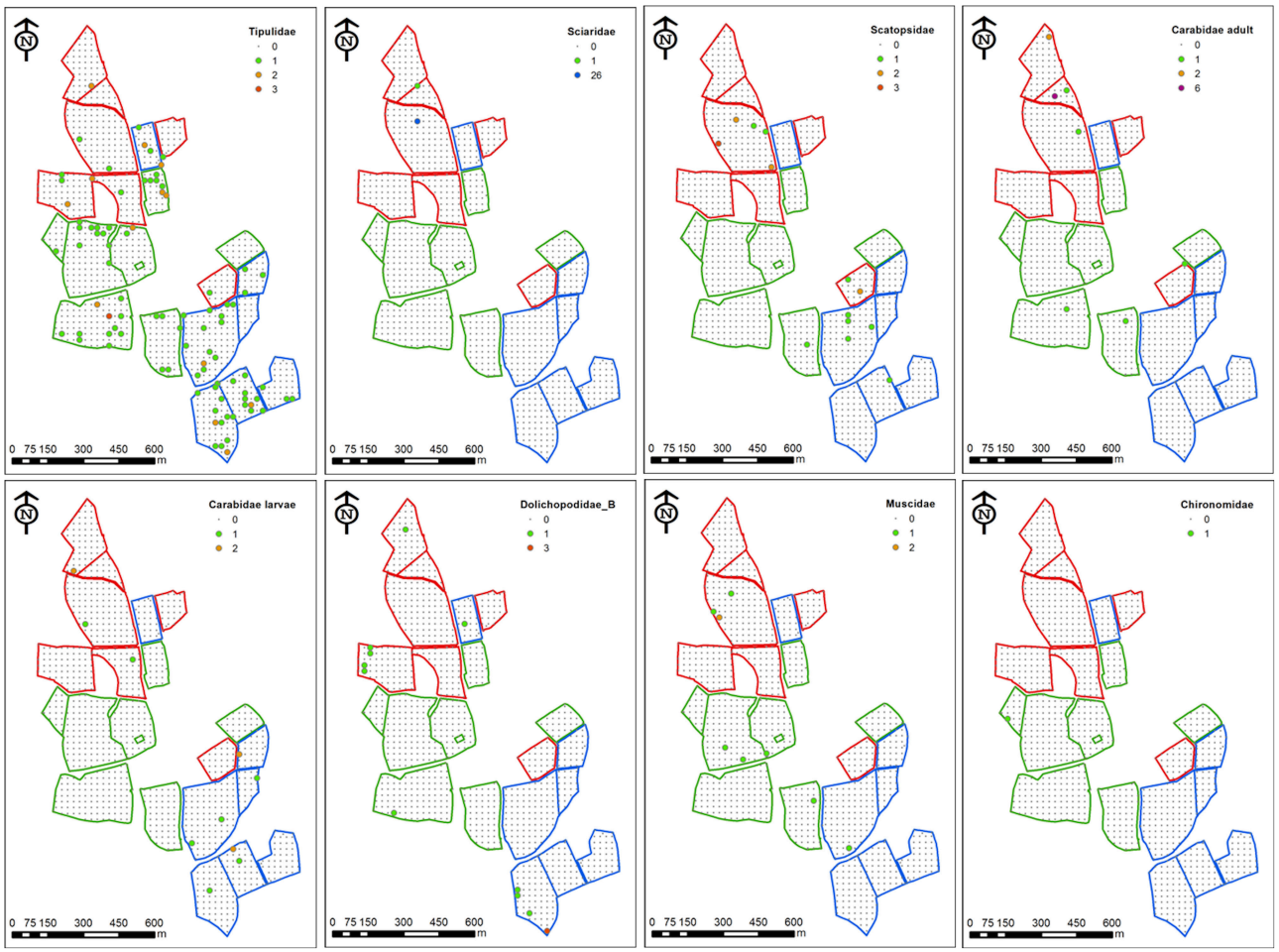


FIGURE 1 | Distribution maps for selected key taxa on the North Wyke Farm Platform in 2012. Individual soil cores were taken (1130 in total) across 19 fields in three farms (red boundaries = Farm 1, green = Farm 2 and blue = Farm 3). Large circles represent sampling points containing individuals, colored by abundance (see individual keys).

3.39, df = 39, $p > 0.05$, 2012; $\chi^2 = 4$, df = 51, $p > 0.05$, 2013), suggesting there was a random distribution at this scale.

DISCUSSION

Abundance and Community Composition

Generally, the abundance of each taxon recovered was relatively low, but the overall community abundance was similar between sampling years. Tipulidae were the most abundant family found in both years, and were spread throughout the NWFP, but presence and abundance of other taxa was variable between the two sampling years (**Tables 1**, **2**). The high relative abundance of leatherjackets is in accordance with the findings of Benefer et al. (2010), who carried out a similar study in an agricultural grassland in south Devon. However, in that study Sciaridae and Bibionidae also shared this dominance, accounting for between 20–28% of the insects recovered. Here, leatherjackets comprised 45.5% (2012) and 41.9% (2013) of the population, but Sciaridae were the second most abundant taxa in 2012 (11.6%) and Bibionidae the fifth most abundant in 2013 (6.1%).

Tipulidae is the biggest Dipteran family in the UK, with more than 300 species, and previous UK studies indicate that they can occur in large populations, but are greatest in parts of northern and south eastern England (Smith, 1989). In grassland soils, leatherjackets have been recorded in relatively high abundances in comparison with other taxa (Buckle, 1923; Staley et al., 2007), as we found in this study; as an extension to the current study we used DNA barcoding to identify the leatherjackets collected (Benefer et al., unpublished), finding that most were *T. paludosa*, in concurrence with earlier surveys by Humphreys et al. (1993). Blackshaw and Hicks (2013) reported finding no adult *T. oleracea* in the agricultural grassland that they surveyed, but in the current study adults of this species were present in aboveground. While adult *T. oleracea* may be found in agricultural grassland, they have different oviposition preferences to *T. paludosa* and do not lay eggs here. *T. oleracea* are strong fliers and therefore may be passing through these fields to more suitable oviposition

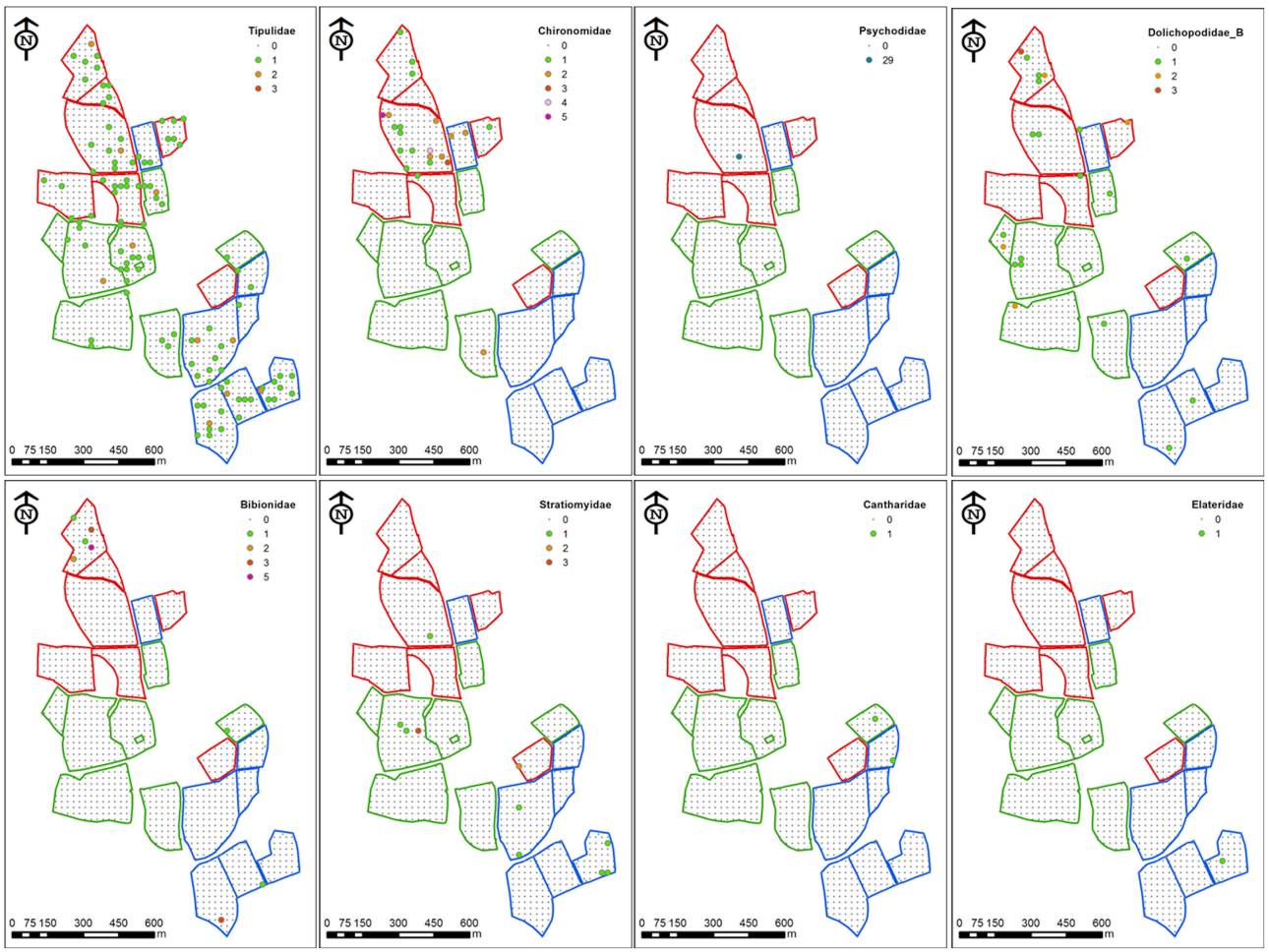


FIGURE 2 | Distribution maps of selected taxa on the North Wyke Farm Platform in 2013. Individual soil cores were taken (1130 in total) across 19 fields in three farms (red boundaries = Farm 1, green = Farm 2 and blue = Farm 3). Large circles represent sampling points containing individuals, colored by abundance (see individual keys).

sites (Blackshaw et al., 1996). Recent studies have shown that populations of *T. paludosa* are regulated by density-dependent feedback at regional and field scales (Blackshaw and Petrovskii, 2007; Bearup et al., 2013), but that populations are relatively stable within and between generations (Blackshaw and Moore, 2012). However, while the population dynamics of leatherjackets have been relatively well-studied, this is not the case for most other soil taxa, with some incongruity in population abundance recorded in grassland soils e.g., Bibionidae and Sciaridae (Frouz, 1999; D'Arcy-Burt and Blackshaw, 1991; Benefer et al., 2010). Taken together, this limited information suggests that while for individual taxa (leatherjackets) populations vary relatively little, soil insect communities as a whole are not necessarily stable over time or space, and that many interacting factors are likely to be driving this.

Only one individual from each of the families Noctuidae and Elateridae were found. While the populations of Lepidopteran larvae are known to be generally low in grassland ecosystems (Curry, 1994), this is a surprising result for Elateridae. Elaterid larvae (wireworms) are traditionally associated with grassland soil in the UK and Europe (Traugott et al., 2015), and adult male pheromone trapping in previous and subsequent years on nearby areas at the North Wyke site suggests that adults at least are present in grass fields (C. Benefer, unpublished data). However, as for the closely related Tipulid species described above, species-specific differences in dispersal ability/oviposition preferences may also be the case for *Agriotes* click beetles and wireworms (the most common agricultural species in the UK and Europe). For example, *A. lineatus* which are trapped in abundance aboveground are not always found belowground in the same fields, in contrast to two other closely related species (Benefer et al., 2012). More information on oviposition preferences of aboveground adult stages in general is required in order to understand how this affects the distribution and abundance of larval stages belowground (Traugott et al., 2015). As with Bibionidae (Blackshaw and D'Arcy-Burt, 1993), the capture of wireworms can be affected by sampling method (Benefer et al., 2012), and as with many other soil taxa, timing

TABLE 3 | The percentage of deviance explained by a GLM with binomial distribution through partitioning the dependent variable (species presence/absence, for taxa with ≥5 individuals) between three groups of explanatory variables (biotic, scale, and space), alone and in combination, and the deviance unexplained by the variables in this study for each taxa in 2012.

Taxa	Biotic	Scale (all)	Scale (farm)	Scale (field)	Space	Biotic & scale	Biotic & space	Scale & space	All	Unexplained
Tipulidae	2	11	3	8	3	10	4	8	10	90
Sciaridae	37	41	16	25	19	55	54	25	56	44
Stratiomyidae	1	19	3	16	2	17	3	18	19	81
Scatopsidae	17	26	4	22	2	35	21	22	37	63
Carabidae (adults)	3	23	2	21	6	23	8	30	31	69
Carabidae (larvae)	3	25	9	16	3	19	5	16	19	81
Dolichopodidae B	5	30	4	26	5	30	9	35	38	62
Muscidae	13	19	2	17	3	26	15	21	29	71
Dolichopodidae A	22	16	1	15	4	32	24	23	41	59
Unknown Coleoptera	2	31	10	21	7	23	10	30	32	68

TABLE 4 | The percentage of deviance explained by a GLM with binomial distribution through partitioning the dependent variable (species presence/absence, for taxa with ≥5 individuals) between three groups of explanatory variables (biotic, scale and space), alone and in combination, and the deviance unexplained by the variables in this study for each taxa in 2013.

Taxa	Biotic	Scale (all)	Scale (farm)	Scale (field)	Space	Biotic & scale	Biotic & space	Scale & space	All	Unexplained
Tipulidae	3	8	1	7	1	10	4	7	10	90
Chironomidae	11	50	20	30	16	37	25	30	37	63
Psychodidae	2	43	14	29	7	33	10	36	42	58
Dolichopodidae B	12	15	3	12	4	22	15	14	24	76
Bibionidae	10	35	4	31	7	38	16	31	38	62
Chrysomelidae	18	23	2	21	2	33	18	22	34	66
Stratiomyidae	7	25	4	21	3	33	13	23	34	66
Muscidae	5	41	14	27	17	34	24	28	34	66
Carabidae (adults)	3	47	15	32	2	34	5	34	36	64
Carabidae (larvae)	9	29	3	26	1	35	11	27	35	65

of sampling can also impact upon the number of individuals recovered since a number of abiotic factors, temperature, and moisture in particular, alter activity and movement in the soil, both horizontally (Lafrance, 1968; Fisher et al., 1975) and vertically (Jung et al., 2014). In comparison to the findings of Benefer et al. (2010) at least, this may account for some of the differences in abundance and composition, since sampling was carried out earlier (from January to March) in that study.

Species Distribution across Spatial Scales and Years

How, and which, organisms interact is key to understanding community ecology in different systems, and one that has received more attention in recent years due to the realization that activity of belowground insects (particularly root herbivores), may play a considerable functional role in processes both below and aboveground, mediated through the host plant (e.g., Johnson et al., 2013). The interspecific competition for habitat is one of the primary biotic factors influencing the abundance, distribution, and diversity of ecological communities (Begon et al., 2009) but these interactions vary between sampling scales. Many of the families collected in this study contain species that are known root herbivores (Tipulidae, Sciaridae, Bibionidae, Chrysomelidae, Stratiomyiidae, Muscidae, Anthomyiidae), and a large proportion of these can be pests, but those in families associated with predatory behavior (Carabidae, Cantharidae, Dolichopodidae) were also collected. The associations of some of these beneficial natural enemies with root herbivores at the farm and field scale (**Figures 1**, **2**) suggests they have the potential to have a regulatory effect on pest populations.

While the presence/absence of the specific taxa found in this study to not appear to have an influence on distribution, other biotic factors, such as the age structure of the population, may be involved. For example, when they reach the fourth larval instar, leatherjackets move to sites with favorable soil moisture and in the process change their spatial pattern (Blackshaw, 1999; Petersen et al., 2013); in the present study Tipulidae had a more uniform distribution at the core scale in 2012 (VMR <1; **Table 3**), and a more random distribution at this scale in 2013 (VMR =1; **Table 4**). In comparison, the opposite was true for Dolichopodidae type B, which was more abundant in 2013. Many insects are able to quickly colonize new habitats because of their high reproductive capacity, which is aided at high populations, but at low populations they may be limited by their limited size, life span and dependence on chemical communication for

locating mates (Schowalter, 2011). Therefore, differences in the abundance of taxa could partly explain some of the differences in distribution observed here between years, and particularly for low abundance taxa suggests caution needs to be applied when interpreting such data.

Apparent co-occurrence of some taxa may indicate similar habitat and environmental preferences, which likely affect distribution at larger scales (Wiens, 1989). For example some taxa, including Muscidae (in both years), Dolichopodidae type A, Chironomidae, and Bibionidae, tended to be found closer to the field margins (**Figures 1**, **2**). Larval stages of Chironomidae and Dolichopodidae are semi-aquatic and prefer high moisture environments (Eisenbeis and Wichard, 1987), and Muscidae and Bibionidae are often found in areas with high organic matter content (Hill, 1987) so common preferences for these and/or other unmeasured factors may have influenced the distributions seen here.

However, it is possible that the taxonomic resolution also affects observed co-occurrences. Here, due to time, expertise and resource limitations (as in many soil ecological studies), the family rather than species level was used as the taxonomic grouping, which may mean that any species-specific differences have been lost. Benefer et al. (2010) identified wireworms and Bibionidae to species level, finding some consistent associations/dissociations between species, but also between Tipulidae and Sciaridae at the family level. In Bibionidae, for example, *Bibio* spp. are found in close proximity to hedgerows whereas *Dilophus* spp. are found across fields (D'Arcy-Burt and Blackshaw, 1991). In Collembola, it has been shown that occurrence-habitat preference relationships were better explained at the family level, where species in the same family with similar habitat preferences congregated, but this was less clear at the genus and species levels, possibly due to competitive exclusion at low taxonomic levels, and convergent evolution/co-adaptation at high taxonomic levels (Ponge and Salmon, 2013). To some extent larval dispersal ability and adult oviposition preferences may be involved in this, but for many soil insect taxa this is largely unknown (Benefer and Blackshaw, 2013). This suggests that there may not be a straightforward relationship between co-occurrence and taxonomic level. Further investigation of the impact of this taxonomic component on soil insect distribution (previous studies have mainly been carried out at the mesofaunal level; e.g., Ponge and Salmon, 2013) would aid our understanding of how biological components, such as phylogenetic relationship and species traits, interact with scale and spatial effects. DNA based techniques, such as metabarcoding, yet to be applied to soil insect ecological studies, may be of use here (Benefer and Blackshaw, 2013), particularly across multiple sites and over time where the large sample size precludes ecological studies using traditional morphological identification and soil processing techniques.

Effects of Scale, Spatial, and Biotic Variables on the Distribution of Taxa

There are several key similarities in the results of this study as compared to that of Benefer et al. (2010) in terms of the extent to which scale, spatial and biotic variables explain the distribution of taxa. In the current study, unexplained deviance was high for most taxa (44–90%; **Tables 3**, **4**) in both years, comparable to the 57–88% of deviance unexplained by the same variables in Benefer et al. (2010) study. Similar levels of deviance were explained by the scale, spatial and biotic variables for leatherjackets (11 and 8% in 2012 and 2013 respectively in this study, 12% in Benefer et al., 2010) and scale was the single most important variable in explaining deviance, accounting for up to 50% of the total deviance in this study, and 36% of total deviance by Benefer et al. (2010). Thus, in broad terms, the present study has confirmed the results and conclusions reached by Benefer et al. (2010). This replication of results (rarely attempted in many ecological studies), across sites and years confirms that these variables (alone and in combination) are ineffective in explaining the distribution of a range of grassland soil insects, and that other unmeasured factors (as discussed) may have a more important role for many.

Some of the differences in the amount of deviance explained by individual variables could give some indication of differences in the biology of taxa that is linked to their observed distributions and abundances. For example, in 2012 the biotic variable for Dolichopodidae type A explained 22% of deviance, but only 5% of deviance for Dolichopodidae type B (**Table 3**), and only Dolichopodidae type B was found in 2013, demonstrating possible differences in, for example, growth, predation, competition and social aggregation (Borcard et al., 1992). On the other hand, that space and biotic variables explained so little of the deviance for Carabidae adults and larvae in 2012 and 2013 suggests that biotic mechanisms have less of an impact than scale and that environmental factors may be more important for these taxa. For example, adult Carabid spatial distribution aboveground is known to be affected by interstitial habitats, which in turn are affected by management (Thomas et al., 2002). It should also be noted that abundances of some taxa, for example unknown Coleoptera in 2012 and Carabidae adults and larvae in 2013, were particularly low, and so the patterns observed here should be interpreted with caution; it is possible that these may change at greater densities, and as Benefer et al. (2010) point out, this could itself be another scaling factor.

In conclusion, the findings of this study in terms of taxa abundance and factors affecting the distribution of soil insect taxa across spatial scales have replicated those of Benefer et al. (2010) in particular, but also others. This lends support to the robustness and reality of the data. Taken together, this suggests that responses by different taxa are variable, and that more complex studies and datasets are needed to fully explain distributions of soil insect communities over time and space. For pest management, in particular, having information on all important influencing factors and their spatio-temporal interactions would help in predictive modeling of abundance and distributions (and ultimately crop damage and reduction of pesticide usage). More sophisticated models are now being produced for some taxa (e.g., wireworms; Jung et al., 2014) that are taking some of these factors into account, but there are still gaps in our knowledge concerning effects across time and space for different species.

AUTHOR CONTRIBUTIONS

RB and PM conceptualized and designed the study. KD collected, processed and identified samples with help from CB with some of the collection. KD and CB analyzed data. HS produced the distribution maps. KD primarily interpreted data with contributions from RB, PM, and CB, and CB wrote the manuscript. All authors were involved in reviewing, revision and final approval of the manuscript.

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Multiple Assembly Rules Drive the Co-occurrence of Orthopteran and Plant Species in Grasslands: Combining Network, Functional and Phylogenetic Approaches

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Understanding the factors underlying the co-occurrence of multiple species remains a challenge in ecology. Biotic interactions, environmental filtering and neutral processes are among the main mechanisms evoked to explain species co-occurrence. However, they are most often studied separately or even considered as mutually exclusive. This likely hampers a more global understanding of species assembly. Here, we investigate the general hypothesis that the structure of co-occurrence networks results from multiple assembly rules and its potential implications for grassland ecosystems. We surveyed orthopteran and plant communities in 48 permanent grasslands of the French Jura Mountains and gathered functional and phylogenetic data for all species. We constructed a network of plant and orthopteran species co-occurrences and verified whether its structure was modular or nested. We investigated the role of all species in the structure of the network (modularity and nestedness). We also investigated the assembly rules driving the structure of the plant-orthopteran co-occurrence network by using null models on species functional traits, phylogenetic relatedness and environmental conditions. We finally compared our results to abundance-based approaches. We found that the plant-orthopteran co-occurrence network had a modular organization. Community assembly rules differed among modules for plants while interactions with plants best explained the distribution of orthopterans into modules. Few species had a disproportionately high positive contribution to this modular organization and are likely to have a key importance to modulate future changes. The impact of agricultural practices was restricted to some modules (3 out of 5) suggesting that shifts in agricultural practices might not impact the entire plant-orthopteran co-occurrence network. These findings support our hypothesis that multiple assembly rules drive the modular structure of the plant-orthopteran network. This modular structure is likely to play a key role in the response of grassland ecosystems to future changes by limiting the impact of changes in agricultural practices such as intensification to some modules leaving species from other modules poorly impacted. The next step is to understand the importance of this modular

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structure for the long-term maintenance of grassland ecosystem structure and functions as well as to develop tools to integrate network structure into models to improve their capacity to predict future changes.

Keywords: coexistence, competition, environmental filtering, functional traits, grasshoppers, grassland-invertebrate interactions, null models

INTRODUCTION

Understanding the rules underlying species assembly is a key challenge in ecology (HilleRisLambers et al., 2012). In a foodweb, species interact hierarchically with species from other trophic levels through trophic interactions (producer-consumer, predator-prey, and parasite-host). Within trophic levels, different theories such as the competition, the environmental filtering and the neutral theories describe species assembly into ecological communities (*sensu* Hubbell, 2001). Practically, assembly rules within and among trophic levels were mainly considered separately. Studies have investigated the importance of among-guild interactions including plant-pollinator (Olesen et al., 2007), trophic networks (Dunne et al., 2002a) or host-parasite networks (Vázquez et al., 2005) without considering within-guild processes. Similarly, studies focusing on within-guild assembly rules have mainly focused on answering which of competition, environmental filtering and neutral processes could explain observed ecological assemblages (Cottenie, 2005). However, a growing body of evidence suggests that different species assembly mechanisms can operate simultaneously and that they should be placed along a continuum (Amarasekare et al., 2004; Mouquet et al., 2005; Gravel et al., 2006; Leibold and McPeek, 2006; Fournier et al., 2016). Understanding this complexity of processes is key to better predict and manage species assemblages and their associated functions and services (Balvanera et al., 2006; Cadotte et al., 2011) and becomes increasingly critical in the current context of global change and biodiversity crisis (Koh et al., 2004). However, it remains difficult to assess multiple assembly rules from species distribution or co-occurrence data (Fournier et al., 2016). Here, we explore to what extent the combination of species co-occurrence network and functional and phylogenetic approaches can provide new insights on how multiple rules interact to shape species assembly.

Phylogenetic relatedness and functional traits are strong determinants of the structure of ecological networks (Cattin et al., 2004; Martín González et al., 2015) that can be used to identify assembly rules (Götzenberger et al., 2012). According to the environmental filtering hypothesis, species lacking specific adaptations to local conditions are filtered from the community (Weiher et al., 1998; Cornwell et al., 2006). As a result, species with similar functional traits co-occur preferentially. If these traits are more similar among closely related species (phylogenetically conserved), closely related species should co-occur more often than expected by chance (Webb et al., 2002). Under the competition theory, the best local competitors are expected to exclude other species resulting in spatial or temporal partitioning of species distribution (Chesson, 2000; Grime, 2006). Eventually, this process can induce a selective pressure forcing the displacement of functional traits where sufficiently different species can coexist (limiting similarity) (MacArthur and Wilson, 1967; Wilson, 2007; Wilson and Stubbs, 2012). When functional traits are phylogenetically conserved, communities are expected to be composed by functionally dissimilar and phylogenetically unrelated species. Neutral drift of species abundance can also occur and support coexistence over extended periods (Hubbell, 2001). In this case, the functional and phylogenetic similarity among species is expected to be random.

In this paper, we explore the possibility of combining functional and phylogenetic analyses of assembly rules with ecological network approaches to go beyond the view of a single mechanism driving a whole assemblage. Our hypothesis is that co-occurrence networks constitute a directly observable outcome of species assembly whose structure results from different assembly rules. We focus here on two well-documented network structure: nestedness and modularity (Fortuna et al., 2010). Nestedness and modularity plays a key role for the stability of species-rich ecosystems and their response to global change (Bascompte and Stouffer, 2009). Modularity refers to the organization of a network into modules or groups where species co-occur more frequently within than among modules (Newman, 2006). Modularity can retain the impact of perturbations or land use changes within few modules thereby minimizing the impact on other modules (Krause et al., 2003; Teng and McCann, 2004). Well-known examples include pollination network in tropical high-altitude grasslands (Danieli-Silva et al., 2012) and the hummingbird–plant networks across the Americas (Martín González et al., 2015). Nestedness describes the organization of a network where species-poor assemblages are a subset of species-rich assemblages. It can make the community more robust to both random extinctions (Memmott et al., 2004; Burgos et al., 2007) and habitat loss (Fortuna and Bascompte, 2006). Nestedness was first described for insular fauna where island size strongly determines the total diversity. Examples for grasslands include temporal nestedness in Californian plant communities (Elmendorf and Harrison, 2009) or spatial nestedness in European butterfly communities (Öckinger and Smith, 2006). Assembly rules can change with the structure of the network (Bascompte et al., 2003; Bascompte and Stouffer, 2009). They can differ among modules in the case of a modular network or from the richer to the poorer assembly in the case of a nested network.

Here, we focus on the co-occurrence network of plant and orthopteran species in the grasslands of the French Jura Mountains. Semi-natural grasslands are biodiversity hotspots that provide important services to human societies such as food production or soil protection. These ecosystems typically host a large number of species over short spatial scales. Before

the Middle Ages, the Jura Mountains were mostly covered by forests. Silvopastoral practices have reorganized species co-occurrence networks leading to the creation of grassland and wood-pasture ecosystems (Buttler et al., 2009). Nowadays, human activities increasingly threaten grassland biodiversity and thereby the organization of ecological networks. Plants and orthopterans are key actors of grasslands ecosystems. Orthopteran communities constitute an important link within grassland food chain. They are important consumers of plant biomass (Deraison et al., 2015) and their richness and abundance can impact higher trophic levels such as birds (Hamer et al., 2006). As such they can mediate trophic cascades and their consequences on element cycling (Strickland et al., 2013). Plants provide resources and habitats to a broad range of species and they fulfill key functions (production of biomass) that sustain important services to human societies (cattle foraging). Studying the co-occurrences of plants and herbivore insects thus provides important information about the functioning of grassland ecosystems. Furthermore, understanding how plant and orthopteran species assemble can provide important insight about how future changes will influence grassland ecosystems.

We first verified whether the plant-orthopteran co-occurrence network has a nested and/or modular structure. As our study encompasses an altitudinal gradient and clear changes in agricultural practices (Mauchamp et al., 2014), we expect the plant-orthopteran co-occurrence network to have a modular structure that reflects this environmental heterogeneity (Olesen et al., 2007). We then verified our main hypotheses that the modular structure of the co-occurrence network reflects a complexity of assembly processes. More specifically, species coexistence in some modules is expected to result from a filtering effect of intensive agricultural practices while species coexistence in other modules is expected to result from biotic interactions such as competition for resources among plants or orthopterans. To do so, we assessed the changes in functional traits and assembly rules (null models of species functional and phylogenetic similarity) among modules. We also expected agricultural practices, soil conditions and spatial variables to have a strong importance for the modular structure of the network. We used variance partitioning to assess the importance of these variables for the whole network as well as for each module individually.

MATERIALS AND METHODS

Study Site and Sampling Design

The study was conducted in the NW part of the French Jura Mountains in an area located between 391 and 1195 m a.s.l. and characterized by a nemoral climate with a strong suboceanic influence (**Figure 1A**). The dominant soils are cambisols developed on limestone. Permanent grasslands cover about 22% of the total surface of the study area. They are mainly used for dairy farming and Protected Designation of Origin cheese production (mainly Comté cheese, a major economic sector, with constraining specifications for agricultural practices). Within this area, we targeted mesic grasslands that have not been plowed and sown at least for the 10 past years and where it was possible to delimit a 1000 m $\times$1000 m rectangular plot located on a flat area. These selection criteria allowed us to avoid potential biases due to slope or extreme soil conditions (excluding wet or dry grasslands) so as to focus on the effect of agricultural practices (grazing, mowing, fertilization) and climatic conditions (elevation) on plant and orthopteran communities. Overall, 24 farmers accepted to participate by indicating two parcels per

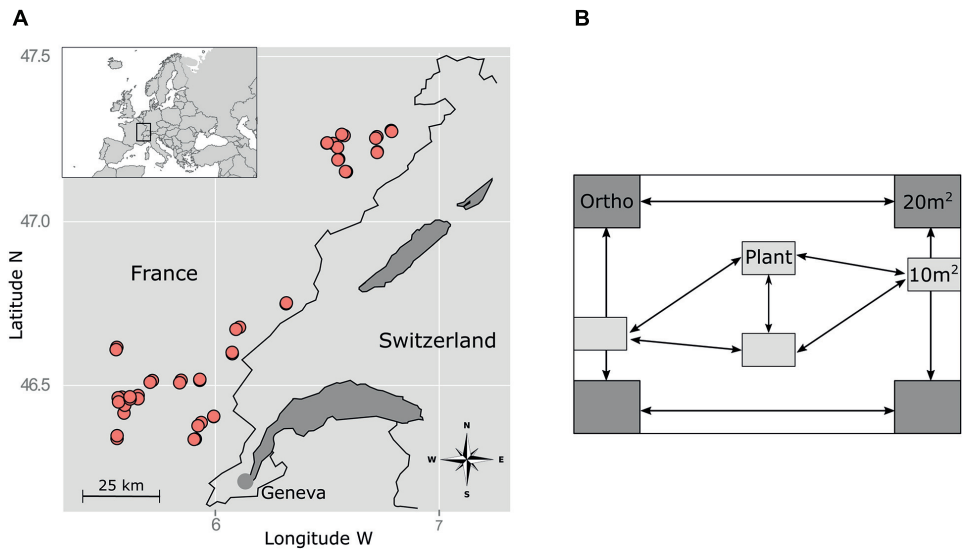


FIGURE 1 | Sampling design. (A) Location of the study area (upper left corner) and spatial distribution of the plots within the study area. **(B)** Sampling design within each plot. Plant and orthopteran communities were sampled in the same year (2011) and in the same plots but at different periods (following the shift in optimal development of vegetation and insect populations) and at different subplot locations to avoid disturbances by observers.

farm, one mainly used as pasture and one as hayfield, resulting in a total of 48 grasslands that met our criteria. The 48 grasslands encompassed a gradient of mowing and grazing practices (from strictly grazed to frequently mown including variations in grazing intensity and mowing frequency) as well as various fertilization regime (from no fertilizers to high input of fertilizers; fertilizer type: liquid or solid manure, organic or industrial fertilizers) (Mauchamp et al., 2014).

Agricultural Practices and Soil Surveys

Agricultural practices were assessed for each plot by interviewing the farmers. We used a questionnaire aiming at defining the defoliation regime and nutrient input regime. Questions concerned grassland management (mowing frequency, forage yield, grazing duration, livestock type, stocking rate) as well as the amount and type of fertilizers (liquid or solid manure, industrial fertilizers) applied during the year preceding sampling as well as during the last 10 years. Defoliation regime was assessed by the mean number of cuts per year (*cutting*, 0 in strictly grazed parcels), and by the stocking rate (*grazing*) expressed in livestock units days per hectare and per year (available for year 2011 only). The fertilization regime was evaluated by the mean amounts of available nitrogen brought per hectare and per year, by all potential sources (liquid and solid manure and industrial fertilizers), averaged over the 10 past years (*fertilizers*).

Soil surveys were carried out in each plot to assess soil texture and chemical composition. A total of eight soil subsamples were taken in each plot to account for within-plot heterogeneity. These samples were then pooled for analyses of total N, C/N and soil cation exchange capacity (CEC).

Insect and Plant Sampling

We sampled orthopterans in August–September 2011 in four 20-m^2 subplots located in the four corners of each 1000-m^2 plot (**Figure 1B**). In each subplot, we conducted 100 sweeps using a standard net of 40 cm in diameter. We then conducted 5 min of hand searching to target the remaining individuals. All adult individuals were frozen and identified to species level (Dehondt and Mora, 2013). Trait data were gathered in the literature (Hendriks et al., 2013; Gossner et al., 2015) (**Table 1A**).

The vegetation of the 48 selected grasslands was sampled in May–June 2011 in four rectangular subplots of 10 m^2 (4 m × 2.5 m). These plots were placed on the flattest area inside the parcel, presenting a homogeneous vegetation physiognomy and far from the parcel's margin. All vascular plant species observed in each plot were listed and the relative cover of each species was estimated using the seven degrees of the Braun-Blanquet's scale. Plant trait data were gathered in various databases (Jäger, 2000; Kühn et al., 2004; Kleyer et al., 2008; Klimešová and De Bello, 2009; Mauchamp et al., 2014) (**Table 1B**).

Phylogenetic Data

For orthopteran taxa, we searched DNA sequences of the cytochrome oxidase subunit 1 (COI) on Genbank for each species observed. When no sequences were available for the target species, we used species from the same genus as surrogate. Species from the same genus were available in all cases and we thus did not need to go to family level. We used the sequences of all species of the same genus as the target species to calculate an average phylogenetic distance between the target genus and the other species. We used ClustalX 2.1 (Larkin et al., 2007) and Se-Al 1.0al software (Rambaut, 1996, University of Oxford, Oxford, UK[1]) to align the sequences. We analyzed these datasets using a combined Bayesian Monte Carlo Markov Chain approach under BEAST 1.5.3 (Drummond and Rambaut, 2007). We then performed AIC-based selection of the model of nucleotide substitution using MrModeltest 2.0 (v 2.0, Evolutionary Biology Centre, Uppsala University, Sweden). Several family relationships were constrained according to Song et al. (2015) in BEAST to calibrate the rates of molecular evolution of each lineage. We consequently assessed the regional phylogeny by building an ultrametric maximum likelihood tree using mantid sequences (*Apteromantis aptera, Tamolanica tamolana* and *Ameles* sp.) as outgroup. We use the obtained tree to calculate the cophenetic distances among all pairs of species. We obtained the plant phylogenetic distance matrix from an ultrametric multiple-genes regional tree for the 197 plant species recorded in the study area (Mauchamp et al., 2014). We searched Genbank for data about two genes encoding chloroplast proteins (rbcL and matK).

[1]http://tree.bio.ed.ac.uk/software/seal/

TABLE 1 | Selected traits of (A) orthopteran and (B) plant species.

Trait	Short name	Values	Definition
(A)			
Habitat specificity	Habitat	0 = narrow, 1 = wide	Range of habitats of a species
Dispersal capacity	Dispersal	0 = limited, 1 = high	Capacity to disperse
Change in feeding regime	Feed_change	0 = no; 1 = yes	Change in feeding regime during life cycle
Egg deposition preference	Egg_deposition	0 = soil, 1 = plants	Preferred location for egg deposition
(B)			
Maximum height	Hmax	[cm]	Maximum height of a plant species
Leaf dry matter content	LDMC	%	% of leaf biomass remaining after desiccation
Seed mass	Seed_mass	[mg]	Seed mass in mg
Specific leaf area	SLA	0–1	Ratio of leaf area to dry mass

Co-occurrence Network

We classified all pairs of species as having positive, negative or random co-occurrences using the probabilistic model of Veech (2013) (**Figures 2A,B**). This model calculates the observed and expected probabilities of co-occurrence of all pairs of species and determines whether the observed values is lower or higher than expected by chance. In the case of a positive co-occurrence, two species are more frequently encountered together than expected by chance. To the contrary, two species have a negative co-occurrence when they are more frequently encountered alone than expected by chance. We focused on positive co-occurrences to build an undirected network. The final co-occurrence matrix included all species in rows and columns and was filled with 0 in the absence of positive co-occurrence and 1 otherwise. Species showing no positive co-occurrence were filtered from the data at this stage.

We assessed the nestedness and modularity of the resulting network (**Figure 2C**). Nestedness was estimated using the weighted index of Galeano et al. (2009) where 1 represents perfect nestedness and 0 no nested structure. The classification of species into modules was obtained using the algorithm of Dormann and Strauss (2014). Modularity was then measured with the Newman's Q index of modularity (Clauset et al., 2004; Newman, 2004) where values above 0.3 are good indicators of significant structuring of the network. We then assessed whether the observed nestedness and modularity values were lower or greater than expected by chance. To do so, we computed 9,999 permutations of the co-occurrence network and computed nestedness and modularity for each iteration. The resulting values provided a null distribution of nestedness and modularity values that was used to compute standard effect sizes and *p*-values. To minimize potential bias related to the chosen methodology, we compared the 'swap' (Gotelli and Entsminger, 2003), 'tswap' (Miklós and Podani, 2004) and 'quasiswap' (Miklós and Podani, 2004) permutation algorithms where row and columns sums are fixed in all cases.

Numerical Analyses

We assessed the contribution of each species to the structure of the co-occurrence network using a knock-out approach (**Figure 2C**). We removed all species one by one from the data and calculated the modularity and nestedness of the network. We subtracted the obtained values (n-1 species) from the initial values of modularity and nestedness ($n = 91$ species) to obtain Δ_i for each species *i*. Δ_i was transformed into Standardized Effect Size (SES_i) according to:

$$SES_i = \left(\Delta_i - \frac{\sum_{j=1}^{n} \Delta_j}{n} \right) \Big/ \sigma$$

where σ is the standard deviation of Δ_j.

We then used the plant and orthopteran functional and phylogenetic distance matrices to investigate whether assembly rules change with the structure of the network (**Figure 2D**). We

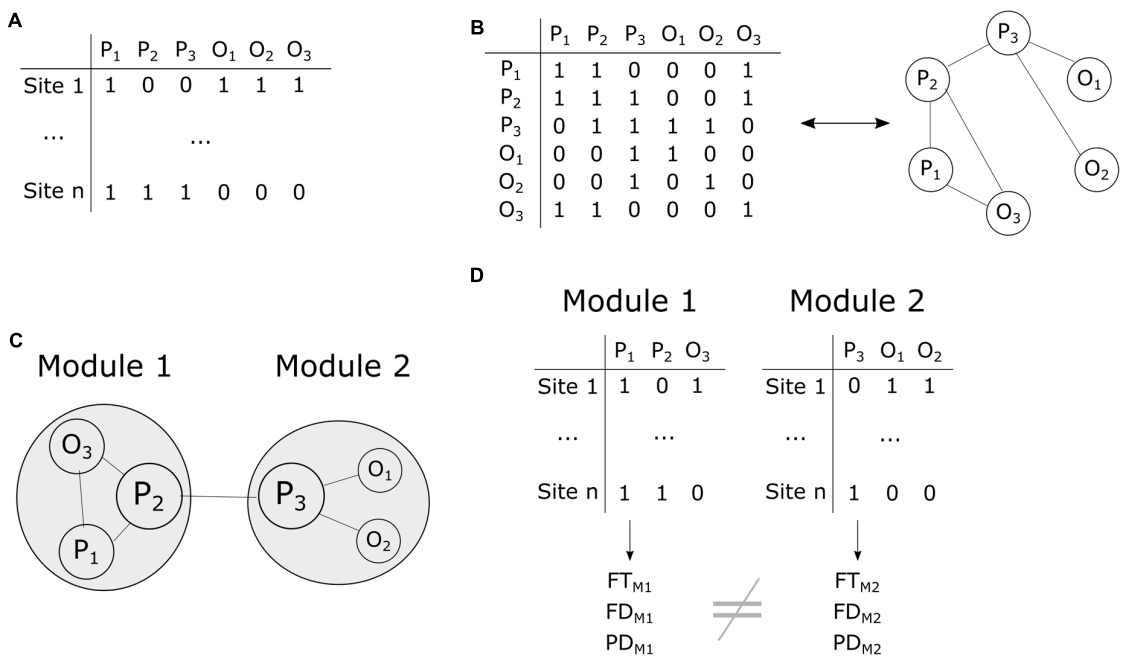


FIGURE 2 | Summary of the different steps of the co-occurrence data analyses. (A) The input site × species matrix contains information about species presence–absence (1-0) in each plot. P_1, P_2, P_3 denote plant species and O_1, O_2, O_3 orthopteran species. **(B)** The presence-absence matrix is transformed into a co-occurrence species × species matrix where 1 means positive co-occurrence. In turn, the co-occurrence matrix is used to build a co-occurrence network. **(C)** Assessment of network modularity and nestedness (here a hypothetical modular network with two modules is shown) as well as species importance for network structure (circle size is proportional to species importance). **(D)** Assessment of functional traits (FT), functional distances (FD) and phylogenetic distances (PD) within modules and comparisons among modules.

tested individually for the different parts of the network (the different modules in the case of a modular network or the species-rich and species-poor assemblies in the case of a nested network) whether functional and phylogenetic distances among species were greater or lower than expected by chance. To do so, we randomly attributed species to the different parts of the network and calculated the mean functional and phylogenetic distances for each network part. This procedure has the advantage of preserving the structure of the distance matrices. We applied a similar procedure to functional distance matrices computed using all traits (multiple trait) as well as to functional distance matrices computed with each trait individually (individual trait). We also used Kruskal–Wallis non-parametric tests to assess whether the distribution of plant and orthopteran functional traits changes with the structure of the network.

We compared this co-occurrence-network approach to an abundance-based approach. The goal here was to assess whether the rules underlying species co-occurrences and abundance are similar. In this case, we used the dispersion index of Laliberté and Legendre (2010) using the functional and phylogenetic distance matrices as input. We thereby obtained an index of functional dispersion (FDis) and an index of phylogenetic dispersion (PDis) that were used for null-model testing. We computed 9,999 permutations of the species abundance matrix using the same procedure as described above and calculated simulated values of FDis and PDis. We used the resulting distribution to calculate SES and *p*-values. This procedure preserves local abundance and diversity, yet attributing random abundance to species.

We also assessed to what extent spatial, soil and agricultural variables explain species abundance data. We computed variance partitioning based on RDA (Borcard et al., 2011) for all species (i.e., ignoring network structure) as well as for each module separately. Species abundance data were Hellinger-transformed prior to analyses (Legendre and Gallagher, 2001). To conduct variance partitioning analyses for each module, we first divided the initial species abundance matrix into five subsets each containing information about the species of a single module only. We then conducted variance partitioning for each of these subsets. We finally compared the proportion of the variance in abundance data explained by the three sets of environmental variables in the whole network to that in the different parts of the network.

Network analyses were done with packages "igraph" (Csardi and Nepusz, 2006) and "co-occur" (Veech, 2013) of R-3.2.1 (R Development Core Team, 2015). Packages "FD" (Laliberté and Legendre, 2010) and "picante" (Kembel et al., 2010) were used for functional and phylogenetic analyses, respectively. Package "vegan" (Oksanen et al., 2015) was used for variance partitioning. Network visual representation (**Figure 3A**) was done in Gephi (Bastian et al., 2009).

RESULTS

We sampled 22 and 197 species of orthopterans and vascular plants, respectively. The dominant and more frequently encountered species in orthopteran communities were

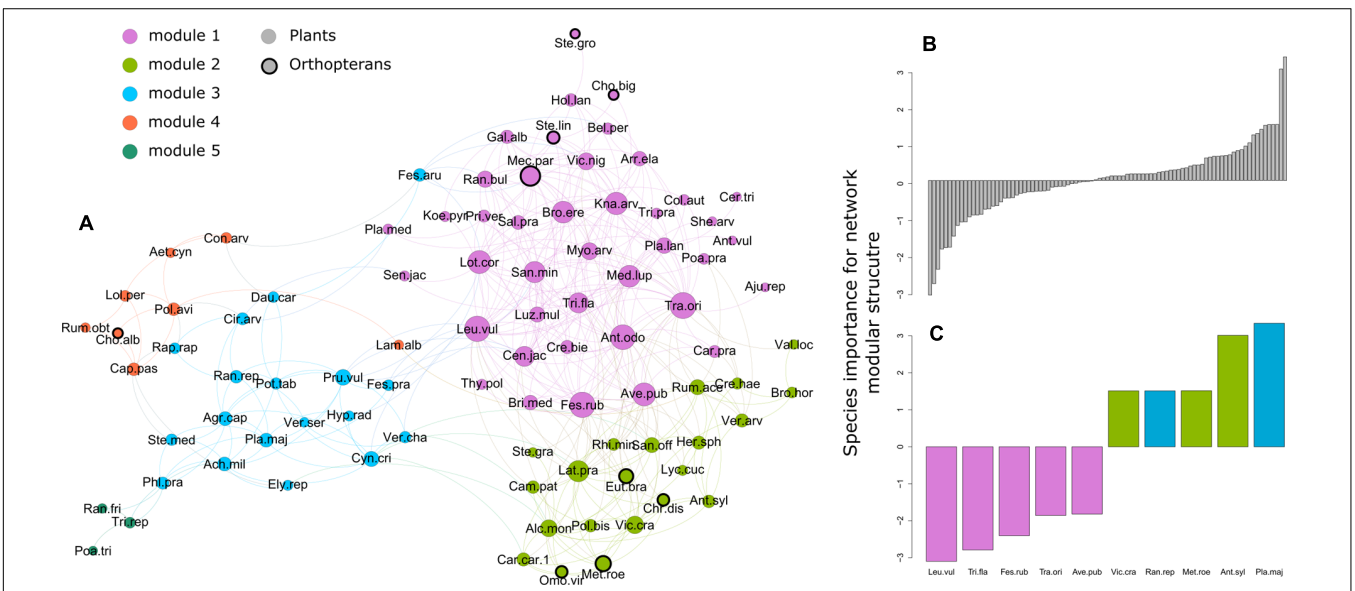


FIGURE 3 | (A) Plant and orthopteran species co-occurrence network in the permanent mesic grasslands of the French Jura Mountains. Points (edges) correspond to species and lines (vertex) to significant positive co-occurrence between two species. Point size is proportional to species degree (i.e., the total number of significant positive co-occurrences with the species). Colors show species membership to the five modules revealed by the modularity analysis. Abbreviation corresponds to the three first letters of the genus and species names following Supplementary Table S1 (online Supplementary Material). **(B)** Species importance for the modular structure of the network. Values on the *y*-axis are standardized effect sizes. Negative values indicate species with negative impact on modularity (i.e., the network becomes more modular when the species is removed) and positive values indicate species with positive impact. **(C)** Zoom on the five species with the highest negative and positive impact on modularity, respectively. Colors show species module membership.

Chorthippus parallelus and *Chorthippus biguttulus*. *Poa trivialis*, *Trifolium repens* and *Taraxacum officinale* were the dominant plant species.

After trimming species lacking positive co-occurrences, 82 plant and 9 orthopteran species remained. These represented 42 and 41% of the total number of plant and orthopteran species, respectively. The resulting network had a relatively high modularity (0.36), but a low nestedness value (0.29) (**Figure 3A**). Our permutation analyses revealed a significantly higher modularity than expected by chance (SES = 18.8; $P < 0.001$) with five groups of co-occurring species. To the contrary, the observed nestedness was significantly lower than expected by chance (SES = −5.2; $P = 0.01$). The most connected plant species (i.e., species having numerous significant positive co-occurrences with other species) were *Tragopogon orientalis, Leucanthemum vulgare, Festuca rubra and Anthoxanthum odoratum* in module 1, *Lathyrus pratensis*, *Vicia cracca* and *Alchemilla monticola* in module 2, *Prunella vulgaris* and *Cynosurus cristatus* in module 3. Orthopterans were present in module 1 (*Chorthippus biguttulus, Mecostethus parapleurus, Stenobothrus lineatus* and *Stethophyma grossum*) and 2 (*Chrysochraon dispar, Euthystira brachyptera, Metrioptera roeselii* and *Omocestus viridulus*) and in module 4 with *Chorthippus albomarginatus*. Species with the strongest positive influence on modularity were found in modules 2 and 3 (e.g., *Metrioptera roeseli*) while those with the strongest negative influence on modularity belonged to module 1 (**Figures 3B,C**).

Null-model tests on co-occurrence data revealed a significantly higher phylogenetic and functional distance among plants than expected by chance in modules 3 and 5, respectively (**Table 2A**). By contrast, the same analysis revealed a significantly lower functional distance than expected by chance in module 4 for multiple trait, SLA and LDMC (**Tables 2A,B**). Plant species in this module had a higher average SLA with a lower variance than any other modules (**Figure 4A**). Similarly, the three species in module 5 were phylogenetically less related than expected by chance. Null-model tests on abundance data without considering network structure revealed a lower functional distance among species than expected by chance for multiple traits, LDMC and seed mass (**Table 3A**). For orthopterans, null-model tests on co-occurrence data were limited to modules 1 and 2 where several species were present and revealed no significant pattern in functional or phylogenetic data (**Tables 2A,C**). The same was true for abundance data (**Table 3B**). This agrees with the lack of significant changes of functional traits among modules except for a marginally significant change in egg deposition strategy (**Figure 4B**). Nevertheless, orthopterans in module 1 tended to be generalists with broad environmental range and good dispersal capacity. In module 2, species were preferentially habitat specialists with intermediate dispersal capacity and

TABLE 2 | Null model analysis of plant and orthopteran functional and phylogenetic distances in the five species groups (Modules 1–5) revealed by the modularity analysis.

(A)	Functional distances				Phylogenetic distances			
	Plant		Orthopteran		Plant		Orthopteran	
	SES	*P*	SES	*P*	SES	*P*	SES	*P*
Module 1	−1.11	0.148	−0.26	0.363	0.33	0.60	−0.75	0.349
Module 2	−0.3	0.394	−0.35	0.375	**−2.20**	**0.03**	0.4	0.737
Module 3	**1.83**	**0.957**			0.07	0.49		
Module 4	**−1.77**	**0.028**			0.05	0.44		
Module 5	−0.02	0.553			**1.32**	**0.98**		

(B)	Hmax		LDMC		Seed_mass		SLA	
	SES	*P*	SES	*P*	SES	*P*	SES	*P*
Module 1	−0.32	0.366	1.02	0.848	−0.91	0.211	−1.46	0.074
Module 2	−0.46	0.351	−0.36	0.361	0.67	0.790	−0.93	0.147
Module 3	−0.38	0.332	1.09	0.853	0.73	0.759	1.88	0.948
Module 4	0.41	0.642	**−2.42**	**0.010**	−0.09	0.580	**−1.56**	**0.015**
Module 5	0.27	0.638	−0.60	0.306	−0.45	0.444	0.94	0.864

(C)	Habitat		Dispersal		Feed_change		Egg_deposition	
	SES	*P*	SES	*P*	SES	*P*	SES	*P*
Module 1	1.12	0.924	0.83	0.820	−0.92	0.271	−2.48	0.061
Module 2	−1.59	0.137	0.00	0.387	1.15	0.785	0.03	0.387

(A) Functional distances based on multiple traits. (B) Null-model analysis of plant functional distances based on individual traits. (C) Null-model analysis of orthopteran functional distance based on individual traits. SES, standardized effect size; P = P-values after 9,999 permutations of the distance matrices. Significant negative or positive SES are in boldface.

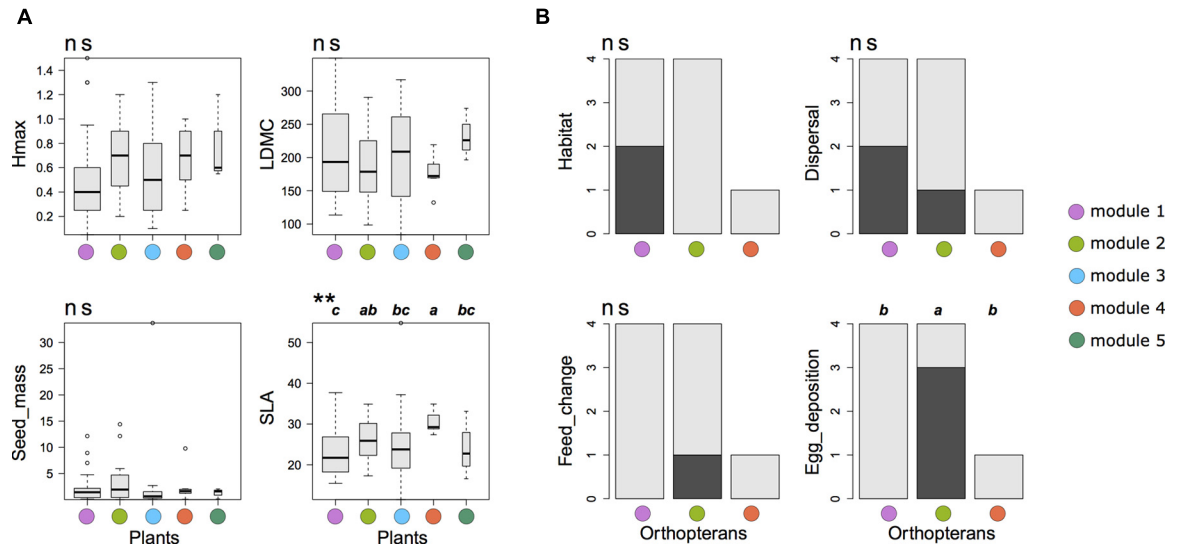


FIGURE 4 | Functional trait variations among the modules of the plant-orthopteran co-occurrence network of the French Jura Mountains. (A) Variation in plant functional traits. Hmax, maximal vegetative height; LDMC, leaf dry matter content; Seed_mass, seed mass; SLA, specific leaf area. Kruskal–Wallis non-parametric tests were used to assess significant differences among modules (**$P < 0.01$; ns $P > 0.05$). **(B)** Variation in orthopteran functional traits. The *y*-axis shows the number of species with a given trait value (light gray = 0 and dark gray = 1). *Chorthippus albomarginatus* was the only species present in module 4. Habitat: range of occupied habitats (0 = narrow; 1 = wide); Dispersal: capacity to disperse (0 = limited, 1 = high); Feed_change: change of feeding habits during life cycle (0 = no, 1 = yes); Egg_deposition: preferred location for egg deposition (0 = soil, 1 = plants). Differences were tested using Fisher's exact test. Only egg deposition showed a marginally significant difference among modules ($P = 0.09$).

TABLE 3 | Abundance-based null model analysis of plant and orthopteran functional and phylogenetic distances for the whole dataset.

(A)			(B)		
	Plants			**Orthopterans**	
	SES	***P***		**SES**	***P***
Multi-traits	**−1.72**	**0.022**	Multi-traits	0.45	0.713
Hmax	−0.56	0.31	Habitat	1.62	0.89
LDMC	**−1.67**	**0.04**	Dispersal	0.56	0.729
Seed_mass	**−1.21**	**0.028**	Feed_change	0.26	0.783
SLA	−0.15	0.505	Egg_deposition	−1.25	0.128
Phylogenetic	−1.06	0.132	Phylogenetic	0.09	0.705

(A) Plants. (B) Orthopterans. SES, standardized effect size; P = P-values after 9,999 permutations of the distance matrices. Significant negative or positive SES are in boldface.

changed their diet during life cycle and preferentially lay eggs in plants. This group was composed by species characteristic of mountain grasslands such as *Metrioptera roeseli*. Finally, *Chorthippus albomarginatus*, the only orthopteran in its module, is a habitat specialist with a low dispersal capacity.

Variance partitioning of plant and orthopteran abundance data revealed that spatial variables were good predictors of species distribution in all five modules as well as for the entire dataset (**Figure 5**). Agricultural practices were significant predictors of species abundance in modules 2, 4, and 5 as well as for the entire dataset. Soil variables were not significant predictors of species abundance within modules but showed a weak yet significant correlation to abundance data for the entire dataset.

DISCUSSION

Structure and Drivers of the Plant-Orthopteran Co-occurrence Network

The organization of the plant-orthopteran co-occurrence network in the grasslands of the Jura Mountains was strongly modular. Other examples of modular co-occurrence networks were found among soil microbes (Barberan et al., 2012; Banerjee et al., 2015). However, such organization was not encountered in plants and arbuscular mycorrhizal fungi (AMF) co-occurrence networks (Encinas-Viso et al., 2016). Contrary to this plant-AMF network where random encounters appear to drive species assembly, our results show that the interaction of multiple assembly rules can explain the modular organization of the plant-orthopteran co-occurrence network. For instance, environmental filtering, limiting similarity and neutral processes alternatively explain plant species coexistence within the different modules. To the contrary, within-guild interactions cannot explain the distribution of orthopteran species among modules. It follows that the distribution of orthopterans into modules most likely reflected that of plants as a result of biotic interactions (herbivory, refuges, reproduction sites). This modular structure is likely to shape the response of plant and orthopteran community assembly to future changes, for example,

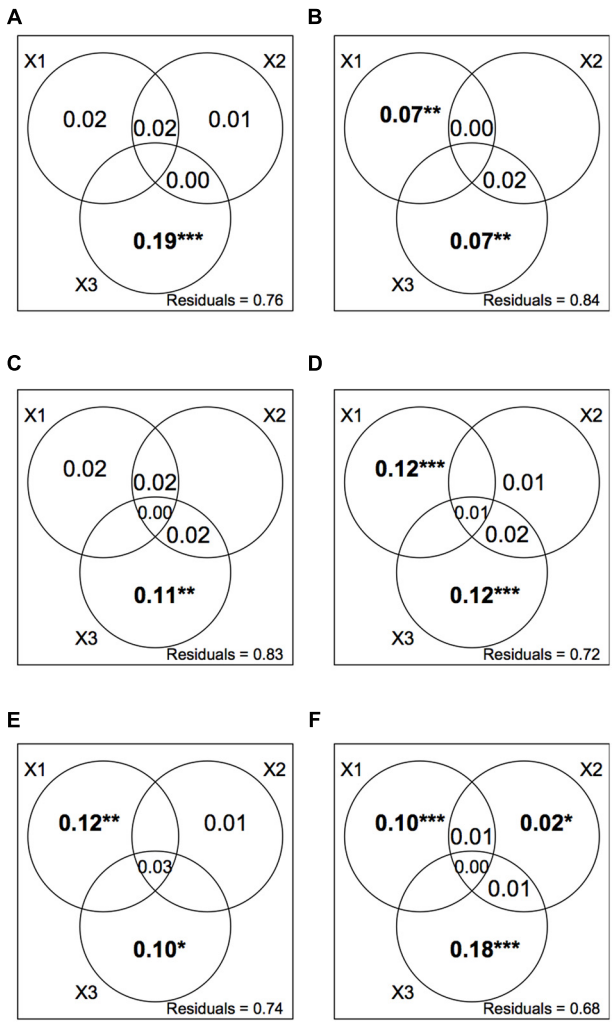


FIGURE 5 | Variance partitioning of plant and orthopteran abundance data explained by three sets of explanatory variables; X1: agricultural practices (*cutting,* the mean number of cuts per year; *grazing,* the stocking rate; *fertilizers,* the total amount of N input); X2: soil conditions (total N, C/N, CEC); X3: spatial location (longitude; latitude; elevation). We show the adjusted R^2 for all non-negative fractions. **(A–E)** Individual variance partitioning for modules 1– 5. **(F)** Variance partitioning for the whole network. Stars and bold typeface indicate significance (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

by limiting the negative impact of land-use changes to individual modules leaving other modules un-impacted. Moreover, a limited number of plant or orthopteran species had a disproportionate positive or negative importance for the modular structure of the network. Species with positive importance for the modular structure were generally less connected to other species than species with negative importance. These species are likely to have particular importance for the structure and functioning of grassland ecosystems (Olesen et al., 2007). Our analyses further revealed a complexity in the processes underlying species co-occurrences. For instance, within-guild processes appear to dominate the co-occurrence of plant species while that of orthopterans is best explained by bottom–up processes (i.e., interactions with plants). Below we provide more detailed explanations about how our results support this conclusion for plants and orthopterans, respectively.

In plants, functional trait analyses revealed that different assembly rules operate in the different modules. In module 4, where the functional distances among species were lower than expected by chance, all species had high SLA and low LDMC. This module was composed by seven plant species including *Rumex obtusifolius* and *Aethusa cynapium*. Species in this module are ruderal species able of rapid resource acquisition and are characteristics of grasslands where grazing intensity is high (Cruz et al., 2010). It is likely that species coexistence within this module results from the interplay of the environmental filtering effect of grazing and the competitive exclusion of species unable of rapid resource acquisition. This agrees with Tilman's Resource Ratio Hypothesis (Tilman, 1982; Miller et al., 2005) where resource acquisition rate determines species coexistence. Species in module 2 were phylogenetically more similar than expected by chance. This result suggests that environmental or biotic filtering forces species to share similar eco-evolutionary features. However, these features were not related to the four investigated traits. To the contrary, plant species co-occurrences were best explained by the limiting similarity process in module 3 where species were functionally less similar than expected by chance. In module 5, the lower phylogenetic relatedness than expected by chance can also be explained by the limiting similarity process. However, this process was not related to the selected functional traits. Species in module 1 show neither significant functional or phylogenetic convergence or divergence nor clear changes in mean functional traits. Such a pattern could result from neutral processes where ecological drift and historical (Fukami, 2015) and spatial contingencies are the main drivers of species assembly. For instance, Lau et al. (2015) showed that phylogenetic founder effect can determine the structure of interaction networks.

In orthopterans, the lack of significant convergence of functional and/or phylogenetic distance among species and the relative low importance of environmental variables suggests that species assembly is not the result of strong environmental filters or competitive interactions. However, changes in functional traits and more specifically in egg deposition strategy suggest that the distribution of orthopteran species into modules results from trophic and other vertical interactions with plants. For instance, species in module 2 tended to lay eggs more frequently in plants as opposed to soil. These species were preferentially encountered in higher elevation grasslands where the microclimate provided by plants could protect eggs from the more constraining environmental conditions (e.g., late freeze). Other types of interactions with plants could also explain the distribution of orthopterans into modules. For instance, plants provide orthopterans with food resources, reproduction and habitat sites and refuge against predators (Pellissier et al., 2011; Ibanez et al., 2013). More generally, co-evolution between plants and orthopterans constitutes another likely explanation for the distribution of orthopterans into modules. For example, it has been shown that the diversification of frugivorous vertebrates

was associated with plant fleshy fruit production (Fleming et al., 1987).

Species Importance for Network Structure

Our analyses further highlighted plant and orthopteran species with particular importance for the structure of the co-occurrence network. Species such as *Metrioptera roeseli* and *Anthriscus sylvestris* had a positive influence on modularity. In other words, the network would become less modular if these species go extinct. These species were found in modules 3 and 4. Species co-occurrence in these two modules was determined by different assembly rules (limiting similarity and filtering, respectively) suggesting that assembly rules are not strong determinant of species role for network structure. However, a common feature of these species is that they share few links with species from other modules. Following the terminology of Olesen et al. (2007) for bipartite interaction networks, these species could be referred to as module hub. Management plans specifically targeting these species are likely to maximize the modularity of the whole system and thereby its capacity to retain the negative impact of perturbations within one or few modules. By contrast, species such as *Leucanthemum vulgare* and *Festuca rubra* had a negative influence on network structure (i.e., the network would become more modular without these species). All of these species belong to module 1. They also tended to have more links than species with positive influence on modularity and were frequently linked to species from different modules. Theoretical studies have shown species interaction networks to be robust to the extinction of poorly connected species but to be sensitive to the loss of highly connected species (Sole and Montoya, 2001; Dunne et al., 2002b). As a result, the extinction of these highly connected species is likely to induce cascading effect within the network.

Differences with Abundance Data

The rules underlying species assembly differed between co-occurrence and abundance data. Null model analyses suggest that plant abundance was strongly determined by seed mass and LDMC. Species with lower seed mass and LDMC such as *Poa trivialis* reached higher abundances. This result likely reflects a strong competition for space where species producing large propagule numbers and capable of rapid colonization and resource exploitation are dominant. To the contrary, phylogenetic or functional patterns could not explain the abundance of orthopteran species. Here, the two dominant species shared lower elevation sites with *Chorthippus albomarginatus* dominating in predominantly grazed grasslands and *Chorthippus biguttulus* dominating in predominantly mowed grasslands. In higher elevation sites, *Metrioptera roeselii* became dominant most likely because of better adaptations to the more constraining abiotic conditions such as a change of feeding regime during its development or its preference for laying eggs in the vegetation. Finally, RDAs showed that the overall abundance of plants and orthopterans was significantly impacted by agricultural practices and spatial variables. This agrees with previous results obtained for plants in the same system (Mauchamp et al., 2014). Interestingly, the impact of agricultural practices was only significant in modules 2, 4, and 5. It is therefore likely that species in these modules will retain most of the impact of changes in agricultural practices such as intensification leaving other species not or poorly impacted (Krause et al., 2003; Teng and McCann, 2004).

CONCLUSION

The combination of co-occurrence network analysis, functional and phylogenetic analyses and multivariate analyses of abundance data constitutes a powerful tool to understand the drivers of species assembly. We highlighted a complexity of processes related to the modular structure of the plant-orthopteran co-occurrence network that differs from those explaining species abundance. We also showed that the modular structure of the network is likely to determine how changes in agricultural practices will influence plant and orthopteran communities. The next step is to understand the importance of this modular structure for the long-term maintenance of grassland ecosystem structure and functions as well as to develop tools to integrate network structure into models to improve their capacity to predict future changes.

AUTHOR CONTRIBUTIONS

FG gathered species data. BF gathered functional and phylogenetic data. AM conducted phylogenetic analyses. BF and FG conducted the numerical analyses of the data. All authors contributed to writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01224

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Impact of Grassland Reseeding, Herbicide Spraying and Ploughing on Diversity and Abundance of Soil Arthropods

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In order to determine the interactive effect of reseeding, herbicide spraying and ploughing on soil fauna communities, we conducted a grassland reseeding experiment combined with pre-reseed management to examine how with the whole reseeding process affects soil faunal composition. Sampling occasions and exact treatments were as follows: (1) before chemical herbicide spray; (2) after spray but before ploughing; (3) after ploughing but before reseeding; and (4) after 1 year of recovery. Our results demonstrate that, Acari and Collembola were the two soil fauna taxa with the highest abundance and accounted for around 96% of the relative total abundance among the various managements. Herbicide application tended to increase soil invertebrate abundance. Conversely, subsequent ploughing significantly reduced soil invertebrate abundance and had an obvious negative effect on soil primary and secondary decomposers, which were mainly due to the variations of Acari (especially Oribatida) and Coleoptera group abundance. Moreover, reseeding also reduced the individual number of the groups mentioned above, and favored those predators with a larger body size and individual weight. After 1 year recovery, Collembola abundance recovered to the pre-treatment levels, while with Arthropod and Acari groups were still fluctuating.

Keywords: soil fauna community, herbicide, ploughing, reseeding, arthropods

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INTRODUCTION

The soil fauna account for a large part of the global biodiversity (Kremen et al., 1993) and play key roles in many ecosystems (García et al., 2010; Carrillo et al., 2011; Basset et al., 2012) because they directly or indirectly influence soil function (Diekötter et al., 2010). For example, approximately 90% of aboveground primary production, in terrestrial ecosystems, enters the belowground system (Gessner et al., 2010) where the soil biota undertake the decomposition and mineralization of soil organic matter (Bernard et al., 2012).

The perennial nature of grasslands means that interactions between the plant and the soil are crucial in regulating soil processes (Murray et al., 2012). The perenniality of grassland ecosystems implies that they generally have a relatively stable and permanent plant cover which provides a secure habitat for abundant and diverse soil invertebrate fauna that contribute to effective soil functioning. Alongside this, grasslands tend to have a high turnover of root and shoot material

than in other ecosystems and this, together with animal inputs, results in a relatively high level of organic matter content. This in turn allows grassland to support numerous and diverse biota, important for soil function (Murray et al., 2012). Soil fauna within grazed grassland break down both the labile and recalcitrant plant compounds releasing the nutrients bound up within them, so that they can be exploited by the plant (Wardle, 1999) and the biogeochemical cycling continues (Wall et al., 2010).

Although grasslands generally provide a stable soil environment, agricultural grassland management practices such as reseeding can affect sward structure and plant species composition (Celaya et al., 2007; García et al., 2010) and, probably more importantly, the soil structure and habitat. Therefore, such interventions in these systems can have a knock-on effect on the associated soil fauna (Gibson et al., 1992; Dennis et al., 1998, 2001, 2008). The soil fauna has been shown to be sensitive to changes in soil conditions (Vasconcellos et al., 2013) and soil management practices can also have an effect on the energy channels (bacterial feeding channel and fungal feeding channel) and soil food webs (Doblas-Miranda et al., 2008; Maharning et al., 2009; Strickland and Rousk, 2010). For example, in cropping systems, conventional tillage is thought to promote the bacterial energy channel in the soil food web by the redistribution of plant residues within the soil during ploughing; in comparison, no tillage systems are thought to promote the fungal energy channel and the immobilization of plant nutrients (Hendrix et al., 1986). Types of tillage also had conflicting effects on soil arthropods, for example, Petersen (2002) showed that conventional ploughing reduced the collembolan population more than the non-inverting tillage does in upper soil stratum, while the two tillage treatments resulted in similar population changes for most collembolan species when take the whole soil horizon into considered. Studies involving herbicide application also had distinct effects. Lins et al. (2007) studied the effects of different herbicides on Collembola, and found that the use of atrazine and 2,4-D significantly decreased Collembola diversity but it depended on the handling time in a no-till soil preparation system. Nevertheless, Greenslade et al. (2010) found that herbicides have no significant effect on surface-active arthropods although Collembola were more affected than Formicidae in the short term. Some soil faunal groups (e.g., spiders, harvestmen) and some ground beetles are known to react strongly to such changes in microhabitat conditions and are subsequently often used as indicators of the effects of management practices (Hillyard and Sankey, 1989; Bell et al., 2001; Rainio and Niemelä, 2003). However, knowledge of such interactions occurring between the faunal community is limited, because the factors responsible for this high diversity of soil animals on small spatial scales are still not fully understood (Maraun et al., 2011). This applies especially to the high α-diversity, which implies the existence of a large number of niches in a very small area (Maraun et al., 2007), possibly because below-ground animal taxa are generalists that inhabit wide niches. In this study we determine the impact of perturbation (herbicide, ploughing), during grassland reseeding on the soil fauna and its subsequent recovery.

MATERIALS AND METHODS

Study Site Description and Experimental Design

The study site was located at the North Wyke Farm Platform (NWFP) in the South West of England (50°46′55″N, 3°55′1″W) and is fully described in Orr et al. (2016). The vegetation is permanent grassland dominated by perennial ryegrass (*Lolium perenne* L.) and creeping bent (*Agrostis stolonifera* L.); the soil is classified as clayey pelo-stagnogley developed from located mainly under gentle low lying slopes (Hallsworth series, Harrod and Hogan, 2008). The main properties of the soil were organic carbon 3.7%, nitrogen 0.5% and phosphorus 0.1%, with a clay content of 38%, bulk density of 0.99 g cm^{-3} and a pH of 5.3 (Harrod and Hogan, 2008). Average annual rainfall at the site is 1085 mm (± 8.5), average air temperature is 9.8°C (± 0.65); yearly total sunlight is 1419 h (± 38) and average soil temperature is 10.9°C (± 0.77; Crotty et al., 2012).

The main aim of this study was to determine the impact of perturbation, during grassland management (reseeding, herbicide, ploughing) on the soil faunal composition. Four field permanent grasslands were chosen (**Figure 1**) were due to be reseeded. Prior to reseeding all the fields were under the same management regime, receiving 200 kg N per annum. In June 2013 the were sprayed with glyphosate (Glyphosate 360, Dow Agrosciences, Hitchin, UK), 6 weeks later the fields were ploughed and a seedbed prepared with subsequent reseeding in August 2013.

Soil Sampling

Soil samples were collected four times during the study: (1) before chemical herbicide spraying (designated S1, 26th June), (2) after spraying but before ploughing (designated S2, 3th July), (3) after ploughing but before reseeding (designated S3, 24th July), and (4) after 1 year of recovery (designated S4, 26th August, 2014). Intact soil cores (8 cm diameter, 10 cm deep; weighing on average 1.2 ± 0.02 kg, wet weight) were taken from the four fields. Overlaying the NWFP is a GPS defined 50 m grid and on each sampling occasion, six grid locations were randomly selected and a single soil core was collected at each point. Each individual core was stored within an individual Sun bag (Sigma-Aldrich, St. Louis, MO, USA). Post-extraction the invertebrates from each field were amalgamated to provide a single sample for each field for each sampling occasion. The soil characteristics for each field are given in **Table 1**.

Invertebrate Extraction and Separation

The cores were placed on a Tullgren funnel system (Burkard Manufacturing, Co., Ltd, Rickmansworth, UK; mesh 5 mm) and were collected in a saturated salt solution. The cores were held in the funnels for 14 days; invertebrate collections were sorted, identified and counted under a microscope (Crotty et al., 2014). These were as follows the four main Collembola orders comprised Entomobryomorpha, Poduromorpha, Neelipleona and Symphypleona, the four main Orders of soil dwelling Acari comprising Astigmata, Mesostigmata, Oribatida and

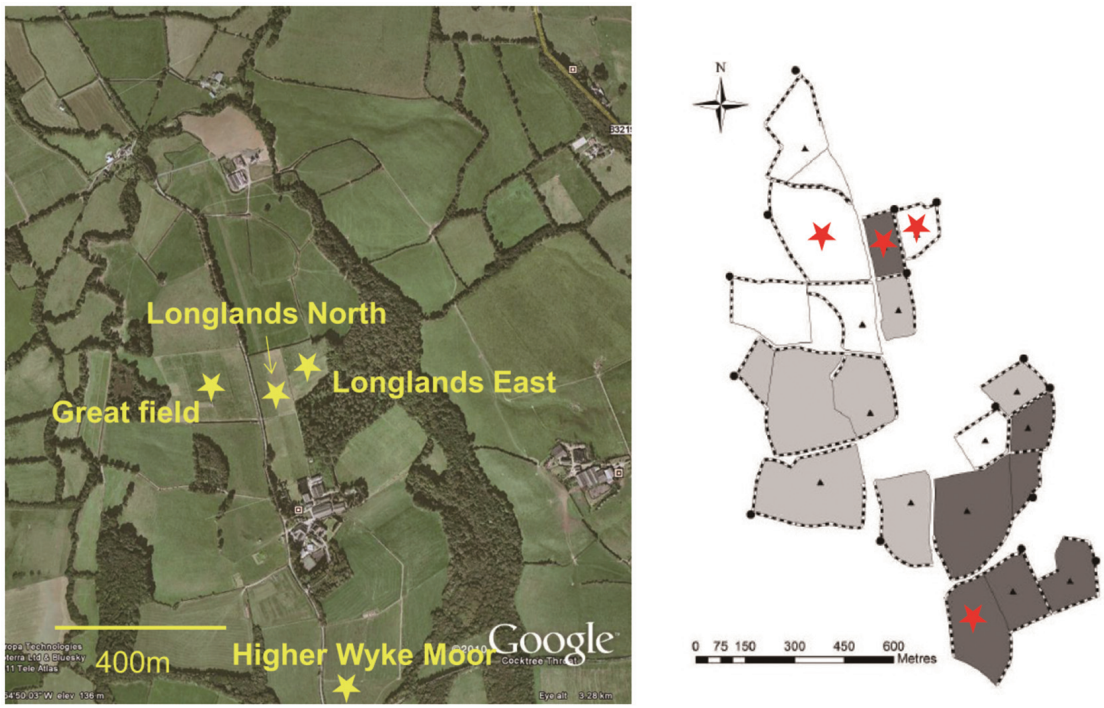


FIGURE 1 | Distribution of grassland management fields of the Rothamsted Research (North Wyke) in South-west UK. Samples were collected from four grassland management field: Higher Wyke Moor (HWM), Longlands North (LN), Great Field (GF), and Longlands East (LE) (Provided by Prof. Philip J. Murray).

TABLE 1 | Soil physicochemical properties of the four different grassland fields.

Field	pH value	Bulk density (g cm^{-3})	Soil organic matter (SOM, g kg^{-1})	Total N (% of DM)	Total C (% of DM)	C/N
Higher Wyke Moor	5.33 ± 0.35 c	0.85 ± 0.04 b	9.30 ± 0.88 a	0.44 ± 0.05 a	3.81 ± 0.52 a	8.73 ± 0.28 a
Longlands North	5.53 ± 0.04 bc	0.91 ± 0.06 ab	6.78 ± 0.41 c	0.43 ± 0.02 a	3.12 ± 0.07 b	7.34 ± 0.20 c
Great Field	5.71 ± 0.19 b	0.97 ± 0.06 a	7.90 ± 0.37 b	0.44 ± 0.02 a	3.52 ± 0.20 ab	7.98 ± 0.21 b
Longlands East	6.15 ± 0.06 a	0.96 ± 0.03 ab	9.35 ± 0.52 a	0.44 ± 0.03 a	3.60 ± 0.32 ab	8.20 ± 0.39 ab

Data are mean values ± SE (n = 6). Significant differences among grassland managements within each variable were tested using Duncan's multiple range test ($P < 0.05$) and are indicated by different letters.

Prostigmata, where possible, these were further identified to a higher taxonomic level (Supplementary Table S1). All other invertebrates collected were identified to Order level, apart from the Coleoptera where the majority were identified to Family level – (i.e., Carabidae, Chrysomelidae, Cuculionidae, Elateridae, Ptilidae, and Staphylinidae. Diptera were sorted to Order level apart from Tipulidae larvae which were analysed separately.

Trophic level and Acari/Collembola grouping were determined according to Crotty et al. (2014). Trophic 0 (T0) represents herbivores. Trophic 1 (T1) represents primary decomposers. Trophic 2 (T2) represents secondary decomposers. Trophic 3 (T3) represents micro-predator. Trophic 4 (T4) represents macro-predator. In this study, for the Acari, the primary decomposers (T1) comprised Ixodida (Ixodes) and Oribatida (Astigmata); Secondary decomposers (T2) contained Oribatida (Brachypyline and Macropyline), Prostigmata (Anystina, Heterostigmata, Parasitengonina, and Raphignathina); Micro-predators (T3) included the Mesostigmata (Gamasina, Uropodidae). The Collembolan fauna was comprised of the T0 Herbivores Symphypleona (Arrhopalitidae, Bourletiellidae, Dicyrtomidae, Mackenziellidae, Sminthuridae, Sminthurididae, Sphyrothecinae, and Sturmiidae); Primary decomposers (T1) Folsomia and Neelipleona (Neelidae); Secondary decomposers (T2) contained Actaletidae, Entomobryomorpha (Entomobryidae and Isotomidae), Poduromorpha (Brachystomellidae, Hypogastruridae, Onychiuridae, Poduridae, and Tullbergiidae).

Data Analysis

Soil faunal taxonomic richness (*S*) was represented by the number of taxonomic groups at each sampling occasion. All data were presented as mean ± standard error, unless otherwise stated. All statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Canonical correspondence analysis (CCA; model choice depending on length of first gradient) was performed to analyse the influence of soil management and environmental factors with respect to the soil fauna community

composition. Principal component analysis (PCA) was explored to analyze the influence of field management and sample time on soil fauna community structure. Ordination analyses and hypothesis testing were conducted in CANOCO for Windows v. 4.5 (ter Braak and Šmilauer, 2002). The species data matrix used in CCA were derived from the original individual number data. To meet the assumptions of normality and homogeneity, the data were transformed if necessary by arcsine, square root, or $\log_{10}(x+1)$.

RESULTS

There was a significant increase in total invertebrate abundance (**Figure 2**) at S2 ($P < 0.05$) followed by a significant reduction post-ploughing (S3, $P < 0.05$) where the lowest abundance was recorded. After 1 year (S4) the total abundance had recovered to pre-treatment levels. Although, the total abundance recovered to previous levels, the total biomass was greater at S4 (**Figure 2**). The Shannon diversity index (H'), Richness (soil fauna Taxon number, *S*) and Evenness index (*E*) were used to analyse impacts of sampling time on diversity of soil fauna communities (**Figure 3**). There was no significant difference in H' over the first three sampling occasions, but it was significantly ($P < 0.05$) greater than at S4 1 year later post-reseeding. There was a significant ($P < 0.05$) increase in *E* after ploughing which was maintained through to the following year. Taxon richness was greatest after the spray application with a significant ($P < 0.05$) reduction due to ploughing, however there was a significant ($P < 0.05$) recovery over the growth of the reseeded pasture back to pre-ploughing levels.

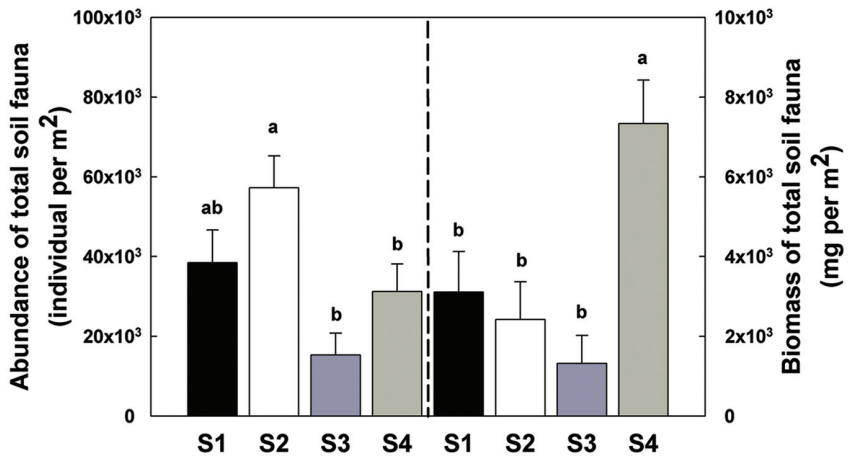


FIGURE 2 | Abundance and biomass of total soil fauna among sample times. Bars represent mean values ± SE ($n = 24$ in abundance; $n = 4$ in biomass). Different lowercase letters represent significant differences among sample times ($P < 0.05$) using one way ANOVA test and Duncan's multiple range test.

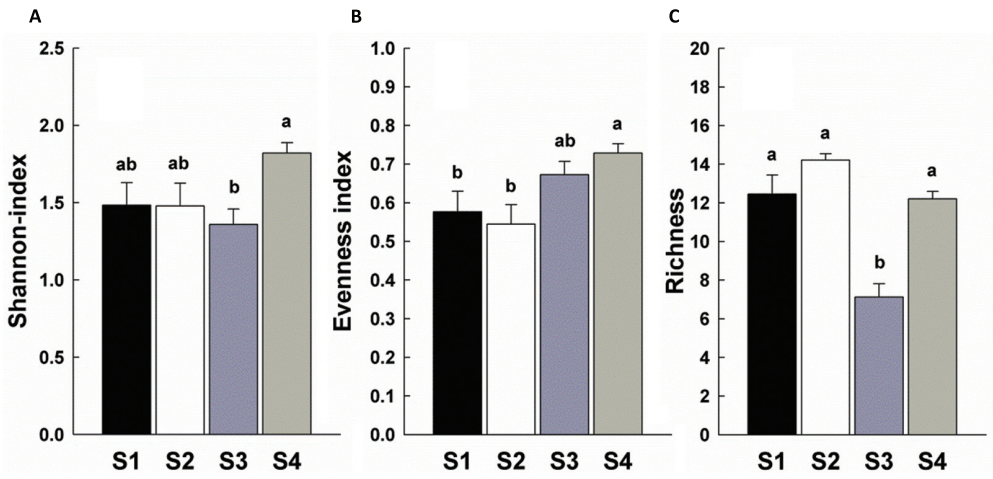


FIGURE 3 | Diversity indices of soil fauna community among sample times. Bars represent mean values ± SE ($n = 24$). Different lowercase letters represent significant differences among sample times ($P < 0.05$) using one way ANOVA test and Duncan's multiple range test. **(A)** Shannon-Weiner index; **(B)** Evenness index; **(C)** Richness.

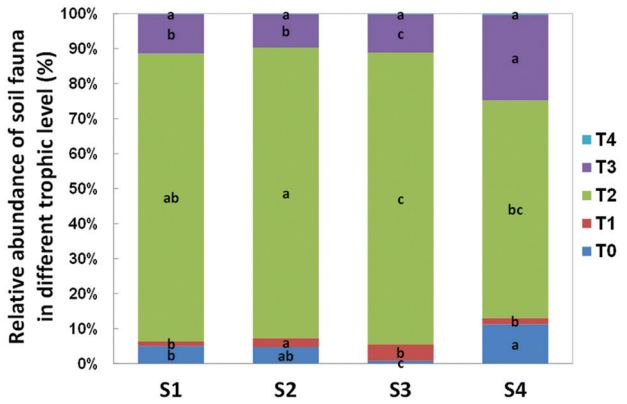


FIGURE 4 | Relative abundance of soil fauna in different trophic level detected in each sample time. T0 represent herbivores; T1 represent primary decomposers; T2 represent secondary decomposers; T3 represent micro-predator; T4 represent macro-predator. S1 represent sample time 1, before herbicide spray; S2 represent sample time 2, after spray and before plough; S3 represent sample time 3, after plough and before seed; S4 represent sample time 4, after seed. Bars represent stacked relative abundance ratio ($n = 24$). Different lowercase represent significant differences among sample times of each trophic level ($P < 0.05$) using one way ANOVA test and Duncan's multiple range test.

In terms of individual number and relative abundance of each trophic level, the secondary decomposers were the most abundant group (**Figure 4**). The micro-predators (T3) were the second most abundant group in this study. At S4, 1 year post-reseed, the proportion of this group was significantly greater than in previous samplings.

Acari and Collembola were the two most abundant soil fauna groups in our study, making up over 90% of the total soil faunal community. The Collembola population was significantly reduced by ploughing, but had recovered 1 year later (S4). The Acari populations were also reduced by ploughing, but different trophic groups showed different recovery patterns. The decomposer population failed to recover, but the predaceous mites did recover previous population levels (**Figure 5**). PCA analysis of the soil fauna community versus sample times showed a significant effect of sample time on soil fauna community and the different taxonomic groups (**Figure 6**). Most data from S1, S2, and S3 were distributed mainly along the x axis, the S4 was separated from most of points along the y axis. Acari had a positive correlation with S2 and S1, Collembola was positive correlated with S2 and S4.

The significance of soil chemical variables in relation to the soil fauna major groups was explored using CCA (**Figure 7**). The Acari were positively correlated with soil pH whilst the Collembola and Coleoptera were positively correlated with soil total carbon content, total C/N ratio and soil bulk density.

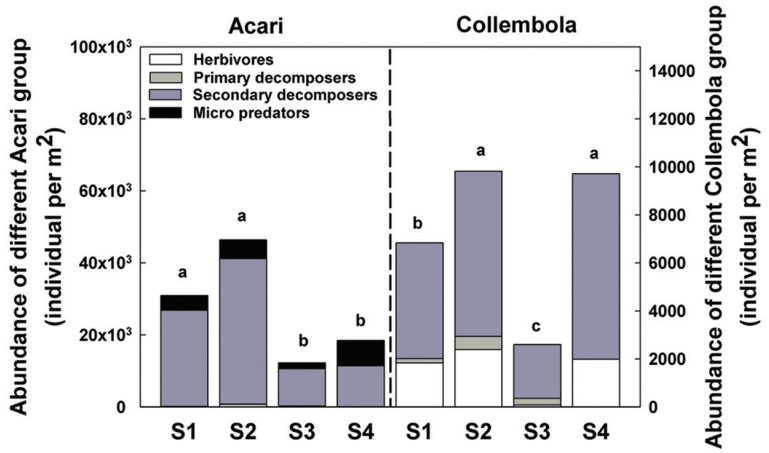
DISCUSSION

Soil invertebrate communities can be affected by fertilization, tillage regimes, or other grassland management (Zhan et al., 2014; Zhu and Zhu, 2015). In this study, the Acari were the numerically dominant soil invertebrates in this study. The Acari covened most trophic groups; primary decomposers, secondary decomposers and micro-predators in this study. This was due to them encompassing a broad range of feeding guilds, including both specialized and polyphagous predators, parasites, herbivores, fungivores, microbivores, detritivores, scavengers, and omnivores (Krantz and Lindquist, 1979; Lindquist, 1979; Walter, 1987). The most abundant groups were the Oribatida and Mesostigmata. The Oribatid Brachychthonioidea were the numerically most abundant and represented between 24.5% (at S4) and 84.5% (at S3) of total soil fauna in this study with a mean value of 58.8% relative abundance. This result was similar to some studies conducted in Canada, which found that the members of Brachychthoniidae dominate the oribatid mite community in fescue grassland of southern Alberta (Clapperton et al., 2002; Osler et al., 2008). The Oribatida have been reported to be one of the most numerically dominant arthropod groups in the organic horizons of most soils (Norton, 1985), and feed on a wide variety of particulate matter including living and dead plant and fungal material, lichens and carrion (Siepel, 1990). The Mesostigmata was the second most dominant Acari group in this study and these have been shown to be the numerically dominant predators in soil and litter of grassland ecosystems (Behan-Pelletier and Kanashiro, 2010; Crotty et al., 2014). Mesostigmata primarily feed on nematodes, Collembola, soft-bodied mites, insect larvae, and small insects, and they respond rapidly to increased prey in the habitat (Behan-Pelletier and Kanashiro, 2010).

Collembola can occupy all the trophic levels in belowground detritus food-webs (Moore et al., 1988) and together with Acari usually account for around 95% of the microarthropods in soils (Seastedt, 1984). Our findings confirmed this and our results showed that plant residue improvement (after herbicide spraying, S2) could lead to an increase in the abundance of the Collembola, possibly due to the fact that, although they can occupy all trophic levels most Collembola tend to be either microphages, feeding on soil microflora, and/or detritivores, scavenging on dead organic matter and plant litter (Bardgett et al., 1993).

At the outset of the study, there had been no gross perturbation for many years and consequently the soil faunal community was in a stable condition. After the herbicide application (S2), the total number of soil invertebrates peaked (approximately 230000 individuals per m^2). Similar to some previous studies. Greenslade et al. (2010) found that herbicides had negligible effects on ants and springtails in an Australian wheat field due to having less exposure in surface soil and hydrophobic structure of fauna body. Lins et al. (2007) pointed out that Acari and Collembola could use herbicides to breed themselves until the decomposition product occurs.

The increasing amount of dead material favored the oribatid mites Brachypyline and Macropyline and the Collembola all of which are among the most important decomposers and provide food source for other soil invertebrates (Norton, 1985; Siepel, 1990). The comminution of the dead plant material by these organisms can impact on the habitat in ways that facilitate microbial activity (Eisenhauer et al., 2007). The increased numbers of herbivores and decomposers, also promoted the



Sample time	Acari Primary decomposers	Acari Secondary decomposers	Acari Micro predators
S1	165.7 b	26673.7 ab	4083.9 b
S2	762.1 a	40391.6 a	5235.3 b
S3	231.9 b	10404.4 b	1598.8 c
S4	74.6 c	11323.9 b	7041.2 a

Sample time	Collembola Herbivores	Collembola Primary decomposers	Collembola Secondary decomposers
S1	1830.7 a	182.2 b	4821.1 b
S2	2385.7 a	555.1 a	6875.5 ab
S3	82.8 b	273.4 b	2244.9 c
S4	1988.1 a	0.0 c	7720.5 a

FIGURE 5 | Abundance of different Acari and Collembola group among sample times. Bars represent stacked sum values of different Acari/Collembola groups. Bars represent stacked mean values ($n = 24$). Different lowercase letters represent significant differences among sample times ($P < 0.05$) using one way ANOVA test and Duncan's multiple range test. Divergence analysis was listed in table, different lowercase of each row represented significant difference among sample times in given Acari/Collembola groups.

abundance of some micro-predators such as Mesostigmatid mites (Supplementary Table S1; **Figure 8**).

After ploughing (S3), there were significant reductions of soil invertebrate individual number and biomass. Total soil invertebrate number decreased from approximately 23000 to 61000 individuals per m^2 and total arthropod biomass decreased from 9680 to 5280 mg per m^2. This could be attributed to the greater impact of ploughing on the larger arthropods such as the Coleoptera and Diptera. Both mites and Collembola have been used as bioindicators of soil quality due to their high sensitivity to disturbances (Prasse, 1985; Hopkin, 1997), and both these groups declined sharply in number with ploughing. Ploughing influences the distribution of resources. Thus, the fauna in the deeper horizon is enriched by a surviving fraction of the surface fauna which to some extent compensates for the mortality due to abrasion, etc. On the other hand, the less abundant fauna of deeper soil layers are translocated to the surface layers where they are more exposed to drought and other microclimatic extremes (Petersen, 2002).

After reseeding (S4), and after all fields had had 1 year of recovery, the total soil invertebrate number and biomass increased (total number from approximately 61300 in S3 to 125000 individuals per m^2 in S4; total biomass from 5280 in S3 to 29300 mg per m^2 in S4). Here, the Collembola populations recovered to their pre-treatment levels. Although, the Acari populations appeared to recover, when broken down the decomposer populations remained low, but the predatory mites significantly increased showing a significant increase in the predator/prey ratio at this time point (**Figure 4** and Supplementary Table S2), indicating the relative instability of the community.

The CCA indicates that soil invertebrate groups as Acari, Collembola, Coleoptera, Diptera, and other taxa are influenced by the soil pH value, bulk density, soil total carbon and nitrogen content, and total C/N ratio. Similar results have been reported by other authors, who concluded that a pH close to neutral is optimal for Acari (Bedano et al., 2006). Soil total carbon content, total C/N ratio and bulk density showed a strong relationship with arthropod groups such as Collembola and Coleoptera. Consistent

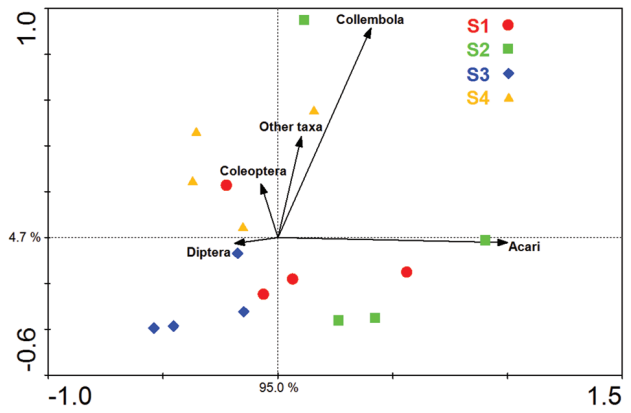


FIGURE 6 | Principal component analysis (PCA) of the soil fauna community and major soil fauna groups. S1 represent sample time 1, before herbicide spray; S2 represent sample time 2, after spray and before plough; S3 represent sample time 3, after plough and before seed; S4 represent sample time 4, after seed.

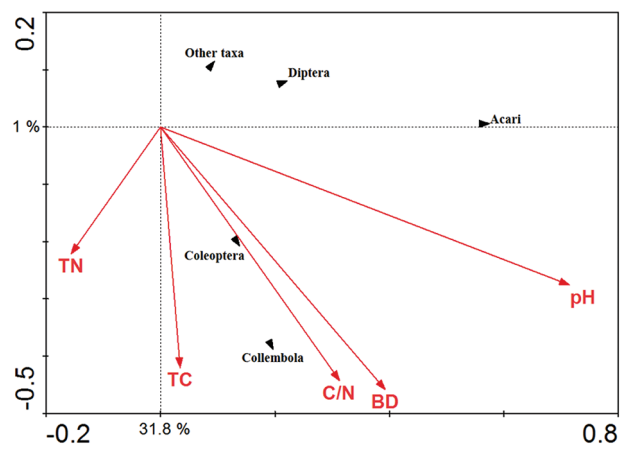


FIGURE 7 | Canonical correspondence analysis (CCA) of the major soil fauna groups in response to vectors of significant soil chemical properties. TN represented total nitrogen content; TC represented total carbon content; C/N represented total carbon content/ nitrogen content; BD represented soil bulk density; pH represented soil pH value.

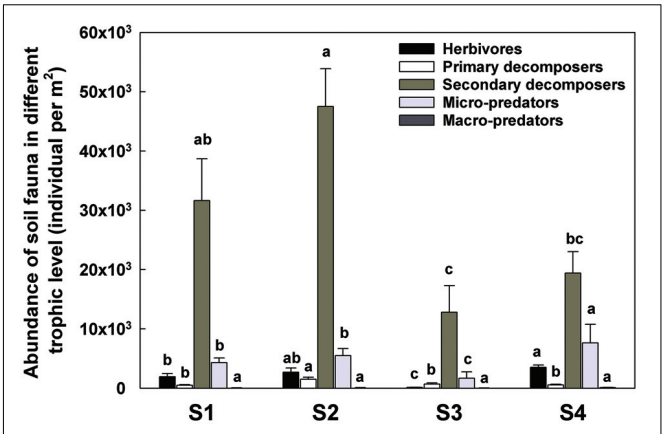


FIGURE 8 | Abundance of soil fauna in different trophic level detected in each sample time. T0 represent herbivores; T1 represent primary decomposers; T2 represent secondary decomposers; T3 represent micro-predator; T4 represent macro-predator. S1 represent sample time 1, before herbicide spray; S2 represent sample time 2, after spray and before plough; S3 represent sample time 3, after plough and before seed; S4 represent sample time 4, after seed. Bars represent stacked relative abundance ratio ($n = 24$). Different lowercase represent significant differences among sample times of each trophic level ($P < 0.05$) using one way ANOVA test and Duncan's multiple range test.

with some previous studies from Europe (Brennan et al., 2006; García et al., 2010).

CONCLUSION

Herbicide application tended to increase soil invertebrate abundance due to the plant residue improvement, less exposure and edibility of herbicide for some soil arthropod. Whereas subsequent ploughing significantly reduced soil invertebrate number and biomass due to its disturbance effects. Ploughing had an obvious negative effect on soil primary and secondary decomposers. This change was mainly due to the Acari (especially Oribatida) and some Coleoptera group abundance variations. Reseeding also reduced individual numbers in groups, and favored those predators with larger body size and individual weight. Over the following year, different arthropod groups responded differently, with Collembola populations recovering to pre-treatment levels. However, the Acari populations still appeared to be in flux 1 year on.

Wagg et al. (2014) showed that loss of soil biodiversity together with the simplification of communities negatively impacts on many ecosystem functions. Thus, maintenance of a healthy soil food web is key to maintaining and in increasing agricultural productivity (Crotty et al., 2015). This study highlights how common agricultural practices impact on soil fauna communities, this impact might cause some ecological function change of soil, and how different components of the community respond differently to the disturbances caused.

AUTHOR CONTRIBUTIONS

PM and JZ conceptualized the study, WL collected, processed, and identified samples with help from SN. WL primarily interpreted the data with contributions from PM and JZ. WL and PM wrote the manuscript and all authors were involved in reviewing, revision and final approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01200

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Detection of Invertebrate Suppressive Soils, and Identification of a Possible Biological Control Agent for *Meloidogyne* Nematodes Using High Resolution Rhizosphere Microbial Community Analysis

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White clover (*Trifolium repens*) is the key legume component of New Zealand pastoral agriculture due to the high quality feed and nitrogen inputs it provides. Invertebrate pests constrain white clover growth and this study investigated rhizosphere-associated fungal controls for two of these pests and attempts to disentangle the underpinning mechanisms. The degree of suppressiveness of 10 soils, in a latitudinal gradient down New Zealand, to added *Meloidogyne hapla* and *Costelytra zealandica* scarab larvae was measured in untreated soil. Most of the soils showed no suppressive activity against these pests but two showed activity against *M. hapla* and two against *C. zealandica*. Rhizosphere fungi responsible for pest suppressive responses were elucidated via next-generation sequencing. In the *M. hapla*-suppressive soils nematode-trapping Orbiliomycetes fungi were present in significantly greater abundance than non-suppressive soils and their abundance increased further with addition of *M. hapla*. A comparison of plant growth and the rhizosphere fungal community between untreated and irradiated soil was carried out on 5 of the 10 soils using *Pyronota* as the scarab larvae. Soil irradiation either: reduced (by 60–70%); increased (16×) or made no difference to white clover growth across the five soils tested, illustrating the range of microbial impacts on plant production. In one of the *M. hapla* suppressive soils irradiation resulted in a significant increase in nematode galling suggesting that Orbiliomycetes fungi were indeed responsible for the suppressive effect. Lack of consistent changes in soil macronutrients and pH post-irradiation suggest these were not responsible for plant or invertebrate responses. The use of next generation sequencing in controlled pot trials has allowed identification of a potential biological control organism and bioindicator for *M. hapla* suppression.

Keywords: *Trifolium repens*, white clover, rhizosphere, grass grub, mānuka beetle, next generation sequencing, Mi-Seq, biological control

INTRODUCTION

New Zealand agriculture is dominated by pastoral grazing systems used to grow animals for their meat, milk, or wool (Statistics New Zealand, 2015). Pastures in these systems largely consist of grass/legume mixtures of various sorts with the majority of mixtures being *Lolium* (mostly *L. perenne*)/*Trifolium* (mostly *T. repens*) (Li et al., 2011). In these mixtures the grass provides the bulk of the grazing ruminant's diet with the legume supplying high protein forage and nitrogen fixation capability. Pastures occur across a broad range of soil and climate conditions the length and breadth of the country (Cullen et al., 2008) so face a range of biotic and abiotic challenges and interact with a wide range of soil microbes. Of the biotic challenges to pastures, invertebrates, including root-feeders, represent a significant check on plant growth (see Goldson et al., 2015).

Invertebrates with contrasting scales and types of impact on *T. repens* include scarab beetles (such as *Costelytra zealandica* and *Pyronota* spp.) and nematodes, such as *Meloidogyne hapla.* Scarab larvae consume plant root tissue (Kain and Atkinson, 1977), reducing root mass and impinging on root function which can result in plant death when they occur at sufficiently high populations densities (East et al., 1980). *M. hapla* juveniles invade root tissue immediately behind the root growing point and establish a permanent feeding site by means of induction of giant cells which are created via plant growth regulator stimulation of the plant tissue (Moens et al., 2010). The resulting galls (or "knots," which give them the common name root-knot nematodes) impair root function but usually only result in plant death if accompanied by other stressors such as low soil nutrient status or water deficit.

The rhizosphere is the area of soil directly in contact with plant roots or their secretions and as such is the site of intense interactions between the plant and soil microbes. This zone is also the first point of contact between plants and invertebrate root-feeders, with plant secretions being used as food signals for root-feeders (see van Dam and Bouwmeester, 2016). It is therefore likely that at least some of the microbes in this zone would be adapted to utilizing soil invertebrates as a food resource (either as closely associated root residents or as recruits in response to plant damage, as occurs in disease responses (see Berendsen et al., 2012), making the rhizosphere an important area to target in the search for biological control (biocontrol) agents against pest invertebrates. Traditionally, invertebrate pathology studies have uncovered microbial biocontrol agents by examining diseased individuals and this has been successful for bacteria against *C. zealandica* in New Zealand (Grimont et al., 1988), and for some *Meloidogyne s*pecies overseas (see Davies, 2009). A rhizosphere-based approach to detecting biocontrol agents would, alternatively, need to consider which microbes increase in abundance in response to herbivore feeding on, or invasion of, roots. Those microbes responding most strongly would then be good candidates for biocontrol agents that are also rhizosphere-competent, an important consideration for persistence of agents in the soil. Along with potential biocontrol agents, a rhizosphere-based approach may identify bioindicators of pest suppression. Such bioindicators could be used to quantify the need, or not, for control measures to be implemented as part of operations such as pasture renewal or crop rotations (e.g., Ophel-Keller et al., 2008).

A culture-dependant method for a rhizosphere-based approach to detecting organisms responsible for soil pest suppression would clearly underestimate the diversity of potential biocontrols and bioindicators. Culture dependant techniques also require either: some prior knowledge of an existing pest suppression mechanism; or that a wide variety of culturing isolations be employed to find any causative mechanism. Culture-independent techniques, by contrast, allow for enumeration of the majority of microbes in soil and would provide clearer evidence of potentially useful biocontrols or bioindicators. Techniques such as high-throughput sequencing are rapidly developing and have recently moved from 454 pyrosequencing to the Illumina Mi-Seq platform (Derakhshani et al., 2016) which can generate up to 150-bp sequencing reads with a total throughput of 1.5–2 Gb per run. Such technologies have facilitated analysis of environmentally derived samples from a variety of ecosystems, including soil, and can measure very small changes in community structure across different samples (Schmidt et al., 2013).

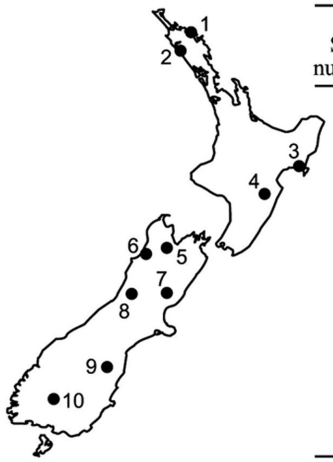
The focus of this study was the below-ground biotic challenge to *T. repens* presented by root feeding invertebrates and the changes that this challenge evokes within the rhizospheric fungal microbiota. We have utilized next generation sequencing to explore the fungal microbiota present in the rhizosphere of *T. repens* across a range of New Zealand soils and in the face of challenges from these invertebrate pests. We hypothesize that near-isogenic *T. repens* plant performance will differ in different soils from across New Zealand and that this will be linked to interactions between components of the rhizosphere microflora and soil dwelling root pests. A substantial comparison of the high resolution microbial communities will form the basis of a separate paper.

MATERIALS AND METHODS

Pot Trials

Soil samples were collected from 10 sites previously characterized by Wakelin et al. (2013) that represented a wide range of geographical locations and soil characteristics, primarily pH and nitrogen levels (**Figure 1**). In addition, half the sites were on brown soil type and half on recent soils, while four sites were dairy farms and six were sheep and beef farms. From each site 200 mm × 200 mm × 200 mm cubes of soil were excised between January and February 2014 or August and September 2014 for Experiments 1 and 2, respectively. The top 15 mm of plant matter and soil was removed from each cube and the remainder stored in sealed buckets at 4°C. Collection and manipulation of soil was performed with gloves and implements rubbed with 70% ethanol to sanitize.

Soil was passed through a 4 mm sieve and mixed by hand to homogenize. Nematodes were extracted and counted from 100 g of each soil using the method of Bell and Watson



Soil number	Locality	Grazing system	Soil sub-group	Elevation (m)	Mean soil temperature[1]	Mean annual rainfall[1]
1	Kaeo	Dairy	Mottled Acid Brown	92	11.4	2143
2	Wekaweka	Sheep & beef	Typic Orthic Brown	209	11.4	1648
3	Nuhaka	Sheep & beef	Mottled Fluvial Recent	8	8.1	1179
4	Waipawa	Sheep & beef	Typic Allophanic Brown	222	7.5	959
5	Tapawera	Sheep	Weathered Fluvial Recent	143	5.8	1070
6	Seddonville	Dairy	Weathered Fluvial Recent	26	7.3	2563
7	Leslie Hills	Sheep	Weathered Orthic Recent	283	5.9	869
8	Rotomanu	Dairy	Acidic Orthic Brown	151	6.6	3601
9	Kurow	Dairy	Weathered Orthic Recent	174	3.5	460
10	Lumsden	Sheep & beef	Typic Orthic Brown	211	4.9	790

[1] according to Wakelin et al (2013)

FIGURE 1 | Location, management, climate, and characteristics of soils used in this study.

(2001). The extraction ran for 72 h, after which the resulting nematode suspension was reduced to a final volume of 20 ml by a combination of beaker settling and aspiration. The total number of nematodes and plant parasitic nematodes were counted in a Doncaster dish (Doncaster, 1962) under a stereo-microscope at 40–80× magnification; plant parasitic nematodes were identified to genera based on the keys of Siddiqi (2000) for Tylenchida and Bongers (1994) for other groups. To allow for valid comparison across treatments, results are presented as nematodes per 100 g of dry soil. Further subsamples from each soil were sent to Hills Laboratory (Hamilton) for soil nutrient analysis [pH, Olsen P, Sulfate S, K, Ca, Mg, Na, total N and cation exchange capacity (CEC), see http://www.hill-laboratories.com/file/fileid/15530 for methods].

Initial moisture of each soil was determined from three 10 g samples oven dried at 80°C for 24 h. Water holding capacity (WHC) was determined by adding 180 g of each soil into small pots (50 mm × 50 mm × 120 mm), weighing the combination of pots with soil, soaking for 24 h, draining for 72 h and re-weighing. The 100% WHC of the system was calculated as the total weight of water held in the system (including initial soil moisture).

Pots were filled with 180–200 g soil per pot, depending on soil, with capillary mat inserted at the base of the pots to assist with water and soil retention. Pots were designated to one of three treatments: clover-only control; clover with *M. hapla* added; or clover with root-feeding scarab larvae added. For the larvae-inoculated pots a plugged hole and wire mesh were incorporated into the pot (**Figure 2**) to allow for introduction of the larva and to prevent larvae consuming the entire clover root system, respectively. Pots were arranged in a randomized split plot design with soils allocated to the whole-plots and treatments to the subplots.

Near-isogenic white clover seed (S9) was obtained from a breeding population developed within AgResearch derived from cv. Crau. A near-isogenic seedline was selected in an attempt

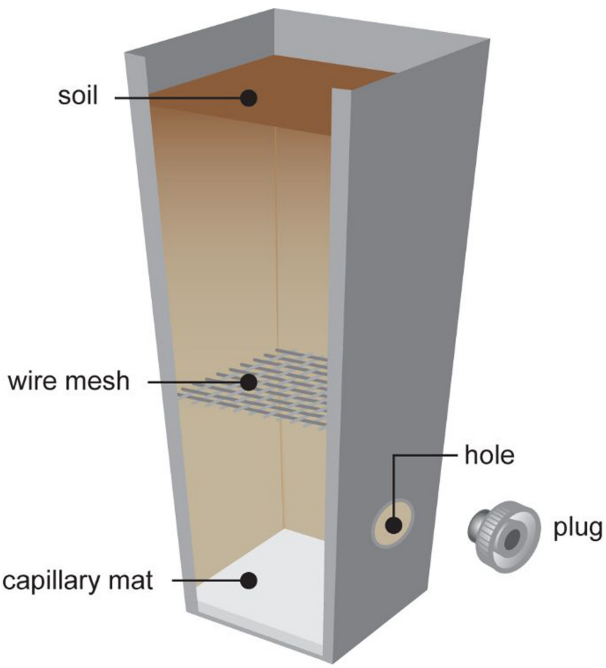


FIGURE 2 | Plant pot with wire mesh as scarab larval excluder and plug for inoculating larvae into pot.

to reduce between-plant variability in growth and reaction to imposed treatments. Seeds were scarified by abrasion with 'wet and dry' sandpaper then surface sterilized by soaking for 15 min in 10N H_2SO_4 containing a drop of Tween 80, then washing five times in sterile milliQ water. Seeds were sprouted on moist sterile filter paper at 20°C for 3 days prior to planting into pots. A single plant was sown into each pot. Aseptic techniques were used during planting to prevent cross contamination between soils. Plants were grown at isothermal 20°C under high pressure

sodium vapor lamp lighting on a 16 h light and 8 h dark cycle. *M. hapla* and scarab larvae were added 5 weeks after sowing clover plants into pots.

MilliQ water was initially added to each pot to bring moisture levels up to 70% WHC. Once a week each pot was reweighed and returned to 70% WHC. Between each weekly weighing pots were checked daily and any that appeared dry were watered with 3–5 ml. Following inoculation of the plants with *M. hapla* or scarab larvae watering was reduced to 60% WHC in Experiment 1 but maintained at 70% WHC in Experiment 2.

Meloidogyne hapla stock populations [confirmed as such by ITS sequencing (555 bp sequence 99% match to *M. hapla* (GenBank reference: LC030360), query coverage 100%, see Rohan et al., 2016 for PCR methodology)] were established and maintained on tomato plants (*Solanum lycopersicum* cv. 'Rutgers') under glass house conditions. After 4 months galled tomato roots were excised from the tomato plants, and nematode eggs were collected following Aalders et al. (2009). The resulting inoculum consisted of 1089 (± 31 SEM) eggs and 20 (± 6) J2 juvenile *M. hapla* per ml with 346 (± 38) of the eggs being embryonated. A 1 ml aliquot of inoculum was added to each nematode-treated pot via a 20 mm deep hole made 10 mm from the base of the plant, followed by addition of 1 ml of sterile water. The hole was then filled in from surrounding soil to prevent desiccation of inoculated nematodes.

Next-Generation Sequencing

DNA was extracted from rhizosphere samples using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsberg, CA, USA). Stored soil fractions were thawed and then centrifuged at 7000 × *g* for 5 min. The supernatant was decanted and discarded. For each sample, 0.25 g of pellet was placed in a sterile 2 ml tube and the contents of one PowerSoil® tube added. DNA was then extracted according to the kit manufacturer's instructions with one exception: a 3 min mix using a bead-beater was substituted for the post-Soln C1 vortexing step. DNA extracts were quantified using a NanoDrop 2000C spectrophotometer (Wilmington, DE, USA) and then stored at −20°C for PCR reactions.

Amplification of fungal ribosomal internal transcribed spacer (ITS) sequences were performed in reaction mixtures that included 0.2 μM of each primer using iProof HF polymerase (Bio-Rad Laboratories, Hercules, CA, USA) and the manufacturers instructions. PCR cycling conditions were: 95°C for 3 min followed by 35 cycles of 95°C/30 s, 46°C/30 s, and 72°C/60 s; followed by 72°C/9 min final extension. For amplifying fungal ITS sequences, ITS3_KYO2F and ITS4R primers (Toju et al., 2012) were used with appended Illumina sequences:

ITS3_KYO2_miseqF TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GATGAAGAACGYAGYRAA
ITS4_miseqR GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCTCCGCTTATTGATATGC

PCR products were purified using a GeneJET PCR Purification Kit (ThermoScientific, Lithuania) and purified product was quantified using a NanoDrop 2000C spectrophotometer (Wilmington, DE, USA). ITS products were then normalized to 0.1 ng/μl using ultrapure water. For each normalized sample, 5 μl aliquots were made up to 25 μl volumes in half-skirt 96-well plates using ultrapure water containing a 467 bp exotic control sequence (synthetic zebrafish sequence appended with Illumina clamps) added to a ratio of 1:50 control sequence-to-sample DNA (i.e., 20 pg:1 ng in 25 μl volumes). Prepared plates were sent to New Zealand Genomics Limited (NZGL, Massey University, Palmerston North, New Zealand) for second PCR amplification and sequencing on an Illumina MiSeq platform.

Experiment 1

For each soil each treatment (clover-only control; clover with *M. hapla* added; or clover with root-feeding scarab larvae added) was replicated 12 times giving a total of 360 pots. Second instar *C. zealandica* larva were collected from soil at a site near Tokoroa (lat/long 175.90, −38.19) in the Waikato district, and stored at 4°C. A single pre-weighed larva was added to each pot (giving an equivalent of 400 larvae/m^2) within 24 h of being collected and larvae were re-weighed at harvest when numbers of live and dead larvae were recorded. *M. hapla* inoculum consisted of 1089 (± 31 SEM) eggs and 20 (± 6) J2 juvenile *M. hapla* per ml with 346 (± 38) of the eggs being embryonated.

Clover plants were harvested after 9 weeks growth (4 weeks after *M. hapla* and *C. zealandica* inoculation). Plants were removed from pots and tipped onto a sterile surface. Soil was gently removed by hand to leave a 2 mm zone of soil around the roots (considered rhizosphere soil). *C. zealandica* larvae were removed, weighed and stored at 4°C. Total plant, shoot and root length were measured then roots were separated from stems and the stem/leaf component dried at 80°C for 48 h and dry weight recorded. All root material and associated soil was placed in a 50 ml Falcon tube with 25 ml sterile saline and vortexed at high speed for 15 s. The partially washed root material was then transferred to 20 ml fresh saline and re-vortexed for 15 s. The two soil-containing wash fractions were combined and a 0.5 ml aliquot from each sample was mixed with 60% glycerol and stored at −80°C for further analysis. The remaining soil suspension was stored at −20°C for rhizosphere DNA extraction.

Root length was recorded and the number of *Meloidogyne* galls assessed microscopically on roots in the control and nematode inoculated treatments. During the course of nematode assessment a score was assigned for degree of root rotting on a 0–3 scale with 0 being no rotting, 1 minor rotting symptoms, 2 moderate rot, and 3 severe rot including roots truncated due to root rotting. No attempt was made to determine the cause of root rots.

Experiment 2

Soil samples for the second trial were collected from five of the sites used in Experiment 1 (Soils 1, 4, 7, 9, and 10). Half the volume of each soil was sterilized by irradiation using 25–32 kGy followed by 15–17 kGy doses (MSD Animal Health, Upper Hutt, New Zealand). A spread plate technique using Tryptic Soy Agar and Potato Dextrose Agar (TSA and PDA) was used to test sterility of soils using a 1 g soil sample in 9 ml sterile water to

prepare the dilution series and agar plates were incubated for 72 h at 20°C.

To limit macronutrient differences between irradiated and non-irradiated samples, soils were mixed with pumice. Initial moisture content was determined using 10 g samples of each soil (three replicates per soil). To determine 100% WHC of each soil: pumice mix, an equivalent of 20 g dry soil and 127 g dry pumice were weighed into pots in three replicates per soil and soaked in water for 72 h before being suspended over a tray to drain for 24 h. Final weights per replicate were averaged and used to calculate 70% WHC.

Six treatments were initiated for each soil: non-irradiated or irradiated soil, each with or without the addition of *M. hapla* or *Pyronota* (mānuka beetle) or an untreated control (*Pyronota* larvae were substituted for *C. zealandica* due to the lack of availability of *C. zealandica* at the time of the experiment. Both are root-feeding scarabs). Each treatment consisted of 10 replicates giving sixty pots per soil. The pots where *Pyronota* were added were as for the *C. zealandica* treatment in Experiment 1. The capillary mats for all sterile pots were autoclaved at 121°C for 20 min while the pots were soaked in 95% ethanol for 1 min. Each pot was filled with a mixture of 20 g dry weight equivalent soil and 127 g autoclaved 4 mm graded pumice, then watered to 70% WHC.

Seed preparation was done similarly to the first trial. The seedlings were aseptically planted, one seedling per pot, and watered with 2 ml of sterile water. Moisture of the soil:pumice mix was maintained at 70% WHC by watering every second day with sterile water for the duration of trial. All *M. hapla* egg methods were as for Experiment 1 with inoculum containing an average of 3,923 eggs (27% of eggs embryonated) and 26 J2 juveniles per ml.

Pyronota sp. beetle larva were collected from a site near Ohakune in the central North Island (lat/long 175.37, −39.39) on 13 November 2014, and individually weighed before aseptically adding them to pots as per *C. zealandica* in Experiment 1. Due to the late stage of larval development (L3), and to ensure sufficient larval feeding and damage to detect soil differences, two larvae were added to each pot. The larvae were added one at a time to each pot, allowing sufficient time for the first larva to bury itself into the soil/pumice mix before adding the second larva so as to minimize the possibility of larval combat. Plants were harvested during week 9 of the trial and processed and analyzed as for Experiment 1.

Data Analyses

All plant and invertebrate data analyses were carried out in GenStat version 16. Unless otherwise stated, data were analyzed by split plot ANOVA with soils applied at whole-plot level and treatments at the subplot level. Shoot dry weight was $\log_e(n+5)$ transformed prior to analysis to stabilize the variance (5 is half the minimum non-zero value). *M. hapla* gall data was analyzed using split-plot REML (GenStat version 16), again with soils applied at whole-plot level and treatments at the subplot level. REML was used as the data is unbalanced with missing data for dead plants and this makes the analysis conditional on plant survival. That is, if galling had affected plant survival the means would be negatively biased. Gall data was $\log_e(n+1)$ transformed prior to analysis to stabilize the variance. For Experiment 1 the gall data was analyzed with root length as a co-variate. The proportion of plants without root rot (i.e., score = 0) was analyzed as split-plot using Generalized Linear Mixed Model – Binomial distribution with Logit link. The proportion of total number of plants without rot was calculated including dead plants, chosen because dead plants are likely to have had root rot (i.e., root rot is not independent of plant death).

For Illumina sequences the reads were joined using PEAR (Zhang et al., 2014) version 0.9.6 using a quality cut off of 30, a minimum joined length of 250 bp and a maximum joined length of 480 bp and a minimum overlap of 10. The sequences were assigned to the different amplicons based on exact matches to the flanking primers (minus the Illumina adapters).

The headers of the sample files were edited to add the sample name to each read. All samples were then pooled into a single file. This file was dereplicated using the Mothur (Schloss et al., 2009) program unique.seqs. In-house Ruby scripts were then applied to modify the headers of the reads to be used with the clustering program Swarm (Mahé et al., 2014). After clustering the putative OTUs were filtered based on abundance and prevalence as follows: an OTU was kept if it was present in 2% of the samples with an abundance >0.1% or in 5% of the samples with an abundance >0.01%, furthermore an OTU was kept if it represented 0.001% of the overall population in all samples together.

The representative sequence for each of the OTUs were annotated using the assign_taxonomy.py script from Qiime (Caporaso et al., 2010), where the uclust consensus taxonomy assigner was used to annotate the 16S amplicon sequences with the greengenes (McDonald et al., 2012) 13_8 database, and BLAST was used to annotate the ITS amplicon sequences with the UNITE (Kõljalg et al., 2005) 02.03.2015 release.

The OTU counts were converted into percentages of total OTU counts less the OTU corresponding to the *T. repens* reads. After treatment and other sample information was added, the OTU percentages were loaded into R (R Core Team, 2015) version 3.2.1 as tab separated values. Boxplots and *t*-test were done on subsets of the data with the default settings.

RESULTS

Experiment 1

There was a wide range of pH and macronutrient levels across the soils used in this study (**Table 1**), with broad agreement between levels measured here and those by Wakelin et al. (2013) from the same soils, with the exception of pH in soil 9. The northernmost soils (1 and 2) had the greatest CEC values while soil 5 was notable for a high level of Mg and soil 8 for low total N.

The nematodes found in the soils prior to sowing were within the ranges normally seen in pasture soil, with potentially plant damaging populations of *Pratylenchus* present in soil 3 in Experiment 1 and of *Heterodera* and *Pratylenchus* in soil 7 for Experiment 2. Sequencing of the ITS region of *Meloidogyne*

TABLE 1 | Macronutrient levels and pH in soils prior to sowing plants for Experiment 1 (soil number, pH and Total N data in parentheses are from Wakelin et al., 2013).

Soil number	pH	Olsen P mg/L	Sulfate S mg/kg	K mg/100 g	Ca mg/100 g	Mg mg/100 g	Na mg/100 g	Total N %	CEC mg/100 g
1 (41)	5.4 (5.7)	6	16	0.30	17.0	2.67	0.16	0.44 (0.66)	31
2 (42)	5.5 (5.5)	9	6	0.39	13.0	2.21	0.23	0.58 (0.79)	30
3 (47)	5.2 (5.2)	23	6	1.38	7.0	2.88	0.22	0.43 (0.39)	21
4 (48)	5.4 (5.6)	79	4	0.98	7.0	0.81	0.07	0.40 (0.56)	19
5 (11)	6.4 (6.8)	3	1	0.21	13.0	6.16	0.05	0.29 (0.35)	21
6 (13)	5.5 (5.4)	58	28	0.24	9.0	0.72	0.09	0.59 (0.74)	21
7 (16)	5.6 (5.3)	5	3	0.31	12.0	0.89	0.12	0.39 (0.27)	19
8 (17)	5.4 (5.2)	4	<1	0.08	3.0	0.54	<0.05	0.17 (0.19)	9
9 (37)	6.3 (5.0)	28	2	1.25	13.0	2.09	0.13	0.40 (0.39)	20
10 (27)	5.6 (5.8)	28	2	1.52	5.0	1.82	0.09	0.43 (0.49)	20

observed in soil extracts prior to sowing (**Table 2**) showed the presence of *M. trifoliophila* in soil 3 and *Meloidogyne fallax* in soil 4. No usable sequence information was obtained from *Meloidogyne* specimens in soil 2, but morphological observations of root galls from clovers growing in the soil at field sampling and of J2 juveniles in the soil extract prior to sowing suggest these were also *M. trifoliophila*. None of the populations of other plant feeding nematode genera were likely to be having a significant effect on clover growth, with the possible exception of the *Pratylenchus* population in soil 3. In addition to the plant feeding nematodes listed in **Table 2**, Criconematidae were observed from soil 10 and *Tylenchorhynchus* from soil 9 (both 10/100 g dry soil).

There was a large range of shoot and root growth across the soils (**Figure 3**) which illustrates the range of white clover productivity across New Zealand. There was no clear pattern for either soil type (brown or recent) or grazing system (dairy or sheep and beef) as drivers for these growth differences. Geographically, there was a tendency for lower shoot and root production in more northerly soils, with the greatest production from the southernmost soils. With temperature and moisture not limiting factors in these pot trials other factors such as soil nutrients and pest invertebrates (e.g., nematodes) and diseases were likely drivers of growth differences. Unsurprisingly, there were positive correlations between soil P and K and clover shoot and root growth but these were not significant. There was a significant negative correlation between soil CEC and root length ($r = -0.736$, $P < 0.05$) (**Table 1**; **Figure 3**).

Addition of *C. zealandica* larva or *M. hapla* eggs to pots significantly reduced clover shoot weight in only one of the soils (**Figure 3**). However, *C. zealandica* feeding significantly reduced root length compared to control plants in all the South Island soils. It is likely that the mesh inserted in the *C. zealandica* pots ensured there was sufficient root system remaining to support similar shoot growth to the control plants. There were some significant differences in the weight of *C. zealandica* initially added to soils (**Table 3**). Soils 5 and 8 were inoculated with

TABLE 2 | Total nematodes and plant feeding genera per 100 g dry soil and soil moisture of subsamples after sieving and prior to being used to fill pots.

Soil number	Total nematodes	*Meloidogyne*	*Heterodera*	*Pratylenchus*	*Helicotylenchus*	*Paratylenchus*	Soil moisture (%)
Experiment 1							
1	644	0	10	0	8	0	30
2	494	11	3	0	31	17	39
3	1508	46	7	459	0	14	16
4	966	13	0	0	0	93	11
5	493	0	11	0	56	0	12
6	1835	0	6	17	188	0	38
7	2164	0	0	33	0	131	17
8	805	0	0	59	208	59	13
9	1576	0	0	22	182	27	11
10	5227	0	9	94	99	260	18
Experiment 2							
1	2957	74	0	0	92	0	85
4	1359	0	115	124	0	53	33
7	1054	0	290	145	0	0	32
9	3394	0	0	293	0	829	28
10	2096	13	238	75	0	213	26

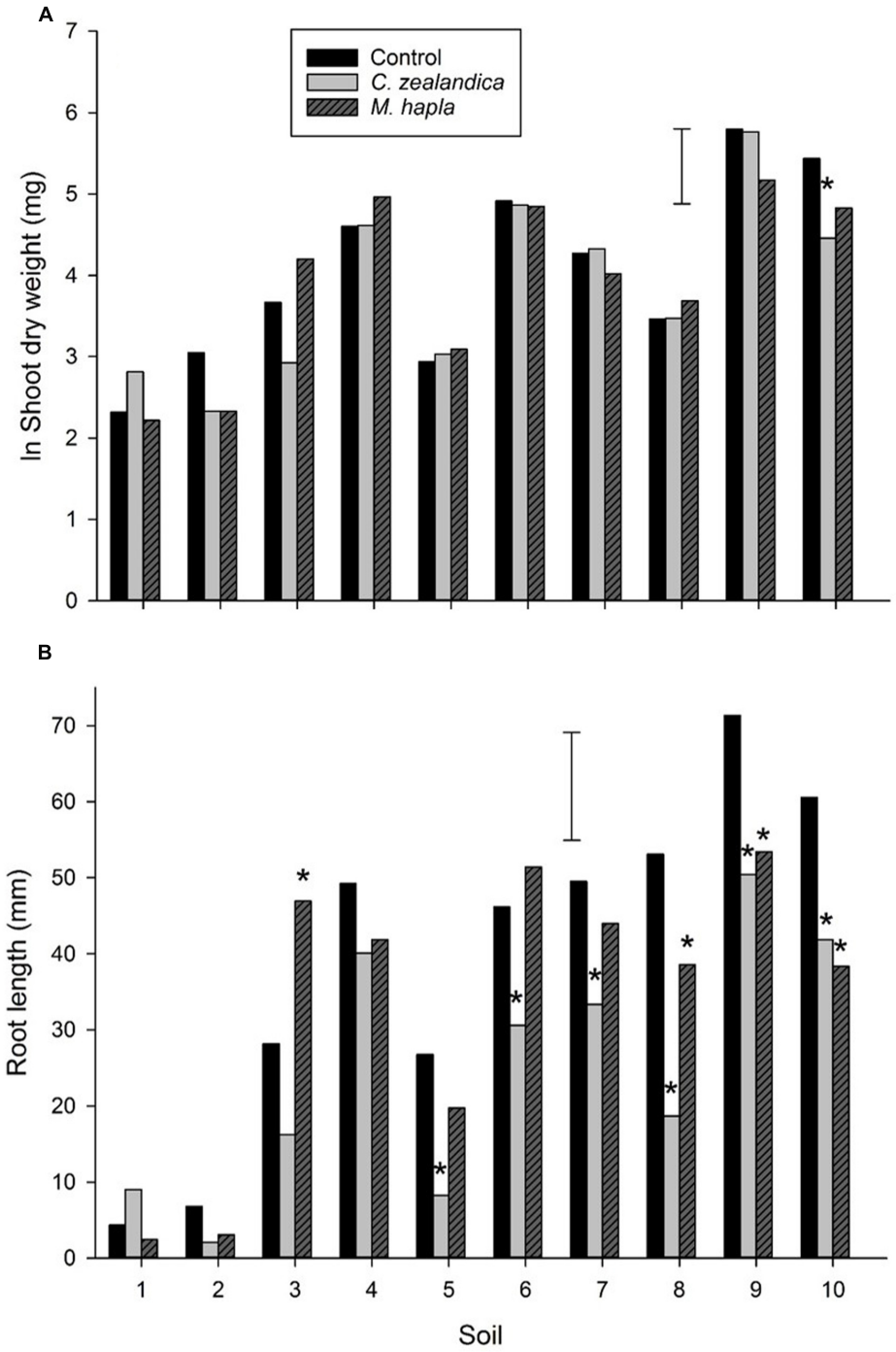


FIGURE 3 | Experiment 1: Growth of white clover shoots (A) and roots (B), without invertebrate pests (control) or inoculated with *Costelytra zealandica* or *Meloidogyne hapla.* Error bars are lsd 5%. Soils are arranged from most northerly (1) to most southerly (10). Astrices denote significant difference to control within each soil.

larvae that were significantly heavier than those in soils 4 and 7. However, those differences had disappeared by the time of harvest. Significantly fewer grubs survived the 4 week duration between inoculation and harvest of the experiment in soils 4 and 10 than most other soils.

Inoculation with *M. hapla* did not result in significant changes in clover shoot growth in any of the soils (**Figure 3**). However, it is notable that the only soils where *M. hapla* inoculation resulted in significant reductions in root length were from the southern South Island where clover-feeding *Meloidogyne* have

TABLE 3 | Experiment 1: Mean *Meloidogyne* nematode root galls (back-transformed data with log_e-transformed data in parentheses) and weight of *C. zealandica* larvae at inoculation into pots (Initial) and at harvest (Final).

	Number galls		*C. zealandica*			
Soil number	**Control**	**Nematode inoculated**	**Initial weight mg**	**Final weight mg**	**Weight change mg**	**No. alive/pot**
1	0.1 (0.13)	4.7 (1.74)*	31.3	37.3	5.9	0.92
2	0.5 (0.38)	2.2 (1.15)*	30.4	45.1	12.9	0.84
3	5.0 (1.80)	11.6 (2.53)*	31.3	35.4	5.7	0.83
4	0.0 (0.03)	2.8 (1.33)*	28.5	40.1	11.1	0.45*
5	1.2 (0.81)	2.9 (1.37)	34.4	43.7	9.0	0.71
6	0.0 (0.01)	7.2 (2.10)*	30.7	40.4	9.7	0.76
7	0.0 (−0.02)	0.7 (0.52)	28.3	46.8	16.6	0.62
8	1.5 (0.93)	6.1 (1.96)*	34.1	39.7	3.8	0.72
9	0.0 (−0.02)	0.6 (0.48)	30.0	46.9	16.2	0.93
10	0.0 (−0.01)	6.2 (1.97)*	33.9	47.8	11.7	0.30*
Lsd 5% $_{soil}$		—	4.95	13.10	9.24	0.348
Lsd 5% $_{treatment*soil}$		(0.647)	—		–	–

Lsd values are to compare: across soils ($_{soil}$) or treatments across soils ($_{treatment*soil}$). Astrices denote significant difference to control for nematode data and significant difference to the soil with the greatest survival rate for C. zealandica.

until recently only been rarely encountered in pastures (personal observation).

Inoculation with *M. hapla* significantly increased root galling in all but soils 5, 7, and 9 (**Table 3**). It is not clear what was responsible for that effect in soil 5 but in soils 7 and 9 fungal community analysis by high-throughput ITS sequencing showed that there were significantly greater abundances of Orbiliomycetes fungal sequences in those than in other soils. Addition of *M. hapla* further increased abundance of these fungi (**Figure 4**).

For root rotting score there was no significant difference between the control and *M. hapla* inoculated soil so results were pooled. Mean root rot scores were generally low to minor severity (**Table 4**). There were significant differences in root rotting incidence between soils, with soils 1 and 10 having the lowest proportion of plants without any signs of rot and this was significantly different to all other soils.

Experiment 2

A second trial was set up to validate the results from Experiment 1 and to directly test the impact of the overall soil microbial content by using a pumice/soil mixture to reduce abiotic effects and comparing biotic with sterilized soil. A different scarab beetle was also used to measure insect effects – in this case a *Pyronota* sp. scarab due to the lack of available *C. zealandica* larvae.

From extractions carried out before sowing clover there was a greater abundance of total nematodes in all but soil 10 compared to the samples collected for Experiment 1 (**Table 2**). These differences in initial nematode population numbers and plant feeding genera present between Experiments 1 and 2 reflect differences in the time of year sampling was carried out and concomitant greater soil moistures. *Heterodera* and *Pratylenchus* spp. were observed at moderate to high populations in all but soil 1. *Meloidogyne* spp. were observed in two soils, neither of which had this genus present in samples taken for Experiment 1. Morphology of J2 juvenile tails and galls suggested the *Meloidogyne* from soil 1 were *M. trifoliophila.* The *Meloidogyne* sp. present in soil 10 was identified from tail morphology as the grass-feeding *M. naasi*. Some of the individuals observed were heavily encumbered by the nematode-pathogenic bacteria *Pasteuria* sp.

The pumice used to dilute soils had notably greater pH and Na levels than any of the soils used in this experiment, so that these parameters were elevated in the soil:pumice mix used (**Table 5**). Irradiation produced no consistent differences in macronutrient levels in the pumice: soil mix. The largest changes due to irradiation were in Olsen P in soils 1, 4 and 10; K in soils 9 and 10; and Ca in soil 1. pH was generally increased after irradiation (by 0.1–0.3), except for soil 10 where no change in pH was observed. Compared to soils alone used in Experiment 1 (**Table 1**) the soil:pumice mix used in Experiment 2 resulted in a reduction in the magnitude of differences in the pH and macronutrient parameters amongst the five soils. Spread plating of irradiated soils showed no microbial growth.

For all soils combined there was a significant reduction in clover shoot weight in irradiated (ln shoot weight = 3.94 g) compared to non-irradiated (4.12 g) soil (lsd 5% = 0.179 g). For control soils irradiation significantly reduced shoot growth in soils 4 and 9 but significantly increased white clover shoot growth in soil 1 and resulted in no significant difference in the remaining two soils (**Figure 5**). Changes in shoot weight in irradiated vs. non-irradiated soil for invertebrate treatments largely mirrored those for the control with the exception of the nematode treatment in soil 7 where plants in irradiated soil produced significantly greater shoot mass than those in non-irradiated soil.

For all non-irradiated soils combined there was no significant effect of invertebrate treatment on shoot growth. However, in irradiated soils there was a significant decrease in white clover shoot dry weight in the mānuka beetle treatment (ln shoot weight = 3.49 g) compared to the control (4.24 g) and *M. hapla*

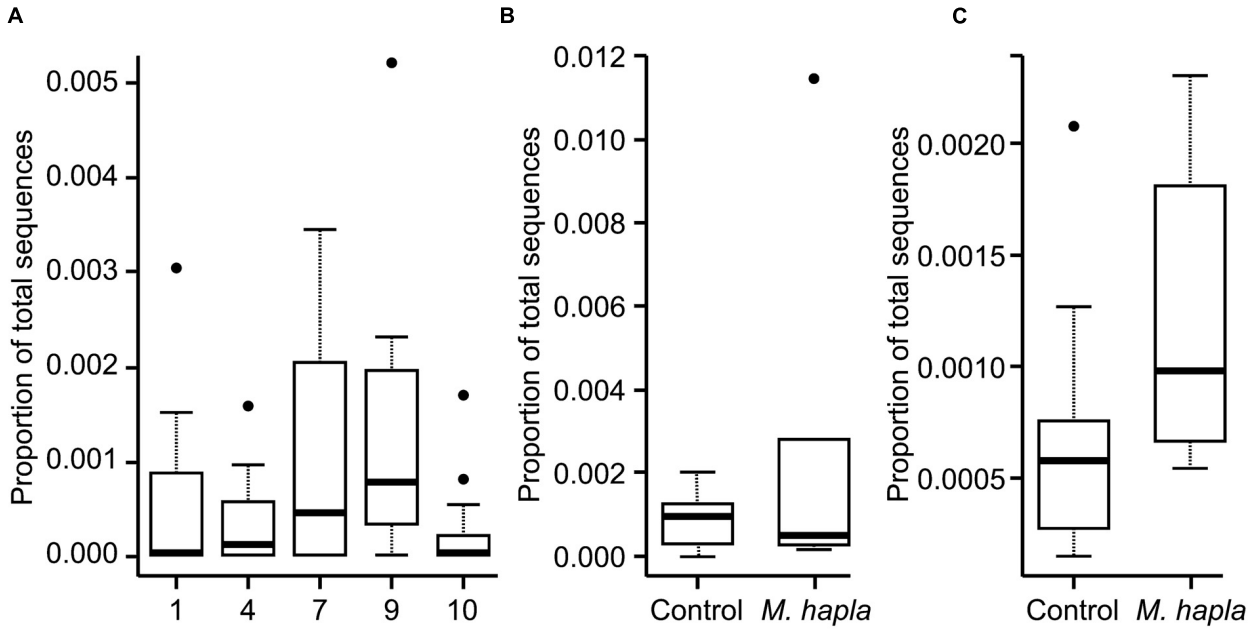


FIGURE 4 | Proportion of all fungal ITS DNA sequences from Experiment 1 that belonged to Orbiliomycetes for: (A) all soils; **(B)** soil 7 with or without added *M. hapla* and **(C)** soil 9 with or without added *M. hapla*.

TABLE 4 | Experiment 1: Arithmetic mean root rot score and back-transformed proportion of control roots without rot (Logit transformed data ± SEM in parentheses) for Experiment 1.

Soil number	Root rot score (0–3 scale)	Proportion of roots without rot
1	1.1	0.12 (−2.00 ± 0.68)
2	0.2	0.46 (−0.17 ± 0.43)
3	0.3	0.71 (0.90 ± 0.47)
4	0.2	0.71 (0.90 ± 0.47)
5	0.3	0.50 (0.00 ± 0.49)
6	0.6	0.55 (0.20 ± 0.47)
7	0.2	0.82 (1.55 ± 0.63)
8	0.1	0.82 (1.55 ± 0.63)
9	0.0	0.88 (2.00 ± 0.68)
10	1.0	0.27 (−0.97 ± 0.51)

(4.10 g) treatments (lsd 5% = 0.309) (**Figure 5**). For individual soils the only significant decreases in shoot growth compared to control occurred in *M. hapla* treatment in non-irradiated soil 7 and in the mānuka beetle treatment in irradiated soils 1, 4, and 7.

Irradiation significantly increased root length over all soils combined (116.0 mm) compared to non-irradiated soil (91.1 mm, lsd 5% = 4.71), which was largely due to the increases in root length due to irradiation of soils 1 and 7 (**Figure 5**). Mānuka beetle significantly reduced root length for all soils combined (99.3 mm), compared to the control (106.9 mm) but this was not the case with the *M. hapla* treatment (104.5 mm, lsd 5% = 5.77), with no interaction due to irradiation ($P = 0.825$). The only significant difference in root length, compared to control soil, due to invertebrate pests for individual soils was in non-irradiated soil 7 where the *M. hapla* treatment gave a decrease in root length. It is possible the combined populations of *Heterodera* and *Pratylenchus* in soil 7 (**Table 2**) had an impact on clover root and shoot growth, in addition to the added *M. hapla*.

For soils 4 and 9 inoculation of plants with *M. hapla* resulted in significantly more nematode galls in both irradiated and non-irradiated soil, compared with the non-irradiated control soil (**Table 6**). In soils 1, 7, and 10 only inoculation into irradiated soil resulted in increased galling. In contrast to the other two soils with this effect, the addition of *M. hapla* to soil 7 also increased root rotting (**Table 7**), and both clover root and shoot growth were significantly reduced in this treatment (**Figure 5**).

The proportion of the sequences that were of Orbiliomycetes fungi was much lower in this experiment that in the previous one (**Figure 6**), with their proportion approximately in line with the soil dilution rate (ca. 6×) for soils 7 and 9 but much lower than that in soil 4. As for Experiment 1 these abundances increased in response to added *M. hapla* but in this case none of the increases were significant.

The initial weights of *Pyronota* larvae added to pots was not significantly different between soils or treatments (data not shown), however final weights and, therefore weight change of larvae at the end of the experiment were significantly different amongst some soils and treatments (**Table 6**). In soils 1, 4, and 9 there was an increase in final weight of larvae in irradiated vs. non-irradiated soil, which was significantly so for soil 4. Significantly less beetle larvae survived in non-irradiated soil 1 (0.9) compared to non-irradiated soil 7 (1.7, lsd 5% = 0.61) but

TABLE 5 | Macronutrient levels and pH in pumice alone, soil alone or soil:pumice mix (Mix) prior to sowing plants for Experiment 2.

Soil number	Soil treatment	pH	Olsen P mg/L	Sulfate S mg/kg	K mg/100 g	Ca mg/100 g	Mg mg/100 g	Na mg/100 g	Total N %	CEC mg/100 g
Pumice alone	—	7.7	3	<1	0.58	2.3	0.57	0.28	<0.04	4
1 Soil alone	—	5.7	6	11	0.59	19.1	3.61	0.16	0.49	36
1 Mix	—	6.3	5	2	0.77	4.6	1.01	0.30	0.06	9
1 Mix	Irradiated	6.6	11	4	0.64	5.9	1.08	0.26	0.07	9
4 Soil alone	—	5.6	65	7	0.57	6.9	0.99	0.08	0.41	19
4 Mix	—	6.0	26	2	0.57	3.4	0.67	0.24	0.08	7
4 Mix	Irradiated	6.3	19	2	0.62	3.2	0.57	0.29	0.08	6
7 Soil alone	—	5.4	5	5	0.32	9.0	0.87	0.09	0.37	19
7 Mix	—	6.0	3	1	0.40	3.6	0.65	0.16	0.10	7
7 Mix	Irradiated	6.2	5	1	0.46	3.2	0.64	0.22	0.10	6
9 Soil alone	—	5.6	15	2	0.58	6.4	1.19	0.07	0.30	14
9 Mix	—	6.5	4	1	0.66	2.9	0.64	0.30	0.08	5
9 Mix	Irradiated	6.6	7	1	0.47	2.8	0.61	0.19	0.08	5
10 Soil alone	—	5.5	10	3	1.07	3.1	1.26	0.06	0.24	17
10 Mix	—	6.0	4	2	0.68	2.7	0.70	0.32	0.08	7
10 Mix	Irradiated	6.0	10	2	0.81	3.1	0.77	0.31	0.08	7

there was no significant difference in beetle survival amongst irradiated soils. Some larvae had developed through to pupae and adults at the end of the experiment and were discounted from the analysis of final weights.

Irradiation eliminated root rot from all soils (mean score = 0.0) which was significantly less than the overall score in control (0.8) which in turn was significantly less than in nematode treated soil (1.0, lsd 5% = 0.2). This effect was significant for soil 7. Soil 9 was the only one of the five soils in which root rot was not observed in any of the treatments (**Table 7**). Similar to root rot the number of rhizobial nodules was significantly reduced in irradiated soil inoculated with *M. hapla* (0.05/ mm root) compared to control (0.16/mm) and *M. hapla* treated non-irradiated soil (0.15, lsd 5% = 0.029). The only individual soil for which this did not hold true was soil 1 where nodules/ mm root in irradiated soil inoculated with *M. hapla* was not significantly different to control or *M. hapla*-inoculated non-irradiated soil (**Table 7**).

DISCUSSION

Invertebrate pests had deleterious effects on *T. repens* root and/or shoot growth in some soils, across both experiments. In a minority of soils, however, invertebrate pests had no significant impacts on plant growth or damage in either experiment. For *M. hapla* it appears Orbiliomycetes fungi were responsible for this effect. The *M. hapla* suppressive effects observed in soils 7 and 9 are, to our knowledge, the first report of nematode suppressive soils from New Zealand. Certainly there has been no reported *in situ* association between Orbiliomycetes fungi and *Meloidogyne* in this country, and few worldwide (Singh et al., 2012). Orbiliomycetes fungi are a monophyletic group which include the genera *Arthrobotrys*, *Drechslerella,* and *Dactylellina* (Li et al., 2005). The use of culture independent next generation sequencing in this study has enabled the relationship between these fungi and a damaging plant-feeding pest to be revealed much more readily than would have been possible with culture-dependant techniques.

Although, many studies have tried to isolate and utilize nematode-trapping fungi as biocontrol agents for plant-feeding nematodes, they have yet to be utilized commercially (Degenkolb and Vilcinskas, 2016). This may be because the fungi that have thus far been worked with have been selected mainly on their ability to be readily cultured which is clearly an important determinant for any potential marketable biocontrol agent. However, because these fungi are often facultative parasites such an approach may overlook those fungi that are actually reacting to the nematodes of interest, and this is an area where next generation sequencing, as shown in this study, can be a useful guide to selection of potential biocontrol agents for specific nematodes. Additionally, the results presented here support the need for future work directed at the use of Orbiliomycetes as bioindicators for nematode suppressive soils. Such bioindicators could be used to inform the decision making process of land managers contemplating actions which could disrupt existing biocontrol mechanisms (e.g., soil cultivation). As mentioned earlier a substantial comparison of the high resolution microbial communities will form the basis of a separate paper.

For soil 7 irradiation removed the nematode-suppressive effect. Although addition of *M. hapla* to pots of this soil did not significantly increase root galling in either experiment it did significantly increase root rotting and this increase was associated with significantly reduced root length. It is possible the invasion of added *M. hapla* into roots created entry points for disease organisms. Once microbes were eliminated by irradiation a significant increase in galling was observed, likely due to elimination of Orbiliomycetes fungi but this was not associated with a decrease in root length. It is possible the impact of eliminating root pathogens was greater than that of the relatively

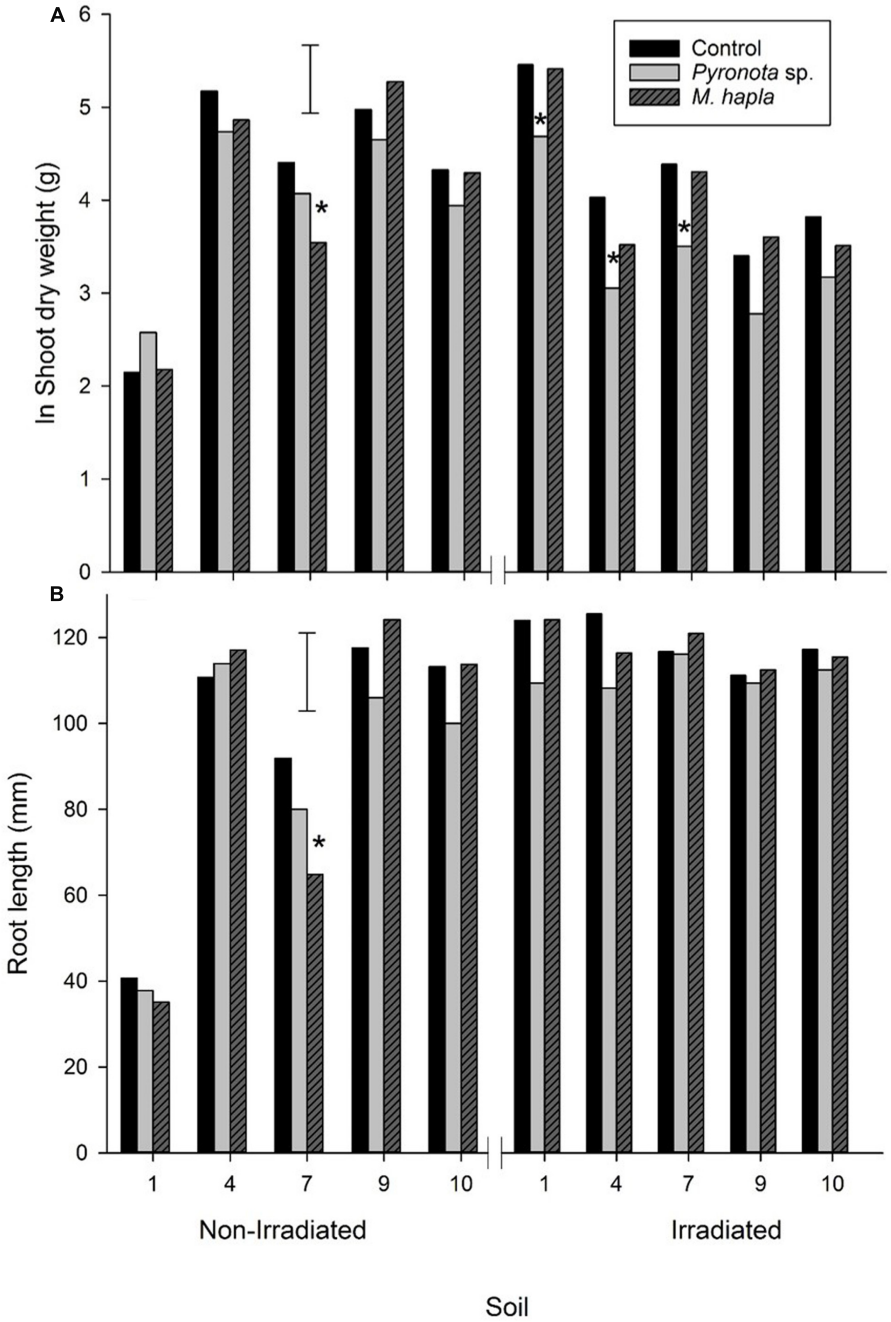


FIGURE 5 | Experiment 2: Growth of white clover shoots (A) and roots (B), without invertebrate pests (control) or inoculated with *C. zealandica* or *M. hapla,* either before or after soil irradiation. Error bars are lsd 5%. Soils arranged as for **Figure 3**. Astrices denote significant difference to control within each soil.

small amount of galling caused by nematodes. White clover is a very good host for the resident *M. trifoliophila* in soil 3 (Mercer and Miller, 1997), as illustrated in this case by the relatively large amount of galling observed on plant roots from the control treatment. The *M. fallax* observed in soil 4 prior to sowing is also hosted by white clover (Rohan et al., 2016) but in this case appears to have been present in too few numbers initially to be able to establish a population causing noticeable galling in the control treatment, as was also the case for the putative *M. trifoliophila* in soil 2.

Along with the resident and added invertebrates, the microbial flora of soils can have a profound impact on plant growth and in this study positive, negative, and neutral soil microbiota impacts on *T. repens* were all observed. Clearly, irradiation had a considerable impact on white clover growth in soil 1, significantly increasing both root and shoot growth. It appears

TABLE 6 | Experiment 2: Mean number of *Meloidogyne* root galls and weight of *Pyronota* larvae at inoculation into pots (Initial) and at harvest (Final).

	Number galls		*Pyronota* sp.			
Soil number	**Control**	***M. hapla* inoculated**	**Initial weight (mg)**	**Final weight (mg)**	**Weight change (mg)**	**No. alive/ pot**
1	8.9	7.9	62.2	43.0	−19.2	0.9*
4	−0.9	7.3*	62.1	46.9	−15.2	1.5
7	0.0	4.6	62.4	44.6	−17.7	1.7
9	−1.0	9.3*	63.2	41.2	−22.0	1.5
10	0.2	5.6	62.6	48.8	−13.7	1.5
1 Irradiated		19.6*	61.9	52.4	−9.3	1.3
4 Irradiated		6.5*	61.8	57.3	−4.3	1.2
7 Irradiated		11.9*	62.3	41.3	−21.1	1.2
9 Irradiated		8.2*	62.6	44.8	−17.8	1.5
10 Irradiated		14.7*	62.0	49.0	−13.0	1.7
Lsd 5%	6.41		1.57	11.11	11.15	0.61

Astrices denote significant difference to control for nematode data and significant difference to the soil with the greatest survival rate for Pyronota.

TABLE 7 | Experiment 2: Arithmetic mean root rot score and nodules/plant.

Soil number	**Root rot score (0–3 scale)**		**Rhizobial nodules/mm root**	
	Control	***M. hapla* inoculated**	**Control**	***M. hapla* inoculated**
1	2.1	2.2	0.16	0.15
4	0.3	0.8	0.22	0.18
7	1.1	1.7*	0.17	0.14
9	0.0	0.0	0.16	0.16
10	0.1	0.5	0.09	0.11
1 Irradiated		0.0*		0.10
4 Irradiated		0.0		0.03*
7 Irradiated		0.0*		0.02*
9 Irradiated		0.0		0.07*
10 Irradiated		0.0		0.02*
Lsd 5% (treatment*soil)	0.51		0.065	

Astrices denote significant difference to control.

pathogenic microbes in that soil were having a large impact on potential productivity with this soil having the greatest root rot scores of any of the soils. There have been few studies of the causes of root rotting in New Zealand pasture plants. The soils with the lowest proportion of rot-free plant roots were from Northland (soil 1) and Southland (soil 10), with root rot incidence likely helping to explain the poor growth of plants in soil 1. Skipp and Christensen (1983) recorded the incidence of potential root-rotting fungi on white clover roots during a survey of New Zealand pastures and found that *Codinea fertilis* predominated in root pieces from two soils in Northland (close to soil 1 in the current study) and that *Bimuria novae-zelandiae* predominated in soils of Southland (close to the current soil 10). Wakelin et al. (2016) found a significant negative association between a *Pythium* clade and *T. repens* shoot growth. Whether, these are the causative agents of the root rotting observed in the current study will be clarified by the next generation sequencing results to be carried out on white clover endosphere samples which will be reported in a future paper.

Other than soil 1 irradiation either significantly reduced (soils 4 and 9) or had no significant effect (soils 7 and 10) on shoot growth for the control treatments. It appears these effects are due to changes in the microbial flora such that in soils 4 and 9 elimination of predominant beneficial microbes reduced plant growth whereas irradiation impacts on both beneficial and deleterious microbes in soils 7 and 10 resulted in no change in plant growth. Changes in soil nutrient levels post-irradiation are not able to explain the plant growth effect observed because they are not consistently associated with either increases or decreases in plant growth. At the rates of irradiation used here (ca. 40 kGy) Buchan et al. (2012) observed a decrease in NO_3-N (from ca. 4 to 0 μg N/g dry soil) and an increase of NH_4-N (from 1 to 8–12 μg) compared to untreated soil, probably due to the elimination of nitrifying bacteria. They also observed that elimination of microbes by irradiation increased pools of labile carbon. It had been expected that these changes would occur in the soils used here and that these would affect plant growth consistently across all soils (NH_4 driving either an increase or decrease in *T. repens* growth depending on its concentration in

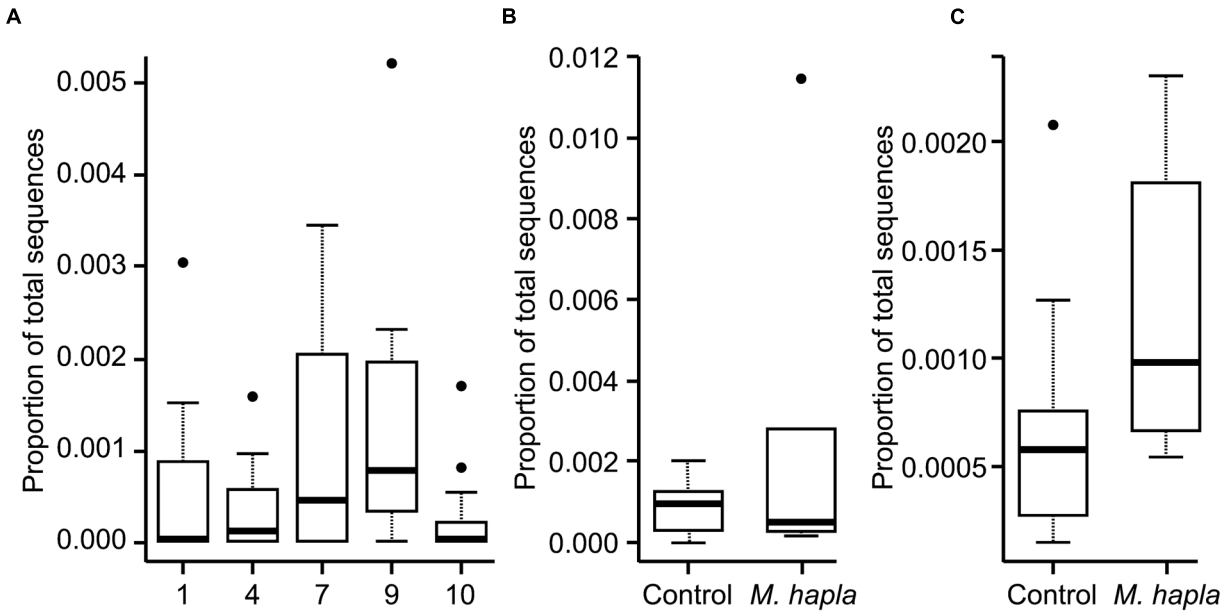


FIGURE 6 | Proportion of all fungal ITS DNA sequences from Experiment 2 that belonged to Orbiliomycetes for: (A) all soils; **(B)** soil 7 with or without added *M. hapla* and **(C)** soil 9 with or without added *M. hapla*.

soil (Britto and Kronzucker, 2002). However, this did not occur which suggests that any effects that occurred due to changes in nutrient status were, at least to some extent, overridden by changes in microbial communities and their effect (positive or negative) on plant growth. The soil dilution method used in Experiment 2 would have minimized differences in nutrients due to irradiation, compared to non-irradiated controls.

Wakelin et al. (2016) used microwaving to sterilize a range of New Zealand soils and found that of 10 soils they tested all but one gave a significant positive response in *T. repens* growth in sterilized soil. Over all soils they found that control soil produced 28.6% more *T. repens* shoot weight than sterile soils. Included in their soil set was soil 9 which, with their sterilization technique, showed a *T. repens* growth increase of ca. 25% in response to soil sterilization. This contrasts with the >200% reduction in growth in irradiated compared to untreated from the current study. There are likely a range of reasons for this difference between studies including temporal (the soils were sampled in different years), spatial (diseases, for instance, can be patchily distributed) and the severity of the treatment imposed. In the current study virtually all microbes were eliminated while the Wakelin et al. (2016) method was less severe and may have retained some beneficial microbes which then minimized the negative effects of the sterilization treatment. Root length in all soils were greater in Experiment 2 than Experiment 1, with many root systems reaching the bounds of the pot depth (120 mm). Dilution of the field soil with pumice appears, then, to have had one of the desired effects of reducing the loading, and therefore impact, of root pathogens or other deleterious microbes.

Although, survival of *C. zealandica* in soil 10 was very low this was not reflected in a significantly lower weight gain of those grubs that did survive, suggesting that quality of the food resource was not the cause of the grub's demise. Indeed, there was a significant reduction in root length of plants growing in soil 10 with grubs which indicates the grubs survived sufficiently long to eat a large proportion of roots but subsequently succumbed. Similarly, there was lower survival of grubs in soil 4 than in many other soils but in this case there was no significant reduction in root length, suggesting grubs may have succumbed more rapidly than in soil 10. Grubs in soil 4 were significantly smaller than those in soil 10 at inoculation to pots which may help explain why they succumbed more rapidly to whatever was the cause of death of these grubs. There was no correlation between *C. zealandica* and any of the fungi sequenced (data not shown) so it is likely these effects are due to other microbes such as the bacteria *Serratia entomophila* which is known to cause disease in these insects (Hurst et al., 2000). There was no similar effect of these two soils on *Pyronota* sp. survival in the second trial, suggesting the host specificity of whatever microbe was responsible for *C. zealandica* deaths did not extend to *Pyronota*. *Pyronota* sp. had no significant impact on either roots or shoots in any of the non-irradiated soils but did significantly decrease shoot growth in soils 1, 4, and 7 post-irradiation suggesting that any control exerted within control soils was eliminated by irradiation.

This study has clearly shown the effects of soil biology on plant growth can be profound. The *in situ* interactions between soil microflora and fauna that occur in the rhizosphere deserve closer attention, especially in New Zealand soils where they are little studied. Insights that can be gained from such studies will form the basis for developments in biocontrol, bioindicators and the agronomic practices that support beneficial interactions.

AUTHOR CONTRIBUTIONS

NB, DF, and RDJ conceived the project and gained grant funding. NB, KA, RJ, RDJ, YM, GB, AP, and DF had substantial contribution to the acquisition and interpretation of data. VC, CC, and PM had substantial contribution to data analysis and interpretation. All authors had substantial contributions to the drafting, revising, final approval, and agreement on the manuscript.

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frontiers in Plant Science

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Grasslands, Invertebrates, and Precipitation: A Review of the Effects of Climate Change

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Invertebrates are the main components of faunal diversity in grasslands, playing substantial roles in ecosystem processes including nutrient cycling and pollination. Grassland invertebrate communities are heavily dependent on the plant diversity and production within a given system. Climate change models predict alterations in precipitation patterns, both in terms of the amount of total inputs and the frequency, seasonality and intensity with which these inputs occur, which will impact grassland productivity. Given the ecological, economic and biodiversity value of grasslands, and their importance globally as areas of carbon storage and agricultural development, it is in our interest to understand how predicted alterations in precipitation patterns will affect grasslands and the invertebrate communities they contain. Here, we review the findings from manipulative and observational studies which have examined invertebrate responses to altered rainfall, with a particular focus on large-scale field experiments employing precipitation manipulations. Given the tight associations between invertebrate communities and their underlying plant communities, invertebrate responses to altered precipitation generally mirror those of the plants in the system. However, there is evidence that species responses to future precipitation changes will be idiosyncratic and context dependent across trophic levels, challenging our ability to make reliable predictions about how grassland communities will respond to future climatic changes, without further investigation. Thus, moving forward, we recommend increased consideration of invertebrate communities in current and future rainfall manipulation platforms, as well as the adoption of new technologies to aid such studies.

Keywords: climate change, drought, insects, invertebrate communities, irrigation, rain-exclusion shelters, rainfall

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INTRODUCTION

Grasses cover more of the earth's surface than any other vegetation type (Tscharntke and Greiler, 1995; Wang and Fang, 2009) and are often of high economic, ecological and biodiversity value, providing forage for livestock and high levels of carbon storage (Lee et al., 2014; Lenhart et al., 2015). Many grasslands exist in seasonal states of water limitation, and are highly responsive to changes in water availability in terms of biomass and composition (Knapp et al., 2002; Fry et al., 2014; Lenhart et al., 2015). Climate models predict changes in precipitation patterns, in terms of

the total amount and the frequency and intensity of rainfall events (IPCC, 2013), therefore leading to alterations in grassland plant composition and primary production.

Invertebrates are the most diverse and abundant constituent of terrestrial ecosystem fauna (Stork et al., 2015). Often overlooked, these organisms contribute to structuring grassland communities, through activities such as pollination and nutrient cycling (Whiles and Charlton, 2006) and contribute to shaping grasslands through top-down processes. For instance, herbivores can modify plant species richness by altering competitive dynamics between plant species (Olff and Ritchie, 1998). Likewise, plant community composition plays a bottom-up role in structuring arthropod communities (Perner et al., 2005; Hertzog et al., 2016), as do abiotic factors like temperature and water availability (e.g., Bale et al., 2002). Thus, both grassland plant and invertebrate communities can be directly impacted by alterations in climate. In addition, precipitation changes can have indirect impacts on both plants and invertebrates as the interactions occurring between the two communities are also climate-sensitive; the effect of herbivory on plant diversity varies across precipitation gradients, for instance (Olff and Ritchie, 1998).

It is in our interest to understand how climate change-driven alterations in precipitation will affect valuable grassland systems and the invertebrate communities they both support and rely on. Over the past 20 years, multiple experiments and observational studies have addressed the responses of grasslands to changes in precipitation, with a subset of these also examining invertebrate responses across a range of precipitation scenarios and spatial scales (summarized in **Table 1**). To our knowledge, there has been no synthesis of the relevant literature examining insect responses to precipitation changes, making a review of these studies timely. In this mini-review, we look at the effects of altered precipitation patterns – including reductions and increases in average rainfall, and changes in rainfall frequency – on grassland invertebrates and the plant communities they inhabit. We focus on findings from field-based/observational studies and precipitation manipulation experiments conducted in grasslands, including steppe and savannah habitats.

INVERTEBRATE RESPONSES TO PRECIPITATION CHANGE

Direct Impacts

In general, terrestrial arthropods are sensitive to changes in moisture, given their high surface-to-volume-ratio (Kimura et al., 1985). Under reduced rainfall, most aboveground arthropods avoid desiccation behaviorally by migrating, hiding in the soil, or, in a few cases, building a shelter (Willmer, 1982; Zalucki et al., 2002; Berridge, 2012). Structurally speaking, soft-bodied arthropods (isopods and myriapods) lack the waxy cuticle found in arachnids and insects that prevents or reduces evaporation (Berridge, 2012). This, in combination with differences in excretion-related water losses (Horne, 1968), suggests that soft-bodied arthropods will be more vulnerable to reductions in water availability, and, in some cases, to increases (Sylvain et al., 2014). Thus, changes in rainfall could be expected to affect hard and soft-bodied groups differently, resulting in shifts in the arthropod community.

On the other end of the spectrum, average increases in rainfall may negatively impact arthropods by disrupting flight, reducing foraging efficiency and increasing migration times (Peng et al., 1992; Drake, 1994; Kasper et al., 2008). Some arthropods can vary their behavior to combat the effects of extreme rainfall events and flooding by shelter-seeking and utilizing submersion tolerance strategies (Lambeets et al., 2008). The effects of increased rainfall on arthropods are also dependent on invertebrate morphology and are group-specific, with larger winged insects like Lepidopterans having a much higher degree of 'unwettability' (i.e., requiring greater volumes of water to become wet) than smaller winged insects (Wagner et al., 1996). Altered rainfall frequency can be positive or negative for invertebrates depending on the size of the event (Nielsen and Ball, 2015), but on the whole is expected to impact more rain-sensitive orders like Lepidoptera (Palmer, 2010).

Arthropods that spend all or some of their life stages belowground have evolved behaviors to manage water stress in times of drought and flood (Verhoef and Witteveen, 1980). Under reduced water availability, most soil invertebrates combat fluctuating moisture by relocating to places that are more favorable within the soil-matrix. Such movement, however, is dependent on suitable soil moisture and texture (Lees, 1943; Brust and House, 1990). Some invertebrates build earthen chambers, controlling the microclimate, similar to shelter-builders aboveground (Haile, 2001; Barnett and Johnson, 2013). Prolonged drought conditions may favor those species capable of such behaviors. Similarly, larvae with morphological adaptations to flooding may fare better in areas predicted to experience increases in rainfall. Species that have evolved in flood-prone environments in particular, like the cranberry root grub, with water-repellent hairs along its body that can trap air (King et al., 1990; Villani et al., 1999), may stand to have competitive advantages over flood-intolerant species. Thus, invertebrates both above and belowground have evolved a range of behavioral and morphological adaptations to alterations in water availability. Differences in the use of such strategies between species and functional groups will likely lead to alterations in invertebrate community composition.

Indirect (Plant-Mediated) Impacts

Invertebrate Responses to Plant Quantity and Quality

There is strong evidence in the literature for resource quantity-driven changes in invertebrate herbivore populations under altered precipitation regimes. Reduced rainfall results in reduced plant biomass, aboveground net primary productivity (ANPP), forage quality and cover, with increases in canopy light penetration and root:shoot ratios (Fay et al., 2003; Wu et al., 2011), leaving less plant biomass to support herbivores; however there is strong evidence that this response is ecosystem dependent (Byrne et al., 2013). Accordingly, declines have been reported – across various ecotypes – in the abundances of Orthoptera (Kemp and Cigliano, 1994); earthworms and scarabs (Davis et al., 2006;

TABLE 1 | A summary of the major precipitation manipulation experimental platforms assessing both plant and invertebrate responses to altered rainfall regimes.

Name; Location; Climate	Manipulation	Ecosystem; Plant groups	Invertebrate groups; Collection method	Method; Shelter design	Outcome	Reference
Silwood (UK) Temperate, cool	+/− water, summer drought and winter increase	Grassland; Forbs and grasses	Auchenorrhyncha, Araneae, Coleopt., Collembola, Dipt., Heteropt., Isopoda; Vacuum	Irrigation with rain water; Removeable roof	Under rainfall and nitrogen addition, plants did not respond. In the third year plant biomass declined in drought plots. Auchenorrhyncha and Araneae declined with plant biomass.	Lee et al., 2014
Wytham – TIGER IV 2c. (UK) Temperate, cool	+/− water, +/− root herbivores	Calcareous grassland; Forb	Lepidopt., Coleopt.; Manual	Manual with deionised water; Mobile shelters	Enhanced summer rainfall increased leaf miner abundance, but not when root herbivores were also present. Root herbivores reduced the parasitism rates of moths above ground (smaller pupal size). Plants under drought were overall less susceptible to leaf-miners regardless of root damage.	Staley et al., 2007
	+/− water, + winter heat	Calcareous grassland; Forbs, legumes and grasses	Auchenorrhyncha; Vacuum sampling		Added water increased plant cover and Auchenorrhyncha abundance; though drought reduced vegetation cover, the abundance of Auchenorrhyncha remained at ambient levels.	Masters et al., 1998
BCNWR[1] (USA) Subtropics, Warm/moderate cool	+ water, natural drought	Mixed-grass prairie and oak savannah; Forbs and grasses	Orthopt.; Manual	Water application method not mentioned; No shelter, natural drought	Water stress reduced plant biomass but not nutrient content and species diversity. Drought reduced forb protein content and grasshopper abundance and diversity. There was increased abundance and species richness of certain grasshoppers in increased precipitation plots.	Lenhart et al., 2015
OCCAM[2] (USA) Temperate, cool	+/− heat, +/− water, +/− CO_2	Old field – fescue; Forbs, legumes and grasses	163 morphospecies; Sticky traps, vacuum sampling	Irrigation with rain water; Fixed roof	No strong trends in terms of water effects; there was greater peak plant biomass in wet compared to dry. Weak effects of soil moisture on invertebrate community composition; more parasitoids in the dry treatment – temperature more important.	Villalpando et al., 2009
Agroscope (Switzerland) Temperate, cool	− water, diversity increments	Calcareous pasture; Forbs, legumes and grasses	Annelida; Mustard extraction	Not mentioned; Temporary shelter: summer only	Measurements were taken 1 year after drought application. Drought significantly increased the biomass of earthworms in plots where subordinate plant species were present. Drought caused shifts in earthworm community in terms of individual species.	Mariotte et al., 2016
Berkeley (USA) Subtropical, cool	+ water, winter precipitation +, spring precipitation +	Grassland; Forbs, legumes and grasses	Coleopt., Hemipt., Hymenopt., Orthopt., Araneae; Manual, pitfall	Irrigation with spring water; No shelter	Spring water addition caused diminishing increases in winter forbs/legumes resulting in lower plant and invertebrate species richness at the end of 5 years.	Suttle et al., 2007
DRIGrass[3] (Australia) Subtropical, warm/moderate cool	+/− water, altered frequency, summer drought	Pasture; Forbs, legumes and grasses	Coleopt., Hemipt., Hymenopt., Orthopt., Araneae; Vacuum sampling, sticky trap	Automatic irrigation with tap water; Fixed shelter	TBD	Power et al., unpublished

Climate classifications follow the Global Agro-Ecological Zones set out by the Food and Agriculture Organization (gaez.fao.org). [1]*Balcones Canyonlands National Wildlife Refuge (Marble Falls, TX, USA).* [2]*Oldfield Community Climate and Atmospheric Manipulation (Oak Ridge, TN, USA).* [3]*Drought and Root herbivore Impacts on Grassland (Richmond, NSW, Australia).*

Mariotte et al., 2016); belowground herbivores (Staley et al., 2007); and across herbivore communities generally (Lee et al., 2014). Plant quality changes could also play a role in these declines. In a feeding experiment, army worm larvae reared on droughted Yorkshire fog grass took longer to develop and had higher mortality rates than those feeding on non-droughted grass, due to lower soluble protein content (Walter et al., 2012). While some trends can be identified in the responses of invertebrates to reductions in rainfall, there is a high degree of variation between species. For instance, gastropod species in a UK study had highly individual responses to changes in water availability, with some benefitting from drought and others instead occurring in greater abundance under supplemented rainfall (Sternberg, 2000).

Increases in average precipitation (to a degree – the negative effects of flooding in grasslands have been reviewed elsewhere – see Plum (2005)) may result in benefits to invertebrate herbivores, except in cases where increased moisture facilitates pathogens and disease (Grant and Villani, 2003). On the whole, increases in precipitation lead to increases in ANPP (Zaller and Arnone, 1999; Byrne et al., 2013). Consequently, studies have reported improved grasshopper nymph survival (Guo et al., 2009) and increased abundance and richness of grasshoppers (Lenhart et al., 2015). However, in contrast to the increases in grasshopper abundance reported in Lenhart et al. (2015) two other studies reported reductions in grasshopper survival under similar increased rainfall treatments (Barton et al., 2009; Guo et al., 2009).

Hence, a recurrent theme in the literature is that the responses of herbivorous invertebrates to altered precipitation will likely be idiosyncratic in nature, making it difficult to make generalized predictions about the directions of their responses under different scenarios (González-Megías and Menéndez, 2012; Nielsen and Ball, 2015). The responses of herbivores to both reduced and increased water availability are likely to be linked to the responses of their individual food-plant(s), as well as the invertebrate specie's own physiological precipitation optimum (Schowalter et al., 1999).

Invertebrate Responses to Plant Community Composition

The responses of a grassland plant community to alterations in rainfall depend on the type of grassland (i.e., average water state – mesic, xeric etc.; Heisler-White et al., 2009), as well as the plant functional types (PFTs) that dominate the system (Collins et al., 2012; Andrey et al., 2014). For example, under altered rainfall frequency, with longer dry periods between more intense rainfall events, mesic grasslands generally experience a decrease in ANPP, whereas xeric grasslands show an increase (Fay et al., 2002, 2003; Heisler-White et al., 2009). In terms of PFTs, grasslands dominated by C_4 grasses tend not to show stimulations in ANPP under increased rainfall, perhaps because they are likely to be less water limited than their C_3 counterparts (Niu et al., 2008; Wu et al., 2011; Wilcox et al., 2015). Indeed, there is evidence that herbivorous insects consume relatively more C_4 plants in years with reduced rainfall frequencies (Warne et al., 2010), possibly due to improved quality or increased quantities of these plants under such scenarios. Thus, we could expect that reorganizations occurring at the plant community level in response to altered rainfall regimes will have consequences for herbivores, particularly for specialist feeders which may be reliant on the presence of just one or two plant species.

So far, experimental evidence directly linking precipitation-mediated changes in plant diversity to changes in the herbivore community is lacking. However, a 5-year field experiment by Suttle et al. (2007) showed that whilst increased summer rainfall enhanced plant biomass, increased dominance and reduced grassland plant species richness had eventual negative consequences for the invertebrate community. Specifically, herbivore and consumer abundance declined and the invertebrate food web became simplified, potentially pointing to the loss of more specialized herbivores. This study demonstrates the importance of longer-term studies in detecting plant community shifts as opposed to more immediate biomass responses. Furthermore, Wilcox et al. (2016) recently showed that short-term plant community shifts in response to increased water availability may be misleading when considering shifts over a decadal time scale.

Secondary Consumer Responses to Altered Rainfall

Alterations occurring in the abundance and diversity of primary consumers can flow up through the food chain to affect populations of predators and parasitoids (Suttle et al., 2007; Lee et al., 2014), which may themselves be more sensitive to climatic change (Voigt et al., 2003). Buchholz et al. (2013) found reductions in semi-dry grassland spider and carabid diversity and abundance under water-limited conditions. However, at a similar site 3 years earlier, the same authors found no change in spider species richness, composition or abundance under precipitation manipulation (Buchholz et al., 2010). Similarly, in a Chinese steppe, reduced rainfall caused declines in herbivore abundance with no corresponding decline in secondary consumers (Zhu et al., 2014). Clearly, as with herbivores, there will be differences in the individual responses of higher trophic levels to changes in precipitation patterns.

Precipitation-Sensitive Species Interactions

The idiosyncratic nature of invertebrate responses can be at least partially explained by complex precipitation-driven alterations in the interactions occurring between species within the system. The handful of studies which have tackled pairwise species interactions under precipitation manipulations have found complex, unpredictable responses with the potential to affect multiple trophic levels. For instance, spatially separated above- and belowground herbivores may influence each other through their effects on the shared host plant, such as by altering secondary chemistry (Johnson et al., 2012). Staley et al. (2007) found that enhanced summer rainfall increased the abundance of leaf mining moths on wild basil, but not when root herbivores were present. The negative effects of root herbivores on leaf miner pupal size reduced the parasitism rates of moths above ground, indicating the potential for precipitation-altered species interactions to have knock-on consequences for higher trophic levels.

In a separate study, detritivorous tenebrionid beetles belowground had negative effects on the abundances of generalist sap sucking and chewing herbivores when summer precipitation was supplemented, similar to the findings of Staley et al. (2007) (González-Megías and Menéndez, 2012). In contrast, the presence of belowground herbivores had positive impacts on aboveground leaf-mining flies, restoring their pupal weight and development time to ambient levels, when reared under drought conditions on milk-thistle (Staley et al., 2008). Taken together, these studies suggest that belowground organisms could serve to moderate the effects of reduced or increased water availability on aboveground herbivores, which may otherwise be expected to decrease or increase in abundance, respectively, in response to such rainfall regimes. As with the responses of individual species, the directions of the responses of the interactions between multiple species may also prove to be species- and system-specific. Further work is needed in the area to determine whether or not generalizations can be made, and to determine whether other interactions such as competition may also be affected by alterations in precipitation.

INVERTEBRATE-MEDIATED FEEDBACKS ON PLANT COMMUNITIES

At the ecosystem scale, invertebrate herbivores generally exert weak control over grassland plant communities (Whiles and Charlton, 2006; Coupe et al., 2009), though their impacts on plant species richness, for instance, may be stronger during herbivore outbreaks (Olff and Ritchie, 1998). Altered precipitation has the capacity to change the relative strength of the interactions occurring between grassland plant and invertebrate communities, by altering the abundance and composition of species within the system. In a Canadian grassland, invertebrates caused short-term reductions in plant cover, increases in root mortality and altered plant composition compared with pesticide treated plots (Coupe et al., 2009). The effects of the invertebrate community only became apparent under naturally occurring drought conditions. This suggests that invertebrate herbivores may exacerbate the negative effects of drought for grassland plants, and that grasslands may become more vulnerable to herbivores under drought.

Trophic level	Increased rainfall magnitude	Reduced rainfall magnitude	Altered rainfall seasonality and frequency
Plants 	▲ ANPP and resistance to herbivory[13]	▲ Light penetration, root:shoot ratio, susceptibility to herbivores[3,6,18]	▲▼ System and scenario specific changes in ANPP, cover, diversity, richness, protein content & C:N ratio[1, 2,4,5,6,8,16,18,19]
	▼ Diversity[17]	▼ ANPP, cover, diversity, richness & protein content[3,6,18]	
Primary consumers 	▲ Altered behaviour (e.g. shelter-seeking), richness, abundance[7,12,13,15,17,20]	▲ Altered behaviour (e.g. moving deeper in soil), mortality & development times[1, 2,4,16,18]	▲ Altered behaviour (e.g. shelter seeking), migration times & disrupted flight[9,10]
	▼ Survival[7,12,13,15,17,20]	▼ Abundance[1,2,4,16,18]	▼ Abundance[9,10,11,14]
Secondary consumers 	▲ Altered behaviour (e.g. shelter seeking)[10,17]	▲ Altered behaviour (e.g. moving deeper in soil)[2]	▲ Altered behaviour (e.g. shelter seeking), migration times & disrupted flight[9,10]
	▼ Abundance[10,17]	▼ Abundance & diversity[2]	▼ Abundance[9,10,11,14]

FIGURE 1 | A summary diagram of the general trends expected or found in the literature (theoretical, experimental, and observational studies) for plant, primary consumers (herbivores, detritivores etc.) and secondary consumers (predators and parasitoids), in response to altered precipitation regimes. Arrows: ▲ indicates increases in the given metric, ▼represents declines and ▲▼ denotes more varied results. References are given by the numbers on the diagram: (1) Barnett and Johnson (2013), (2) Buchholz et al. (2013), (3) Coupe et al. (2009), (4) Davis et al. (2006), (5) Fay et al. (2002), (6) Fay et al. (2003), (7) Guo et al. (2009), (8) Heisler-White et al. (2009), (9) Kasper et al. (2008), (10) Lambeets et al. (2008), (11) Lee et al. (2014), (12) Lenhart et al. (2015), (13) Masters et al. (1998), (14) Palmer (2010), (15) Staley et al. (2007), (16) Staley et al. (2008), (17) Suttle et al. (2007), (18) Walter et al. (2012), (19) Warne et al. (2010), (20) Zhu et al. (2014).

In an American temperate old-field study, experimentally increased rainfall caused declines in grasshopper abundance, which translated into a 15% reduction in grasshopper-inflicted plant damage for every 1 cm of increase in mean monthly precipitation (Barton et al., 2009). Conversely, in a limestone grassland, the plant community sustained an increase in biomass under supplemented rainfall scenarios, despite a significant increase in the abundance of Auchenorrhyncha herbivores (leaf, plant, and frog hoppers; Masters et al., 1998). Assuming that this greater abundance of insects inflicted comparable levels of damage on a per capita basis as those in ambient plots, this would suggest that grassland plants may be able to maintain increased growth despite higher levels of herbivory under increased rainfall scenarios. These two studies demonstrate that the strength of indirect, invertebrate community-mediated effects of altered precipitation on grasslands will depend on the identities of both the plant community and invertebrate species involved.

CONCLUSION AND FUTURE DIRECTIONS

Depending on the underlying water-status of the ecosystem, alterations in rainfall may have generally negative direct and indirect consequences for invertebrates (summarized in **Figure 1**). Changes in precipitation will also have the potential to cause impacts spanning multiple trophic levels, moderating the outcomes of species interactions. Reductions in rainfall may exacerbate the negative effects of herbivores for the plant communities they inhabit, though other players in the system might alter this response (e.g., Staley et al., 2008). In order to better understand how grassland invertebrates – and the important ecological processes they underpin – will respond to altered precipitation, we highlight the following four areas for future research:

(1) The incorporation of invertebrate responses in the design of current and future precipitation manipulation experiments: invertebrate responses remain under-studied in rainfall manipulation experiments, with the majority of studies considering only the responses of plants to short-term rainfall alterations of limited scope – altered frequency scenarios, for instance, remain critically under-represented (Johnson et al., 2016). This under-representation, coupled with the idiosyncratic nature of the responses detailed to date, makes it difficult to identify solid trends and predict how grasslands will respond to a wide range of precipitation scenarios. Achieving such a goal will require an increased number of studies from which to draw patterns from, across a broader range of precipitation scenarios.

(2) A focus on long-term studies: aside from the identities of the different components of the system, the timescale over which precipitation alterations are studied may also be important. The relatively short term nature of many field experiments to date obfuscates our ability to make more realistic predictions about how grassland communities will respond to future changes (Beier et al., 2012). Such studies are needed in order to capture changes in the direction of responses over time and lags in the manifestation of responses – particularly given the potential for short-term studies to have misleading results compared with those over longer timescales (Suttle et al., 2007; Wilcox et al., 2016).

(3) Greater geographical representation: this should be prioritized to determine the extent to which findings can be extrapolated across different biomes (the studies we review here are mostly from the UK and USA; Beier et al., 2012; White et al., 2012). Given how many plant and insect responses are likely to depend on the underlying water-status of the system, research across ecotypes will be an essential target for enabling progress the field.

(4) Examination of multiple climate factors at once: there is a need for experiments reflecting the reality of global change which will involve the simultaneous alterations of many factors (Villalpando et al., 2009; Beier et al., 2012). This is especially important given the potential for synergisms between factors, as may be expected between increased temperatures and reduced water availability. On the other hand, the effects of one factor may serve to moderate those of another (e.g., Lee et al., 2014).

Such studies will not be without logistical difficulty, though future developments in technology will help to ease this, including improvements in long-term, sensor-based data gathering. Continued development of DNA-based methods like metabarcoding will assist community-level studies by reducing time-consuming work and taxonomic expertise (Cristescu, 2014). Results from studies like those suggested above will provide critical information about grassland community responses for use in theoretical modeling approaches such as structural equation modeling, enabling the testing of theories at scales not yet possible experimentally. Such experimental and modeling approaches, carried out with broader geographical and precipitation-scenario representation, will be necessary in order for us to form more accurate predictions about the fate of ecologically important grassland ecosystems under climate change.

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KB and SF contributed equally in drafting, writing, and approving the final manuscript.

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OPINION
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The Importance of Testing Multiple Environmental Factors in Legume–Insect Research: Replication, Reviewers, and Rebuttal

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Keywords: atmospheric change, biological nitrogen fixation, climate change, insect–plant interactions, legumes, pastures

THE CASE FOR TESTING MULTIPLE ENVIRONMENTAL FACTORS

Investigating the impacts of predicted changes in our atmosphere and climate change on insect–plant interactions is a widely pursued area of research. To date, the majority of experimental studies have tested the impacts of single environmental factors on insect–plant interactions, but meta-analyses have clearly illustrated the importance of investigating multiple factors in tandem (Zvereva and Kozlov, 2006; Robinson et al., 2012). In particular, environmental change factors often interact with each other which can either strengthen or mitigate the effects of environmental factors acting alone (Robinson et al., 2012). For example, the positive effects of elevated atmospheric carbon dioxide concentrations (e[CO_2]) on plant growth are stronger under high nitrogen (N) conditions compared to low N conditions (+32 and +19%, respectively; Robinson et al., 2012). Likewise, from the limited number of studies available, Robinson et al. (2012) showed that e[CO_2] had different impacts on plant nitrogen, plant biomass, and secondary metabolites under elevated air temperature (eT) conditions. This does not invalidate single factor studies, of which we have published numerous examples, but this is an important consideration for making realistic predictions about how plants and insects will respond to future climates (Facey et al., 2014).

LEGUME–INSECT INTERACTIONS

A key feature of legumes is their capacity for biological nitrogen fixation (BNF), which they accomplish via symbiotic relationships with soil bacteria which associate with the plant in discrete root nodules. Given that insect herbivores are frequently nitrogen limited (Mattson, 1980), concentrations of N in legumes derived from BNF are likely to be crucial determinants of plant–herbivore interactions. Legumes differ markedly from non-legume plants in their responses to environmental change because BNF is often significantly affected (Robinson et al., 2012). Moreover, e[CO_2] and eT appear to have contrasting effects on BNF; e[CO_2] tends to promote BNF via several mechanisms (Soussana and Hartwig, 1996), including larger numbers of N_2-fixing symbiotic bacteria in the rhizosphere (Schortemeyer et al., 1996), increased nodulation (Ryle and Powell, 1992) and enhanced nitrogenase activity (Norby, 1987). In contrast, eT tends to have an inhibitory effect on BNF because of the low tolerance of N_2-fixing bacteria to higher temperatures (Zahran, 1999; Whittington et al., 2013). These generalizations are, of course, contingent on nutrient availability in the soil (e.g., Edwards et al., 2006).

Given this, one might assume that e[CO_2] and eT might have contrasting impacts on insect herbivores of legumes since they affect nitrogen concentrations in the plant

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tissues in a divergent manner. This seems to be the case, with e[CO_2] either having no adverse effects (e.g., Karowe and Migliaccio, 2011) or, more often, a beneficial impact on herbivore performance (e.g., Johnson and McNicol, 2010), particularly for aphids (Guo et al., 2013, 2014; Johnson et al., 2014). However, our recent work with lucerne (*Medicago sativa*) has shown that the positive impacts of e[CO_2] on pea aphids (*Acyrthosiphon pisum*) were negated under eT because eT caused decreases in nodulation and amino acid concentrations in the foliage (Ryalls et al., 2013, 2015). Testing multiple environmental factors, including soil nutrients, therefore seems to be particularly relevant for investigations into how legume herbivores will respond to atmospheric and climate change research.

THE CHALLENGES: REPLICATION AND REVIEWERS

Why are there so few multi-factorial experiments in climate change research? Put simply, constraints on replication are the biggest obstacles faced by investigators. Pseudoreplication (a term first coined in Hurlbert, 1984) is particularly common in climate change research (Newman et al., 2011). For example, 49 of the 110 climate change studies reviewed by Wernberg et al. (2012) had pseudoreplication issues. This usually arises because when environmental factors are applied to controlled chambers, glasshouses, or FACE (Free Air CO_2 Enrichment) rings, the unit of replication for those treatments is the chamber, greenhouse, or ring, respectively (Lindroth and Raffa, in press). Subunits (e.g., individual plants) are not independently subjected to the treatment, and therefore not true replicates. As a result, statistical tests are based on artificially high degrees of freedom, resulting in a larger F statistic, potentially leading to type I errors (i.e., false positives; Lindroth and Raffa, in press). For this reason, many reviewers for scientific journals automatically reject manuscripts if any part of an experiment is pseudoreplicated without necessarily considering whether the biological conclusions of the study are really compromised by pseudoreplication (Davies and Gray, 2015). This is possibly an overzealous interpretation of the case by Hurlbert (1984), the authority on the subject, who states that "there should be no automatic rejection of [such] experiments" (Hurlbert, 2004). In a recent and comprehensive article, Davies and Gray argue convincingly that reviewers erroneously and dogmatically reject papers that have pseudoreplication issues which is slowing the pace of ecological research. While Davies and Gray (2015) focussed on non-manipulative experiments in natural systems, many of the points were germane to multi-factorial climate change research. In particular, many contemporary statistical tests, such as nested designs and random/mixed effect models, account for the lack of independence between pseudoreplicates so may help in some cases (Chaves, 2010; Leather et al., 2014; Davies and Gray, 2015). Of course, such statistical approaches could only help where a treatment combination was repeated in more than one chamber, glasshouse, or FACE ring.

COMPARING EXPERIMENTAL APPROACHES—POTENTIAL FOR REBUTTAL?

How do researchers attempt to overcome the pseudoreplication problem experimentally? The simplest way is to avoid it altogether by fully replicating environmental treatments. However, using even the bare minimum of replicates (e.g., $N = 4$) would require 16 separate chambers, glasshouses, or rings for an e[CO_2] × eT experiment. Many researchers cannot readily access this number of identical facilities or monopolize them for that matter. Repeating the experiment several times and using experimental run as the source of replication is another approach (e.g., Johnson et al., 2011), but this can be logistically demanding in time and cost. Even when fully replicated, the degrees of freedom in these studies are often so low that they are susceptible to type II errors, whereby "real responses" are not statistically detected (e.g., the "false negative").

Another approach that researchers sometimes use is "chamber swapping", whereby experimental units (e.g., plants) are moved within, and then between, chambers with attendant changes in environmental conditions (e.g., Bezemer et al., 1998). This does not eliminate pseudoreplication, but rather serves to minimize its effects by equalizing any unintended "chamber effects" across all experimental units. While this approach might be criticized because chamber effects might affect plants differently during different stages of their development (Potvin and Tardif, 1988), researchers have addressed this by staggering experiments so plants are exposed to particular chambers at the same stage of development (e.g., Vuorinen et al., 2004a,b).

How do results from a "chamber swapping" experiment compare with replicated experiments? We can answer this question, in part, using three comparable published studies that examined the impacts of environmental change on interactions between lucerne and the pea aphid. One experiment was replicated using multiple chambers (Johnson et al., 2014), one replicated using multiple experimental runs (Ryalls et al., 2015) and one adopted the chamber swapping approach (Ryalls, 2016). The first of these only examined e[CO_2], whereas the other two experiments also included eT. **Figure 1** shows the increase in dry mass of plants (with and without aphids) grown under e[CO_2] and eT relative to plants grown under ambient conditions. This response was selected for comparison since it was evidently measured the same way in each experiment. Despite using very different approaches, in most cases we obtained very similar responses whether the experiment was fully replicated or conducted with regular chamber swaps (c. every 10 days). Analysis of variance suggested that study type had little impact on the response we measured [$F_{(2,\ 219)} = 0.20$, $P = 0.82$]. This is a crude

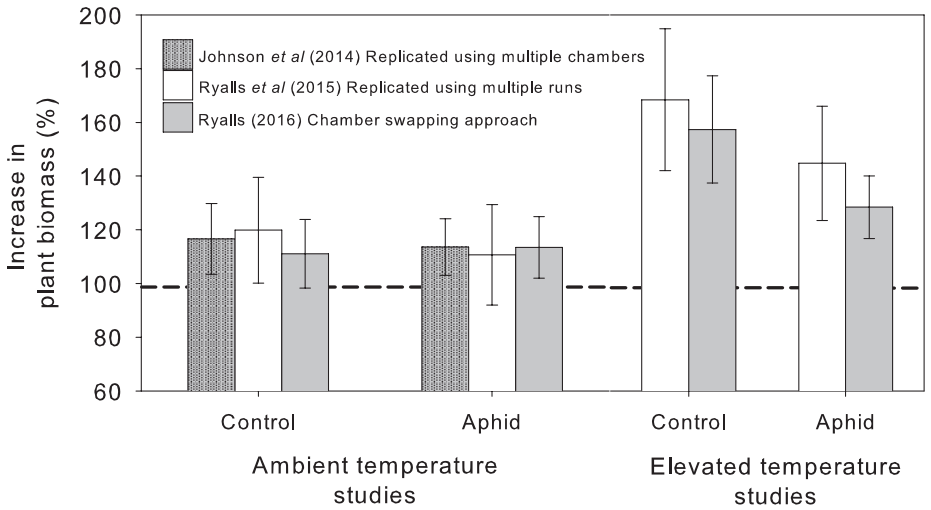


FIGURE 1 | Relative change in plant biomass at elevated [CO_2] compared to plants grown at ambient [CO_2], indicated with the dashed line, with and without (control) aphids (mean ± *S.E.* shown). Data from three experiments using replication with multiple chambers (Johnson et al., 2014) and multiple experimental runs (Ryalls et al., 2015) compared with the "chamber swapping" approach (Ryalls, 2016). All experiments used the same cultivar (Sequel) and similar levels of [CO_2] (400 vs. 600–640 ppm) and temperature (25–26 vs. 30°C).

comparison, but it is reassuring that we obtained similar data and reached identical conclusions using the chamber swapping approach.

CONCLUSIONS AND RECOMMENDATIONS

While incorporation of multiple environmental factors is desirable in many climate change studies of plant–herbivore interactions (clearly advocated by Robinson et al., 2012), we argue here that it is especially relevant to legume–insect research. Nitrogen status in legumes is shaped by BNF, which is highly affected by atmospheric and climatic change, often in divergent directions. This will inevitably affect legume quality for herbivores (i.e., especially primary metabolites, but possibly secondary metabolites too), and likely affect herbivore abundance and performance. Nonetheless, experimental manipulation of multiple factors is challenging and prone to pseudoreplication. "Chamber swapping" does not eliminate this problem, but it appears to minimize "chamber effects" and give comparable results to fully replicated experiments—at least in the lucerne-aphid system. We recommend that researchers working in other systems also take a cautious approach with regard to careful replication until they can develop confidence that their observed effects are real and repeatable. The statistical significance of numerical differences remain inflated, however, so it would be judicious to treat any marginally significant results with caution and rather interpret effect sizes rather than *P*-values *per se* (see discussion by Ellison et al., 2014). Davies and Gray (2015) make the similar arguments and suggest that conclusions can be phrased as new hypotheses if necessary. In conclusion, we agree with Newman et al. (2011) on this issue that "as long as authors are clear about the use of pseudoreplicates, and the readers appreciate the potential problems interpreting such results, then such studies are valuable despite their pseudoreplication."

AUTHOR CONTRIBUTIONS

SJ conceived and drafted the article with significant intellectual input from all authors. JR conducted the majority of the experimental work described in the article, with SJ, AG, and AF overseeing collection of further data used in **Figure 1**.

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METHODS
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DRI-Grass: A New Experimental Platform for Addressing Grassland Ecosystem Responses to Future Precipitation Scenarios in South-East Australia

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Climate models predict shifts in the amount, frequency and seasonality of rainfall. Given close links between grassland productivity and rainfall, such changes are likely to have profound effects on the functioning of grassland ecosystems and modify species interactions. Here, we introduce a unique, new experimental platform – DRI-Grass (**D**rought and **R**oot Herbivore **I**nteractions in a **Grass**land) – that exposes a south-eastern Australian grassland to five rainfall regimes [Ambient (AMB), increased amount (IA, +50%), reduced amount (RA, −50%), reduced frequency (RF, single rainfall event every 21 days, with total amount unchanged) and summer drought (SD, 12–14 weeks without water, December–March)], and contrasting levels of root herbivory. Incorporation of a belowground herbivore (root-feeding scarabs) addition treatment allows novel investigation of ecological responses to the twin stresses of altered rainfall and root herbivory. We quantified effects of permanently installed rain shelters on microclimate by comparison with outside plots, identifying small shelter effects on air temperature (−0.19°C day, +0.26°C night), soil water content (SWC; −8%) and photosynthetically active radiation (PAR; −16%). Shelters were associated with modest increases in net primary productivity (NPP), particularly during the cool season. Rainfall treatments generated substantial differences in SWC, with the exception of IA; the latter is likely due to a combination of higher transpiration rates associated with greater plant biomass in IA and the low water-holding capacity of the well-drained, sandy soil. Growing season NPP was strongly reduced by SD, but did not respond to the other rainfall treatments. Addition of root herbivores did not affect plant biomass and there were no interactions between herbivory and rainfall treatments in the 1st year of study. Root herbivory did, however, induce foliar silicon-based defenses in *Cynodon dactylon* and *Eragrostis curvula*. Rapid recovery of NPP following resumption of watering in SD plots indicates high functional resilience at the site, and may reflect adaptation of the vegetation to historically high variability in rainfall, both within- and between years.

DRI-Grass provides a unique platform for understanding how ecological interactions will be affected by changing rainfall regimes and, specifically, how belowground herbivory modifies grassland resistance and resilience to climate extremes.

Keywords: climate extremes, community ecology, drought, NPP, plant-herbivore interactions, rainfall manipulation, root herbivory

INTRODUCTION

Grasslands cover more than 40% of the Earth's land surface (LeCain et al., 2002). They support tremendous biodiversity, underpin grazing and animal production, and store more than one-third of global terrestrial carbon stocks (Trumper et al., 2009). Given the close relationship between rainfall and both the productivity and diversity of grasslands (Sala et al., 1988; Walter et al., 2012), future changes in rainfall regimes are likely to have a substantial impact on the ability of grasslands to provide these important ecosystem services.

Climate models predict changes in the overall amount and seasonality of rainfall, and increased intervals between rain events (i.e., reduced rainfall frequency; Easterling et al., 2000; Fischer et al., 2013; Intergovernmental Panel on Climate Change [IPCC], 2013). Of particular note is the expectation that prolonged and more intense droughts, in combination with warmer temperatures, will combine to expose ecosystems to more frequent extreme climates, pushing today's ecosystems into uncharted climate territory (Kayler et al., 2015). The seasonality of rainfall inputs is also a crucial determinant of grassland dynamics, with seedling establishment, productivity and senescence all influenced by the amount and timing of growing season rainfall (Huxman et al., 2004). Indeed, even small increases in winter rainfall have been shown to influence the functioning of grassland ecosystems in the following spring (Fry et al., 2014a). Furthermore, there is a growing body of evidence that reductions in the frequency of rainfall events are at least as (and sometimes more) important as reductions in the size of events, in terms of their effects on key ecological processes (Fay et al., 2003; Knapp et al., 2008; Heisler-White et al., 2009; Peng et al., 2013).

Shifts in rainfall regimes are not only expected to have a major impact on the composition and functioning of grasslands (Fry et al., 2016), but are also likely to modify interactions between plants and their associated herbivores (Staley et al., 2007; Johnson et al., 2011; Lee et al., 2014). Invasive root-feeding scarab beetles were accidentally introduced to Australia in the first part of the 20th century (recently reviewed by Frew et al., 2016) and, in pastures, their collective mass can exceed that of mammals grazing aboveground (Britton, 1978). Because root herbivory is hidden and occurs by attrition, losses in primary productivity are less conspicuous than those due to aboveground herbivory, but can be up to 25% in grassland systems (Seastedt and Murray, 2008). Even minor root herbivory can damage plants and alter their physiology by: (i) decreasing nutrient and water uptake, (ii) causing disproportionate resource losses by severing roots, (iii) diverting assimilates away from shoot growth for root re-growth, (iv) imposing leaf water deficits, and (v) causing infection (Johnson and Murray, 2008; Zvereva and Kozlov, 2012). The resulting effects on plant biomass and metabolism are often larger (Meyer et al., 2009) and differ from those caused by aboveground herbivores (Zvereva and Kozlov, 2012). Impairment of root function via root herbivory has parallels with water stress imposed via periods of drought. Indeed, a recent meta-analysis has shown that root herbivory and drought reduced plant growth to a greater extent than any other combination of biotic and abiotic stresses (Zvereva and Kozlov, 2012). Moreover, root herbivory can change plant community composition in grasslands via preferential feeding on certain plants (Schallhart et al., 2012).

DRI-Grass (**D**rought and **R**oot Herbivore **I**nteractions in a **Grass**land Ecosystem) is a new experimental platform designed to examine ecosystem responses to the twin stresses of altered rainfall and root herbivory. Uniquely, DRI-Grass includes shifts in the size, frequency and seasonality of rainfall events, and incorporates a factorial belowground herbivore addition treatment to investigate interactions between these abiotic and biotic stresses. It joins a new generation of drought experiments (*sensu* Thompson et al., 2013) that incorporate realism in terms of both future rainfall scenarios (e.g., Jentsch et al., 2007; Hoover et al., 2014; Knapp et al., 2015) and also trophic complexity (Johnson et al., 2011, 2015; Zhu et al., 2014). Despite the clear importance of root herbivores for the functioning of grassland ecosystems (Frew et al., 2016), their role in moderating grassland resistance and resilience under changing rainfall regimes has rarely been examined in long term field-scale experiments.

Here we introduce DRI-Grass, presenting microclimatic data that demonstrate the impacts of shelter infrastructure on the physical and biotic environment. We also present data on early vegetation responses to test the hypotheses that: (1) reduced rainfall amount and summer-long drought, will reduce aboveground productivity to a greater degree than a shift in rainfall frequency toward fewer, larger events (with annual rainfall amount unchanged); and (2) root herbivory will alter plant quantitative (e.g., ANPP) and qualitative (e.g., chemical) responses to altered rainfall regimes. In focusing on our approach and methodology, this paper aims to provide the methodological detail that will assist other researchers interested in constructing experimental platforms that incorporate both biotic and abiotic stressors. Presentation of selected early results is intended to provide a broad indication of the ecosystem responses that can be measured using this multi-stressor, multi-trophic approach.

THE DRI-GRASS EXPERIMENTAL PLATFORM

The study site is located in Richmond, NSW, Australia (S33 36′35, E150 44′18), at an elevation of 25 m a.s.l. Mean annual rainfall at the site is 806 mm (Australian Government Bureau of Meteorology, Richmond – UWS Hawkesbury Station[1]), with summer being the wettest season and winter generally the driest. Seasonal mean maximum/minimum temperatures are 29.4/18.8°C in summer and 17.3/3.2°C in winter. The soil is a Blackendon Sand, with a sandy loam texture and a water holding capacity of 20–22%. There is a mineral hardpan present at approximately 90 cm depth. **Table 1** summarizes the soil characteristics of the site.

The experiment is situated within a former pasture grassland, comprising a total of 62 plant species (Supplementary Table S1), of which ∼12 species are common. The most abundant species include the C_4 grasses *Axonopus fissifolius, Cynodon dactylon, Cymbopogon refractus, Eragrostis curvula,* and *Paspalum dilatatum,* the C_3 grasses *Microlaena stipoides* and *Lolium perenne*, and the C_3 forbs *Hypochaeris radicata* and *Plantago lanceolata*. The site had been under grazing management until 2001; since this time grazers were removed, the site was fenced and subsequently mown every 2–3 months, until the experiment commenced in June 2013.

TABLE 1 | Soil properties at the DRI-Grass field site.

Soil property	Value (units)
Texture	84.4% sand
	7.4% silt
	9.2% clay
SOM content	2.4%
pH	6.4
Total N	0.011 mg g^{-1}
Total P	0.0016 mg g^{-1}
*Exchangeable NO_3	17.1 μg cm^{-2} 90 $days^{-1}$
*Exchangeable NH_4	3.6 μg cm^{-2} 90 $days^{-1}$
*Exchangeable PO_4	1.55 μg cm^{-2} 90 $days^{-1}$
Bulk density	1.66 g cm^{-3}
C:N ratio	12.98
Water holding capacity	0.21 ml ml^{-1}

*Exchangeable nutrient concentrations obtained using ion exchange membranes (Plant Root Simulators)®; values measured at 0–10 cm depth.

RAINOUT SHELTER DESIGN

Shelter frames are made from 25 mm galvanized steel tubing and covered with a single sheet of clear Acrylic cast Perspex (1.88 m × 2.49 m, Mulford Plastics, Silverwater, NSW, Australia). Roofs are at a maximum height of 140 cm, sloping at a 20° angle down to a low-end height of 70 cm (**Figure 1**). Shelters are orientated along a SW-NE axis, with the low end facing into the direction of the prevailing wind. All rainfall is intercepted and directed away from the plots. Water treatments are applied following each rainfall event, using an irrigation system controlled by a Campbell logger (CR1000) and a series of 16-Channel AC/DC Relay Controller units (SDM-CD16AC units; Campbell Scientific, Thuringowa, QLD, Australia) that control solenoid valve opening/closure, and thus regulate delivery of water to individual plots. To simulate rainfall patterns that reflect actual rainfall events, the amount of water delivered is proportionate to the amount of precipitation that has fallen in the previous 24 h (i.e., AMB receives the same amount of rainfall as measured at the site in the previous 24 h; IA receives 50% more; and RA receives 50% less than the ambient amount). Target amounts of water are set using a calibrated flow meter. Water is delivered to each plot via a network of polyethylene pipes and four 90° spray heads per plot, mounted at a height of 30–45 cm (moveable, depending on vegetation height) at the corners of each shelter. An impermeable root barrier is installed within each plot, just inside the roof footprint, to a depth of 30 cm, giving an actual plot size of 1.8 m × 2.0 m (i.e., 3.6 m^2). This barrier prevents incursion of roots from outside the experimental plots and minimizes horizontal water flow between plots and the surrounding grassland area.

ENVIRONMENTAL MONITORING

Rainfall is measured using a tipping bucket rain sensor (0.2 mm graduation, ICT International, Armidale, NSW, Australia) and air temperature is measured on site every 5 min (model 107 sensor, with radiation shield, Campbell Scientific, Thuringowa, QLD, Australia). Photosynthetically active radiation (PAR) is recorded at 15 min intervals (Apogee sensors, model SQ-110, ICT International, Armidale, NSW, Australia), under three shelters and in three unsheltered (outside) plots.

Soil moisture TDR probes (CS616, Campbell Scientific, Thuringowa, QLD, Australia) with 30 cm long prongs are installed at an angle of 30°, to integrate moisture readings for the top 15 cm of the soil profile, in half of the plots ($n = 3$ per treatment combination). Regular (approximately every 4–6 weeks) measurements of soil moisture are also conducted manually in all plots, using a theta probe (Delta T Devices, UK), to determine whether automatically logged moisture readings from permanently sensored plots are representative of the respective treatments.

Given the open-sided nature of the shelters and the potential for rain ingress under windy conditions, edge effects on soil moisture were quantified under a range of conditions, including during dry periods and after small, medium and large rainfall events. Soil moisture (0–10 cm depth) was measured using a theta probe inserted in a 5 × 5 grid system, covering 25 points per plot, evenly spaced at a distance of 40 cm from the plot boundary and 40 cm from the next grid point. These within-plot measurements were compared with readings taken immediately outside of the shelters (eight replicates – two along each side of the plot).

[1]http://www.bom.gov.au

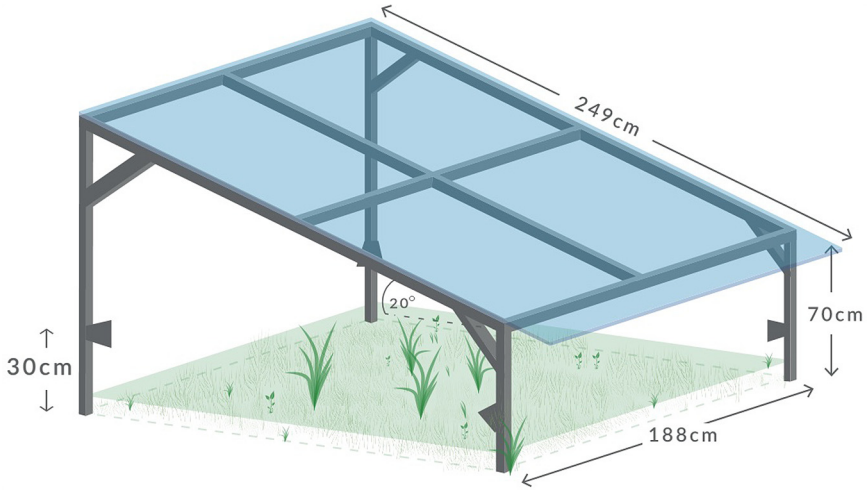


FIGURE 1 | Schematic of DRI-Grass rainout shelter design.

EXPERIMENTAL DESIGN

The experiment comprises five different rainfall treatments, three of which are crossed with a root herbivory treatment (detailed below). All treatment combinations are replicated six times, in a fully randomized block design [$n = 48$ (i.e., 8×6) for sheltered plots]. There are also 12 unsheltered plots [hereinafter referred to as "Outside Plots (OP)"] – six with herbivore additions and six without the addition of herbivores – making a total of 60 experimental plots.

Rainfall treatments comprise: (a) sheltered control (AMB), (b) reduced rainfall amount (RA: 50% reduction of ambient), (c) reduced rainfall frequency (RF: ambient rainfall amount, as a single application once every 21 days), (d) increased rainfall amount (IA: 50% increase of ambient), and (e) summer drought (SD: complete removal of all rainfall for a 12–14 weeks period, December–March, with ambient rainfall thereafter). Unsheltered (outside control) plots (OP) receiving ambient rainfall were also included to evaluate the magnitude of shelter effects. Rainfall treatment effects were assessed by comparing the four altered scenarios (RA, RF, IA, and SD) to the sheltered control plots (AMB). Rainfall treatments commenced on June 21, 2013.

Root herbivore treatment: Three of the rainfall treatments (AMB, RA, and RF) and OP also include a belowground herbivore addition treatment ($n = 6$ for each treatment combination). To impose the herbivore addition treatment, 27 g of locally collected adult scarab beetles (Coleoptera: Scarabaeidae) were added to the herbivore addition plots in December 2013, and an additional 9 g of adult beetles were added to each plot in February–March 2014. Adult beetles were added to plots by placing them within mesh enclosures in the plots, and allowing them to oviposit for a period of 3 days on each occasion, before mesh enclosures were removed. In order to control for the effects of the mesh enclosures on vegetation, identical structures were placed on paired (herbivore-free) plots at the same time. We verified the efficacy of herbivore treatments 18 months after beetle additions (October 2015) via destructive, within-plot soil excavation and associated sampling. This involved excavating two holes (25 cm × 10 cm) per plot to a depth of 20 cm; samples were separated into two depths: 0–10 and 10–20 cm, and sieved. Macro and mesofauna were collected, identified under a dissecting microscope and counted.

COORDINATED SAMPLING CAMPAIGNS

We undertake regular, coordinated sampling campaigns, both above- and belowground, to determine treatment impacts on plant, microbial and invertebrate communities, and associated changes in ecosystem properties and processes. Details of these sampling campaigns are outlined below, with selected data presented in this methods paper; further data characterizing above- and belowground responses will be presented in subsequent publications.

Vegetation Monitoring

Non-destructive vegetation cover measurements are conducted approximately every 4 months by placing a 1 m^2 quadrat with 25 sub-divisions in the center of each plot and recording species level presence/absence data in each sub-division. Since October 2013, twice-yearly harvests (April and October) of all aboveground plant material have been undertaken. For this, vegetation is cut to ground level within the central 1 m^2 of each plot and, in a randomly selected subsample (20–40% of the harvested material), live (green) material is sorted to species level and separated from dead biomass. All plant material is oven-dried at 80°C for 48 h, and weighed to provide a measure of growing season (October–April) and cool season (April–October) productivity for all plots.

Invertebrate Monitoring

Immediately prior to the harvests in October 2013, April 2014, and October 2014, aboveground invertebrates were sampled from

each of the plots using a 'G-Vac' suction sampler (SH 86C, Stihl AG & Co. KG, Germany). The device was passed over the plots in a zigzag pattern for 20 s, with all dislodged material and invertebrates captured in a fitted organza bag. In addition, quarterly from October 2013 until April 2015, yellow sticky card traps (Bugs for Bugs, Mundubbera, QLD, Australia) were suspended from the center of each shelter roof (or at the same height for unsheltered controls) for a period of 1 week to capture flying invertebrates. Invertebrates from both suction samples and sticky traps were identified to at least Order level (except for two groups taken to Subclass only – Acari and Collembola).

To quantify belowground invertebrate responses to altered rainfall regimes, two composite soil samples, each composed of two soil cores (3 cm diameter, 10 cm depth) are collected at the beginning (October) and end (April) of each growing season for extraction of soil nematodes and microarthropods. We focus on these two groups as they are the two most abundant soil invertebrate groups. Nematodes and microarthropods are extracted using standard techniques (Baermann, 1917; Tullgren, 1918). Nematodes are classified to trophic level based on morphology under an inverted microscope, and counts converted to individuals per kg dry soil. Microarthropods are initially sorted into springtails, oribatid, mesostigmatid and other mites (for more detail see Nielsen et al., 2016). More detailed analyses will be undertaken on archived samples over the course of the experiment. Further assessments of soil invertebrate groups that require more destructive sampling campaigns will be undertaken at a later stage in the experiment to avoid substantial disturbance.

Plant, Soil, and Microbial Analyses

Leaf material was sampled from three grass species (*C. dactylon*, *E. curvula,* and *M. stipoides*) in November 2014 and analyzed for silicon concentrations. Ground plant material was pressed at 11 tons into 5 mm thick cylindrical pellets with a manual hydraulic press using a 13 mm die (Specac, Orpington, UK). Si concentration (% dry mass) was determined using a commercial P-XRF analyser (Niton XL3t900 GOLDD analyser: Thermo Scientific Winchester, UK) held in a test stand (SmartStand, Thermo Scientific, Winchester, UK; Reidinger et al., 2012).

Since April 2014, we have carried out regular sampling campaigns to investigate treatment effects on bulk soil properties (e.g., chemistry, nutrient availability) and processes (e.g., enzyme activities). Soil samples comprise 8–10 cores (0–10 cm deep, 1 cm wide) per plot. Analyses for soil chemistry, microbial and enzyme activity are conducted using fresh soil samples; molecular analyses (qPCR and MiSeq Illumina high-throughput sequencing) are carried out on DNA extracted from frozen samples, using the PowerSoil kit® (MoBio). Results of soil and microbial analyses will be presented in a subsequent paper.

STATISTICAL ANALYSIS

All analyses were carried out using linear models in R (Version 3.2.4, R Core Team, 2016). Shelter effects on PAR and air temperature were evaluated for month-long periods in summer (November 2014) and winter (August 2014), to compare differences between AMB (sheltered) control plots and outside (unsheltered) control plots. Data from all 48 sheltered plots were used to evaluate rainfall treatment effects on plant biomass. Data were first inspected for homogeneity of variances and normality of errors and, where necessary, log, box-cox or arc-sine transformation was carried out prior to analyses (Crawley, 2012). Treatment effects were evaluated by first fitting the full model (rainfall treatment, herbivore addition and their interactions) and then model simplification was undertaken by removing non-significant terms. When neither the interaction between rainfall treatment and herbivore addition, nor herbivore addition on its own were significant (i.e., $P > 0.10$), herbivore-added plots were retained in the analysis to assess rainfall treatment effects. When overall treatment effects were significant, Tukey's HSD *post hoc* tests were used to determine significance between treatment levels; results were considered significant if $P < 0.05$.

Soil moisture data (November 27, 2013 to November 25, 2014) obtained from automatic sensors were averaged per week and the effects of rainfall treatment were evaluated with a repeated-measures linear mixed-effects model [lme in the nlme package (Pinheiro et al., 2016)] with plot nested within treatment as a random effect. In order to test for the effect of root herbivore addition, generalized linear mixed models were constructed with the lmer() function in the lme4 package (Bates et al., 2015), and Chi square (χ^2) values between models with and without the herbivore interaction were compared (Faraway, 2006). *Post hoc* comparisons were performed with glht() in the multcomp package with a 'Benjamini–Hochberg' correction (Hothorn et al., 2008).

The effect of watering treatment on aboveground invertebrate abundance was assessed using linear models on square-root transformed abundance data. Watering treatment was included in the model as an independent variable along with scaled plot biomass, given the documented effect of underlying plant structure on sampling efficiency (Facey and Torode, 2016). Effects of root herbivore addition on the presence/abundance of scarabs in the soil were analyzed with a zero-inflated-poisson model in the *pcsl* package, and model significance evaluated using a likelihood ratio (lr) test (Jackman, 2015).

RESULTS AND DISCUSSION

Shelter Effects on Microclimate

Differences in air temperature between unsheltered and sheltered plots varied diurnally and between seasons (**Table 2**). On average (24 h mean), sheltered plots were 0.04°C warmer than unsheltered ones, representing non-significant daytime cooling and nighttime warming associated with shelter roofs; this phenomenon is well known from previous studies using permanently installed shelter infrastructure (Fay et al., 2000; Beier et al., 2004; Vogel et al., 2013). Whilst temperature was only minimally affected by the presence of shelter roofs, effects on PAR were more substantial. On average, PAR was significantly lower under shelters than in OPs (−15.9%; $F_{1,2} = 145.3$, $P < 0.01$). Interception losses averaged 17.4% during summer months ($F_{1,2} = 139.5$, $P < 0.01$) and 13.1% in winter ($F_{1,2} = 198.9$,

TABLE 2 | Shelter effects on canopy air temperature and photosynthetically active radiation (PAR).

		Air temperature (°C)			PAR (mean daily mol m^{-2})		
		Outside	Shelter	Diff (°C)	Outside	Shelter	Diff (%)
Overall	24 h	15.73	15.77	+0.04	–	–	–
	Daylight hours	19.43	19.24	−0.19	34.98	29.43	−15.9%
	Night time	12.04	12.30	+0.26	–	–	–
Summer (November)	24 h	20.46	20.48	+0.02	–	–	–
	Daylight hours	23.70	23.55	−0.15	41.54	34.30	−17.4%
	Night time	17.23	17.42	+0.19	–	–	–
Winter (August)	24 h	11.15	11.21	+0.06	–	–	–
	Daylight hours	15.30	15.08	−0.22	27.41	23.80	−13.1%
	Night time	7.01	7.35	+0.34	–	–	–

TABLE 3 | Mean seasonal and annual volumetric soil water content (SWC, %) and seasonal rainfall (mm), 2013–2014.

Treatment	Winter	Spring	Summer	Autumn	Annual
Ambient (sheltered)	14.0 (0.49)[a]	10.3 (0.45)[a]	10.0 (0.32)[a]	13.0 (0.34)[a]	11.8 (0.22)[a]
Reduced amount	12.2 (0.46)[b]	9.2 (0.48)[a]	8.6 (0.20)[a]	10.0 (0.23)[b]	9.9 (0.19)[b]
Increased amount	13.0 (0.51)[a]	10.2 (0.55)[a]	9.8 (0.43)[a]	13.0 (0.43)[a]	11.4 (0.25)[ab]
Reduced frequency	11.1 (0.43)[b]	7.7 (0.40)[a]	8.6 (0.45)[a]	12.5 (0.40)[a]	10.0 (0.23)[ab]
Summer drought	13.8 (0.50)[a]	10.9 (0.51)[a]	8.7 (0.30)[a]	7.3 (0.03)[b]	10.0 (0.22)[ab]
Treatment effects (df = 1,4)	$\chi^2 = 23.5$, $P = 0.0001$	$\chi^2 = 7.85$, $P = 0.097$	$\chi^2 = 8.06$, $P = 0.089$	$\chi^2 = 21.4$, $P = 0.0003$	$\chi^2 = 15.3$, $P = 0.009$
Outside plots (unsheltered)	14.3 (0.55)	11.4 (0.55)	10.5 (0.36)	15.3 (0.45)	12.8 (0.26)
Ambient rainfall (mm)					
06/2013–05/2014	80.4	230.9	124.7	160.6	596.7
30-years mean	137.6	182.4	280.7	205.6	806.3
30-years CoV	77.3%	41.4%	43.4%	60.8%	26.1%

Values in brackets represent ±1 SE. Rainfall treatment effects on SWC are evaluated for all sheltered plots (i.e., excluding unsheltered control plots). Ambient rainfall means and coefficients of variation (CoV) also summarized by season, for the past 30 years (1982–2012). Different letters indicate significant differences between treatments.

$P < 0.01$). This is directly comparable to light interception values reported for similar studies in Germany (15%, Vogel et al., 2013) and the USA (21%, Fay et al., 2000) where, like ours, shelter roofs cover the entire plot area. Lower levels of PAR interception have been associated with shelter infrastructure where roofs cover a smaller proportion of the plot area. For example, Gherardi and Sala (2013) report reductions of just 3 and 6% for shelters covering 50 and 80% of the plots, respectively, while Yahdjian and Sala (2002) found a 10% reduction in PAR associated with roofs covering 80% of the plot area.

Light interception is an unavoidable artifact of field experiments involving fixed roofs. Unless within-shelter PAR is above light-saturation levels for much of the growing season (e.g., Fay et al., 2000), shelter-induced reductions in PAR are likely to have implications for photosynthesis and, depending on other resource constraints, potentially also productivity. Whilst we only measured PAR, it is worth noting that other shelter-associated changes in spectral characteristics can also influence other photosensitive ecosystem processes. For example, Vogel et al. (2013) attributed differences in litter decomposition rates and plant metabolic profiles to contrasting levels of UV radiation associated with shelter roofs in a recent rainfall manipulation experiment, advocating for the need to include roofed controls in shelter-based studies.

Outside plots had slightly higher soil water content (SWC) compared to sheltered AMB plots (**Table 3**), although differences were not statistically significant ($\chi^2 = 0.254$, df = 1, $p = 0.614$). Given the link between canopy transpiration rates and SWC (Patrick et al., 2007), these differences may be due to slightly higher transpirational water loss associated with greater vegetation biomass in AMB compared to OP (see below). A second possible explanation for these differences could be the method for water delivery to plots. The relatively small droplet size of water applied via sprinklers increases the chance of both spray drift and higher levels of canopy interception (and subsequent evaporation; Moss and Green, 1983), both of which could result in lower SWC for a given water application, compared to natural rainfall.

Soil water content within 25 cm of the edge of RA, RF, and SD plots was typically 0–0.5% higher than in the center of the plot. Immediately after heavy ambient rainfall episodes, differences of up to 2.8% were noted, but overall differences in SWC between the center and outside 25 cm of the plot area were small. The biggest differences were observed in SD plots, following a large rainfall event during the period of total rainfall exclusion, when within-plot SWC was particularly low. At this time, average SWC was 23.9% outside of these shelters, while values within SD plots ranged from 2.5% in the plot center, to 3.3 and 6.3% at distances

of 50 and 25 cm from the outer edge of the plots, respectively. In the context of ambient rainfall incursion, we estimate the size of the edge effect to be approximately 25 cm. This is directly comparable with values reported for similar shelters elsewhere (e.g., 20 cm; Gherardi and Sala, 2013), and confirms that the combination of roof interception, impermeable root barrier and a well-drained, sandy soil provide effective hydrological isolation of our experimental plots under all but the wettest/windiest conditions.

Shelter Effects on Plant Productivity

The differences in SWC, air temperature and PAR between AMB and OP were associated with modest differences in ANPP. Growing season ANPP was 10.8% higher, and cool season ANPP was 29.7% higher in AMB compared to OP (**Figure 2**), although neither of these differences were statistically significant. The larger shelter effects on cool season productivity were driven by a significantly greater accumulation of dead plant material in AMB plots (+51%; $F_{1,22} = 7.87$, $P < 0.001$). Although shelter impacts on ANPP were not statistically significant, the biological relevance of 10–30% differences in productivity is arguably high and emphasises the need to compare treatment effects to sheltered controls (AMB). The importance of controlling for shelter artifacts has been raised in rainfall manipulation studies elsewhere, with shelter infrastructure associated with altered net primary productivity (NPP), decomposition and carbon fluxes (Fay et al., 2000; Vogel et al., 2013). Based on information on how shelters modify the microclimate in our study, and associated biological responses, all rainfall and herbivore treatment effects are evaluated against sheltered AMB plots, with unsheltered plots used to provide a context for interpreting these effects.

Treatment Effects and Seasonal Patterns in Soil Water Content

Ambient rainfall at the site for the 12-months period from June 2013 to May 2014 was 597 mm, lower than the 30 years mean of 806 mm. During the 1st year of the experiment, summer rainfall was particularly low, with less than half the long-term seasonal average falling in the local area. Temporal trends in SWC are illustrated in **Figure 3**. Treatment differences reflect both the timing of ambient rainfall and that of imposed treatments with, for example, the 3-weekly periodicity of the RF treatment, and the summer-long water withholding in the SD treatments, clearly reflected in soil moisture patterns.

Table 3 summarizes overall and seasonal treatment effects on SWC for the first 12 months of the experiment. The biggest differences were seen during the summer (December–February), corresponding to the period of maximum plant growth and the timing of the SD treatment. There was a significant overall effect of rainfall treatment on SWC but no effects of herbivore addition, nor an interaction between the two treatments. *Post hoc* analyses revealed that moisture levels were higher in AMB compared to RA plots; RF experienced greater variation in soil moisture, with periods where SWC was higher and others where it was lower than the other treatments, during the 21-day watering cycle. The lowest seasonal mean SWCs were associated with different treatments in different seasons; in winter and spring RF plots had the driest soils, while in autumn SD had the lowest SWC.

Annual mean SWC was consistent between all reduced rainfall treatments (RA, RF, and SD) and clearly demonstrates that contrasting rainfall regimes can result in similar long-term mean SWC, despite highly contrasting patterns both within- and between- seasons. Increasing rainfall variability (i.e., longer inter-pulse intervals) has been associated with increased (or decreased) mean SWC, depending on background climatic conditions and soil type (Zeppel et al., 2014). Under mesic conditions, reducing the frequency of rainfall events (with no change in total rainfall amount) has been found to lower mean SWC (Harper et al., 2005; Fay et al., 2011), but in arid systems similar reductions in frequency can actually increase mean SWC, particularly in deeper soil horizons (Heisler-White et al., 2008, 2009). With a long-term mean rainfall of 806 mm for the local area, SWC in the RF treatment in our study parallels that at other mesic sites and highlights the importance of changes in the pattern, as well as the amount of rainfall for ecosystem hydrology under climate change.

Unlike field-based rainfall manipulations elsewhere (Fay et al., 2000; Gherardi and Sala, 2013), differences in seasonal means (**Table 3**) and temporal patterns (**Figure 3**) in SWC between AMB and IA treatments at our site were minimal. This likely reflects greater transpirational water loss associated with higher plant biomass in IA, and the high drainage capacity and relatively low soil water-holding capacity (Atwell et al., 1999) of our sandy soils, compared to other studies (e.g., silty clay loam; Fay et al., 2000). It also emphasizes that impacts of future shifts in rainfall regime will be contingent not only on the nature of the change, but will also depend on the climate context and soil conditions at a given site.

Early Vegetation Responses to Rainfall and Root Herbivore Treatments

Total ANPP in the first growing season (October 13–April 14) was significantly affected by rainfall treatment ($F_{4,43} = 7.70$, $P = 9.03e^{-05}$), but there was no effect of herbivore addition, nor interactions between rainfall and herbivore treatments at this time. *Post hoc* comparisons reveal that rainfall effects on ANPP were driven primarily by a significant reduction (−62.3%, $P = 0.0004$) in biomass in SD plots (168.4 ± 46.2 g m^{-2}) compared to AMB (446.6 ± 49.4 g m^{-2}, **Figure 4A**). ANPP in IA and RA treatments were not significantly different from AMB, but there was a clear gradient in productivity, increasing from 370.9 (±35.8) g m^{-2} in RA to 556.3 (±74.7) g m^{-2} in IA. This represents a positive linear relationship between ANPP and water inputs for these treatments, despite the absence of a clear relationship with mean SWC. ANPP in RF plots was similar to AMB, despite a somewhat higher mean SWC in RF plots.

Treatment effects on live (green) harvested biomass in April were very similar to those for total aboveground productivity, with a significant overall effect of rainfall ($F_{4,43} = 6.20$, $P = 0.0005$) but not herbivore addition, nor interactions between the two treatments (**Figure 4B**). The amount of dead plant

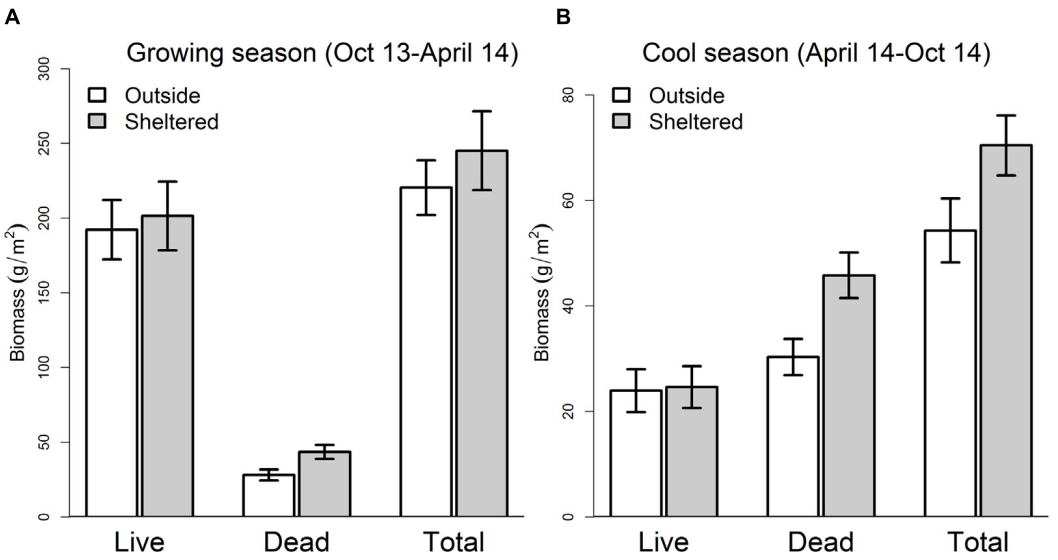


FIGURE 2 | Harvested plant biomass in sheltered ambient and outside (unsheltered) plots in (A) April 2014 (growing season) and (B) October 2014 (cool season).

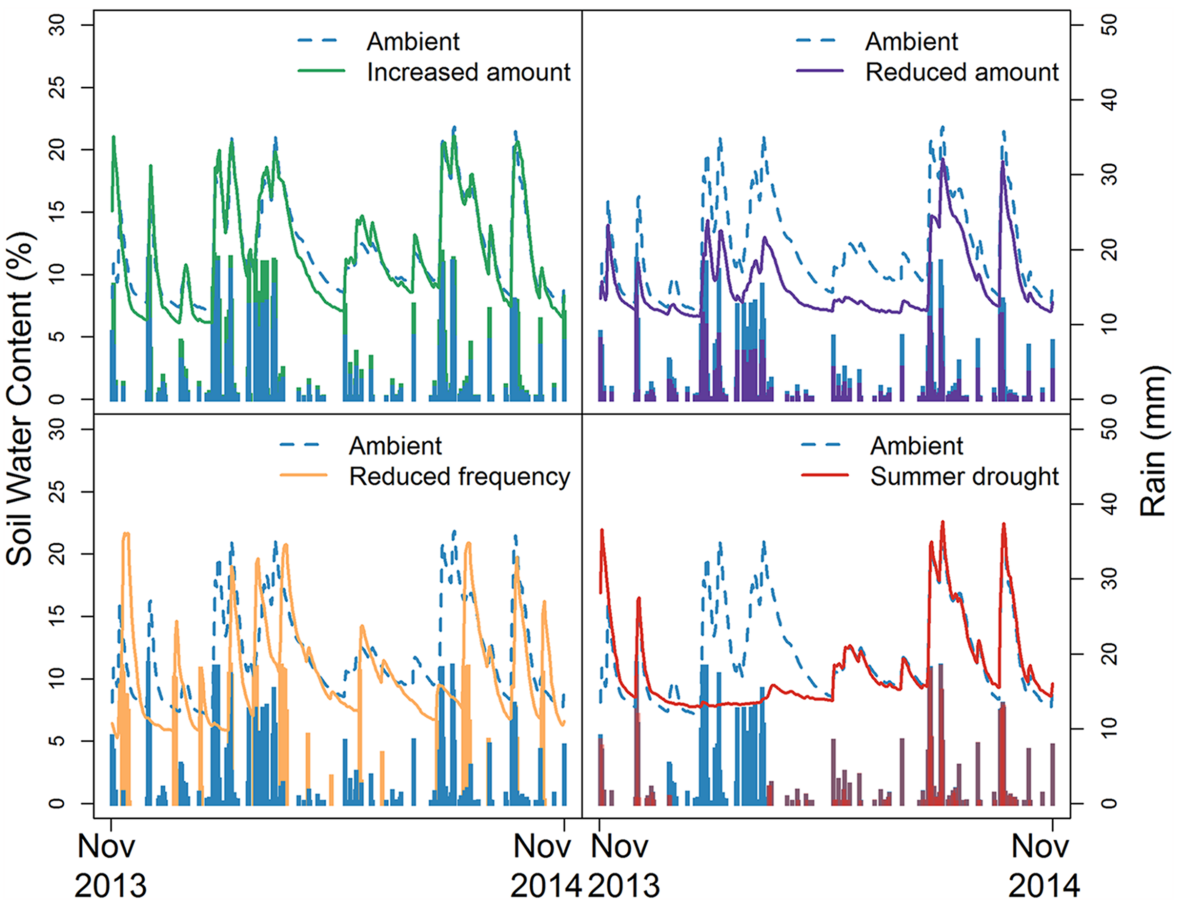


FIGURE 3 | Temporal trends in soil water content, by treatment, from November 2013 to November 2014.

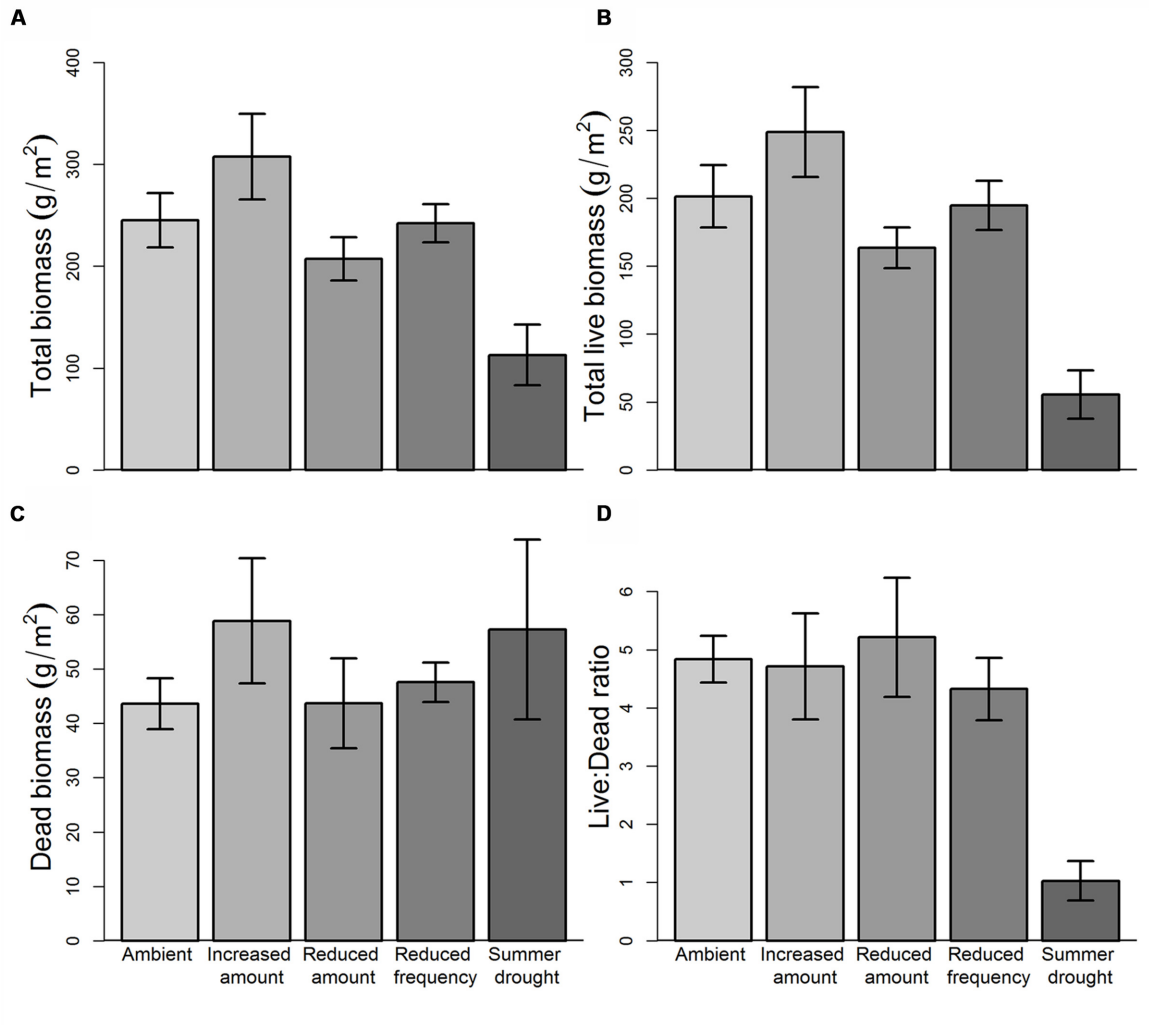


FIGURE 4 | Rainfall treatment effects on growing season biomass (April 2014 harvest). (A) Aboveground NPP (October–April), **(B)** live biomass, **(C)** dead biomass, **(D)** live:dead biomass ratio. Values are means ±1SE.

material harvested at the end of the growing season was fairly consistent across plots, with no significant treatment effects (**Figure 4C**). However, the ratio of live to dead material differed significantly ($F_{4,43} = 3.76$, $P = 0.0104$) between contrasting rainfall regimes, with dead material representing 17.8% of total aboveground biomass in AMB plots, but 58.8% in SD plots (**Figure 4D**).

Taken together, these early data indicate that the total amount of growing season rainfall is a more important determinant of vegetation productivity at our site than the frequency of those inputs. Close relationships between rainfall amount and plant growth are well established (Sala et al., 1988; Hsu et al., 2012; Southon et al., 2012). However, the lack of biomass response to altered rainfall frequency contrasts with recent studies that report negative impacts on species productivity, cover and nutritional quality (Walter et al., 2012; Jones et al., 2016), as well as greater impacts on ecosystem processes, than reducing total rainfall amount in both mesic (Heisler-White et al., 2009; Fay et al., 2011) and (semi-) arid grasslands (Heisler-White et al., 2008; de Dios Miranda et al., 2009). In our study, plant community resistance to altered rainfall frequency may reflect the high variability in rainfall; coefficients of variation in seasonal rainfall are naturally high (particularly during spring) at our site compared to other sites (e.g., Walter et al., 2012) and it is likely that the vegetation has adapted to historically high levels of rainfall variability. The potential for changes in plant community composition to buffer changes in ecosystem functioning under more variable rainfall conditions (Fry et al., 2013, 2014b; Gherardi and Sala, 2015) may also explain the lack of biomass response to RF treatment in our study, and will be a subject for future investigation.

Cool-season (April–October) ANPP and live biomass were not affected by either rainfall or herbivore addition treatments, or their interactions (**Figure 5**). Treatment effects on dead

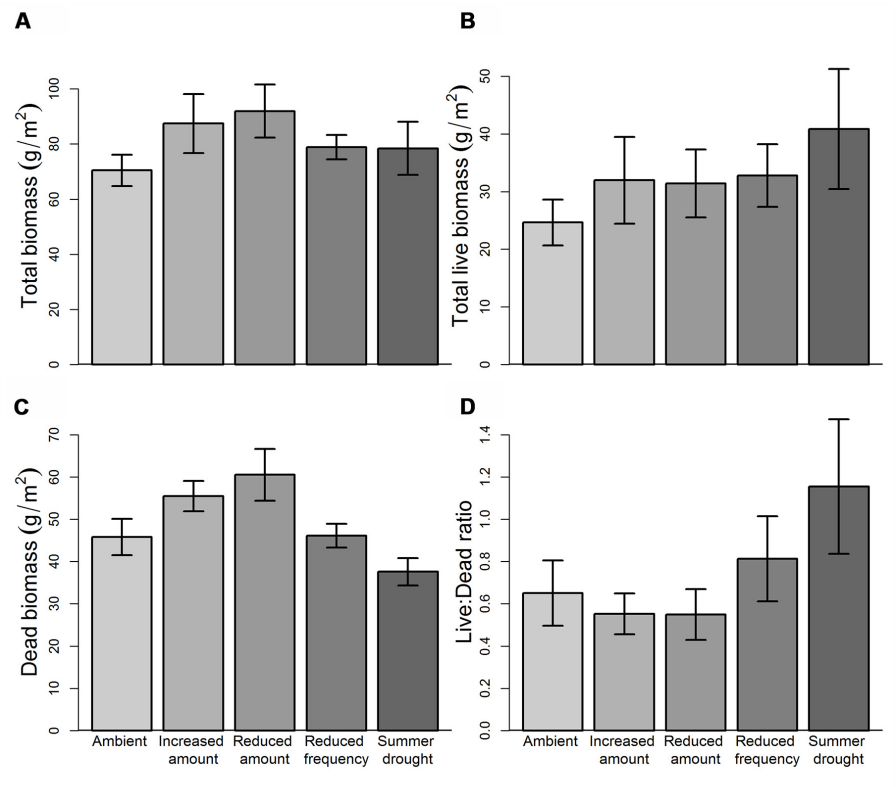


FIGURE 5 | Rainfall treatment effects on cool season biomass (October 2014 harvest). (A) Aboveground NPP (April–October), **(B)** live biomass, **(C)** dead biomass, **(D)** live:dead biomass ratio. Values are means ±1SE.

biomass were only significant for rainfall ($F_{4,43} = 3.329$, $P = 0.018$), with more dead plant material in RF (+32.1%, $P = 0.017$) than AMB at this time. Although not statistically significant, there was nearly twice as much live plant material in SD plots in the October harvest as in AMB ($P = 0.096$), demonstrating very rapid vegetation recovery once the summer-long drought was released. This, together with levels of cool-season productivity in all water-manipulated treatments that were higher than AMB plots, implies a high degree of climate resilience at our site. The ability for water-stressed ecosystems to recover is likely associated with rapid recovery of formerly dominant species, or compensatory growth by other (previously sub-ordinate or newly recruited) species within these plots. Previous rainfall manipulation studies have shown contrasting rates of recovery, with evidence of both rapid return to pre-drought levels of ANPP (Hoover et al., 2014) and legacy effects persisting for many years (Haddad et al., 2002; Sala et al., 2012). Shifts in plant community composition represent a key mechanism by which physiologically driven decline in NPP under drought can be offset (Hoover et al., 2014; Gherardi and Sala, 2015). Compositional change will, therefore, be closely monitored at our site over the next 3–5 years to establish the relationship between diversity, community-weighted functional traits and both resistance and resilience to rainfall change.

The absence of effects of root herbivore addition on plant productivity responses is not surprising, given the timing of additions (December 2013 and February/March 2014) in relation to the first growing season (October 2013–April 2014). Furthermore, given scarab preferences for grazing on more nutritious grass species (e.g., C_3 species; Johnson et al., 2014), shifts in community composition may be more likely than impacts on plot-level productivity. Other studies (e.g., Schallhart et al., 2012) report root herbivore-associated plant community change, and this may become more apparent in our study over time.

Invertebrate Responses

Root herbivore treated plots contained significantly higher abundances of root-feeding insects (mostly scarabs) than those that were not inoculated [23.3 $m^{-2} \pm 9.9_{(0-20\ cm\ depth)}$ and 5.6 $m^{-2} \pm 2.7_{(0-20\ cm\ depth)}$, respectively] (Log-likelihood$_5$ $= -39.6$, $P = 0.0052$).

Preliminary results from the aboveground invertebrate sampling campaigns found that invertebrate abundance was not significantly influenced by the imposed rainfall regime (**Table 4**). However, this lack of response in the invertebrate community regimes is not surprising after only four months of treatments and may change as more data become available from subsequent sampling campaigns. In particular, we may expect

TABLE 4 | Mean total aboveground invertebrate abundances (individuals) from the first sampling campaign (October 2013).

	Mean total aboveground invertebrate abundance	
Rainfall treatment	**Sticky traps**	**Vacuum samples**
Ambient	216.8 (20.2)	133.8 (36.8)
Increased amount	209.3 (18.9)	225.5 (61.9)
Reduced amount	233.4 (19.0)	165.8 (43.4)
Reduced frequency	227.8 (15.2)	396.5 (143.0)
Summer drought	237.5 (27.0)	230.0 (59.7)
Rainfall	$F(4,42) = 0.391$	$F(4,42) = 0.962$
	$P = 0.814$	$P = 0.438$

Values in brackets represent ±1 SE.

invertebrate abundance aboveground to be negatively affected by the reductions in plant material occurring when the SD treatment is imposed (December–March).

No effects of altered precipitation were observed in terms of the abundances of nematodes, nematode trophic group or microarthropods after more than 1.5 years' climate manipulation (i.e., April 2015; Nielsen et al., 2016). However, there were subtle, significant changes in nematode feeding guild composition and diversity in SD plots, suggesting that nematodes are sensitive to extreme events in this grassland (Nielsen et al., 2016). Similar results have been observed in other studies (e.g., Cesarz et al., 2015). These responses will be investigated in depth later in the experiment, to determine if belowground invertebrate responses are amplified or ameliorated over time.

A number of plant chemical characteristics have been measured, but here we focus on silicon (Si) concentrations because grasses typically accumulate high levels of Si and this has been shown to increase their resistance to both abiotic (e.g., drought) and biotic (e.g., herbivory) stress (Epstein, 1999; Cooke and Leishman, 2011). In particular, Si has been demonstrated to be an inducible defense against aboveground herbivores (Massey et al., 2007). We found similar patterns of induction in two of our three sampled grasses, *C. dactylon* and *E. curvula,* in response to belowground herbivore addition (**Figure 6**). To our knowledge, this is the first example of belowground herbivores inducing this defense in grasses. Future work will report whether this effect persists and whether rainfall treatments moderate the induction of this important plant defense.

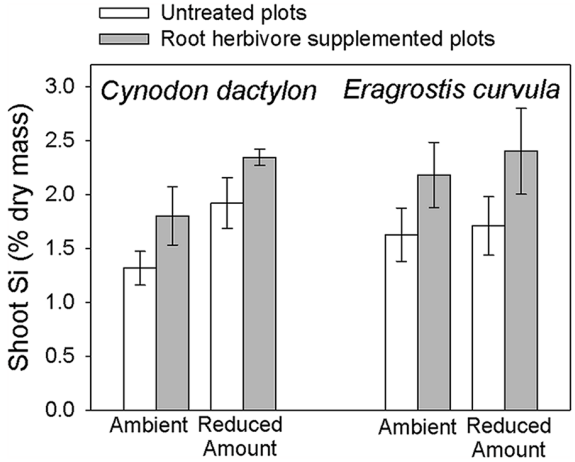


FIGURE 6 | Effects of root herbivore addition treatment on foliar silicon concentrations in *Cynodon dactylon* and *Eragrostis curvula.*

CONCLUSION

This paper introduces a new experimental platform that, uniquely, combines multi-level rainfall manipulation with contrasting levels of root herbivory. Early data clearly identify the importance of shelter controls in rainfall manipulation experiments of this type, in order to assess potential shelter artifacts that may otherwise obscure treatment effects. This SE Australian grassland exhibited relatively high resistance of NPP to changes in the size and frequency of rainfall inputs, except under extreme SD. The rapid recovery of NPP in SD plots after ambient rainfall inputs were resumed indicates that low ecosystem resistance to climate extremes is not necessarily associated with low functional resilience. This may reflect adaptation of the plant community to the naturally high variability in rainfall that can occur both between- and within- years in Australia, with annual inputs at our site varying by as much as 66% below and 114% above the long-term mean. The absence of a productivity response to herbivore addition may be a consequence of the timing of this treatment in relation to the first growing season, compensatory growth by affected plant species and/or changes in plant community composition. This research platform will allow ongoing monitoring of ecological responses to novel combinations of abiotic and biotic stresses, and identification of mechanisms underlying observed above- and belowground responses.

One of the biggest challenges in ecosystem ecology today is to improve our understanding of the mechanisms by which plant physiological and morphological responses to climate change affect interactions within- and between- trophic levels, and ecological feedbacks (Van der Putten et al., 2010). The DRI-Grass experimental platform provides the opportunity to gain important new insight into how ecological interactions are affected by changing rainfall regimes and, specifically, how belowground herbivory modifies grassland resistance and resilience to climate extremes.

AUTHOR CONTRIBUTIONS

SP, SJ, UN, and DT designed and led set up of the experimental facility. SP contributed to data collection and analysis, and led overall data interpretation and writing. UN led nematode/microarthropod data collection, analysis and interpretation and assisted with writing. SJ led plant Si data analysis and interpretation and assisted with writing. DT

contributed to data interpretation and writing. KB implemented root herbivore treatments, led collection of vegetation and scarab data, contributed substantially to data analysis and also writing. RO-H and EG-F contributed to field data collection and assisted with writing. SF led aboveground invertebrate sampling and analysis, and contributed to writing. SH ran foliar Si analyses, and contributed to data interpretation and writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01373

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Altered Precipitation Impacts on Above- and Below-Ground Grassland Invertebrates: Summer Drought Leads to Outbreaks in Spring

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Climate change is predicted to result in altered precipitation patterns, which may reshape many grassland ecosystems. Rainfall is expected to change in a number of different ways, ranging from periods of prolonged drought to extreme precipitation events, yet there are few community wide studies to accurately simulate future changes. We aimed to test how above- and below-ground grassland invertebrate populations were affected by contrasting future rainfall scenarios. We subjected a grassland community to potential future rainfall scenarios including ambient, increased amount (+50% of ambient), reduced amount (–50% of ambient), reduced frequency (no water for 21 days, followed by the total ambient rainfall applied in a single application) and summer drought (no rainfall for 13 weeks during the growing season). During Austral spring (September 2015), we sampled aboveground invertebrates, belowground macro invertebrates and nematodes. Aboveground communities showed a significant response to altered rainfall regime with the greatest effects observed in summer drought plots. This was mostly due to a large increase in sucking herbivores (658% higher than ambient plots). Plots experiencing summer droughts also had higher populations of parasitoids, chewing herbivores and detritivores. These plots had 92% more plant biomass suggesting that primary productivity increased rapidly following the end of the summer drought 5 months earlier. We interpret these results as supporting the plant vigor hypothesis (i.e., that rapid plant growth is beneficial to aboveground invertebrates). While belowground invertebrates were less responsive to altered precipitation, we observed a number of correlations between the abundances of above- and below-ground invertebrate groups under ambient rainfall that dissipated under altered rainfall regimes. Mechanisms underpinning these associations, and reasons for them to become decoupled under altered precipitation regimes (we term this 'climatic decoupling'), remain speculative, but they provide the basis for formulating hypotheses and future work. In conclusion, we predict that shifts in rainfall patterns, especially summer drought, will likely have large, but probably short-term, impacts on grassland invertebrate communities. In particular, sucking herbivores show sensitivity to precipitation changes, which have the potential to cascade through the food chain and affect higher trophic levels.

Keywords: aboveground–belowground interactions, arthropods, climate change, multi–trophic interactions, rainfall extremes, root herbivores, soils

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INTRODUCTION

Invertebrates make up 96% of known terrestrial species, with an estimated contribution to ecosystem services worth $60 billion per year in the US alone (Losey and Vaughan, 2006). Invertebrates are the main faunal component of grassland ecosystems and key to ecosystem functioning, contributing to services such as soil formation, pollination and population control (Curry, 1994; Weisser and Siemann, 2004). Grasslands are globally important ecosystems, underpinning livestock production and regulating our climate (Gibson, 2009), with carbon sequestration by grasslands estimated to be worth over $200 per hectare (Daily, 1997).

Climate change models predict altered precipitation patterns and an increased number of extreme precipitation events (IPCC, 2014), which will likely impact grasslands and the invertebrates within them. Changes in rainfall could be highly variable. For example, Australian rainfall records have shown recent increases in wet and dry extremes as well as greater seasonal variation, thought to be partly explained by climate change (Garnaut, 2011). Altered rainfall patterns can have direct effects on invertebrates, such as heavy rainfall events causing physical damage during flight, to reducing foraging efficiency and increasing migration times (Barnett and Facey, 2016). On the other hand, droughts can cause desiccation of above- and below-ground invertebrates, often decreasing the viability and survival of the eggs and larvae (e.g., Johnson et al., 2010; Gantz and Lee, 2015). Predicting the impacts of changes in rainfall on invertebrate communities is complicated by the fact that altered rainfall is unlikely to affect all invertebrates in the same way. For example, the impact of drought on soft bodied invertebrates, such as isopods and myriapods, is likely to be greater than the effects on arachnids and insects which have a waxy cuticle that serves to reduce water loss (Berridge, 2012).

Indirect effects of water stress, such as those mediated by plants, can also affect invertebrates differently (Koricheva et al., 1998). Sucking herbivores, for example, may benefit from an increased concentration of nitrogenous compounds in plant foliage following water stress, but only when plants enter a recovery phase (i.e., turgor pressure returns). Huberty and Denno (2004) termed this the 'pulsed-stress hypothesis,' but also noted that increased levels of plant defensive compounds following water stress often reduce the abundance of chewing herbivores. In contrast, the plant vigor hypothesis has been proposed based on observations that some phytophagous insects choose vigorous, fast-growing parts of plants to feed on (Price, 1991). Fast growing plant tissue is thought to have higher nutrient availability, greater osmotic potential and be relatively low in fiber and lignified tissue (Price, 1991, 2003). Decreased rainfall or altered patterns of rainfall, causing plant dieback, might subsequently stimulate such vigorous re-growth of some plant species when precipitation patterns return to ambient levels.

The group or guild specific responses of invertebrates to altered precipitation patterns are likely to alter the interactions occurring between species, and subsequently the structure of communities (Voigt et al., 2003). Yet, relatively few studies explore the impact of climate change at the community level, with still fewer including both above- and below-ground invertebrates (McKenzie et al., 2013). Altered rainfall can have variable effects on the interactions between invertebrates. For example, Staley et al. (2007) showed that a root chewer can negatively affect the performance of an aboveground leaf miner, but this relationship breaks down under drought conditions. In contrast, some interactions only become apparent under drought conditions; belowground herbivores have been shown to have positive effects on leaf mining flies under reduced rainfall compared with ambient conditions (Staley et al., 2008). The nature of the water stress can also differentially impact above-belowground interactions; Tariq et al. (2013) showed that under a moderate drought stress, root herbivory increased plant chemical defenses, reducing the performance and abundance of a specialist herbivore. In highly drought stressed plants, however, this response did not occur.

Future precipitation regimes may therefore have a range of impacts on above- and below-ground invertebrates, and potentially modify linkages and interactions between these two groups. Experiments often use simplified pot and lab experiments, which are useful for teasing apart causal relationships but cannot fully incorporate synergies between the direct and indirect effects of altered precipitation across multiple trophic levels. Above- and below-ground linkages observed in such experiments, for instance, do not necessarily represent what happens in field situations (e.g., Vandegehuchte et al., 2010; but see Johnson et al., 2013). Community-level field experiments that incorporate background climatic variation and simulate a range of precipitation regimes, looking beyond just reduced and increased rainfall scenarios (e.g., seasonal and frequency related changes), could prove provide a more realistic simulation of such climatic change (Jentsch et al., 2007). Nonetheless, measuring changes in both above- and below-ground communities is destructive and imposes legacy effects so these experiments provide a 'snapshot' of above- and below-ground community changes rather than detailed temporal information.

We aimed to test how above- and below-ground grassland invertebrate populations were affected by contrasting future rainfall scenarios. To achieve this, we used a unique field-based community experiment in southeast Australia that applied ambient levels of precipitation together with four predicted precipitation patterns in a grassland ecosystem. We identified the effects of altered rainfall patterns on the abundance of invertebrate taxonomic groups, feeding guilds and the structure of the community as a whole. Additionally, we explored potential associations between above- and below-ground invertebrate communities and tested whether these were affected by altered precipitation patterns.

MATERIALS AND METHODS

Experimental Site and Shelters

We used the DRI-Grass (Drought and Root herbivore Impacts on a Grassland ecosystem) experimental platform for this research. This platform applies different rainfall regimes in a grassland ecosystem based in Richmond, New South Wales at

the Western Sydney University Hawkesbury campus (33°36′40″ S, 150°44′26.5″ E). The DRI-Grass experiment consists of 48 permanent rain exclusion shelters (1.8 m × 2.0 m area; i.e., 3.6 m × 3.6 m) constructed from stainless steel frames and clear acrylic Perspex roofs. These shelters allow five rainfall scenarios to be simulated comprising 12 plots with (i) ambient (water applied in "real time" immediately after rainfall events), (ii) reduced amount (−50% of ambient), (iii) reduced frequency (no water for 21 days, followed by the total ambient rainfall applied in a single application) as well as six plots with, (iv) increased amount (+50% of ambient), and (v) summer drought (no rainfall for 13 weeks in the summer, 17 December to 27 March). Six plots of each of the 12 ambient, reduced amount and reduced frequency plots had been inoculated with scarab larvae as part of a concurrent, but separate, experiment which we accounted for as a covariate factor in our analysis (see Statistical analysis below). To assess differences between watering regimes, soil moisture readings are automatically taken every 15 min from TDR probes, and a daily mean was calculated (see Power et al., 2016 for full details of the DRI-Grass platform).

The grassland community beneath each shelter typically consisted of *Axonopus fissifolius* (C_4 grass), *Cymbopogon refractus* (C_4 grass), *Eragrostis curvula* (C_4 grass), *Hypochaeris radicata* (forb), *Microlaena stipoides* (C_3 grass), *Paspalum dilatatum* (C_4 grass), *Plantago lanceolata* (forb) and *Setaria parviflora* (C_4 grass). The soil is characterized as a sandy loam of moderate to low fertility, with a low organic matter content and low water holding capacity (see Barton et al., 2010 for full details). DRI-Grass was constructed in May 2013 and irrigation regimes commenced in June 2013. Aboveground plant material is harvested at the end of both the cool (i.e., October) and growing season (i.e., April) and plant biomass is estimated for each plot.

Invertebrate Collection

Three sampling methods were used to collect aboveground invertebrates, belowground macro-invertebrates and nematodes during 11–21 September 2015, prior to harvesting aboveground plant material in the following week. Vacuum ('G-vac' device) sampling, a proven quantitative technique (Brook et al., 2008), was used to capture aboveground invertebrates. The 'G-Vac' sampler was an adapted petrol-powered device (SH 86C, Sithl AG & Co. KG. Germany) fitted with an organza bag to catch invertebrates. This was passed under each rain exclusion shelter for 20 s in a zigzag pattern on full throttle. Samples were placed in zip lock bags and frozen until identification in the laboratory under a dissecting microscope (SZ51, Olympus, Japan). Belowground macro invertebrates were sampled by excavating two 25 cm × 10 cm × 20 cm (length × width × depth) trenches under each rain exclusion shelter. Macro invertebrates were stored into ethanol and frozen until identification in the laboratory. Both above- and below-ground samples were identified to at least order level, other than two groups which were identified to sub-class only (Acari and Collembola). A number of groups were also identified to sub order or family level, for more accurate identification of feeding guilds (see Supplementary Tables 1 and 2 for the groups identified and guild classifications).

Two additional soil cores (3.5 cm diameter, 10 cm depth), one from each side of the plot, were collected for extraction of nematodes, at the same time as sampling belowground macro fauna. These were combined per plot to form a composite sample, gently homogenized and subsampled, with nematodes extracted from 50 g soil (wet weight) using a modified Baermann funnel technique (Baermann, 1917), over 3 days. The nematodes were then counted, assigned to feeding groups using morphological characteristics, and the numbers converted to individuals per kg dry soil. Nematode samples were collected as part of ongoing monitoring for interactive responses to herbivore addition and rainfall manipulations (i.e. Ambient, Reduced amount, and Reduced frequency treatment plots only), but were used in this study to investigate above- and below-ground linkages further.

Statistical Analyses

Statistical analyses were undertaken in R, version 3.2.2 (R Core Team, 2016). To confirm the effect of the applied watering treatment on soil moisture, we tested the effects of the applied rainfall regimes on soil moisture data from October 2014 to October 2015, using a repeated measures linear mixed effects model [package nlme, lme(); Pinheiro et al., 2016]. Repeated measures were used because of the recurring nature of soil moisture measurements, which were recorded as a proportion (%) and therefore values were transformed prior to analysis using the logit() function (package *car*; Warton and Hui, 2011). The model used rainfall treatment and month as interactive fixed effects, with month nested within plot which was included as a random (mixed) effect. Interactive effects were tested first, followed by individual effects (Crawley, 2013). Custom *post hoc* comparisons were performed based on visual inspection (**Figure 1**) for significant factors [glht(), *multcomp*; Hothorn et al., 2008].

The effect of rainfall treatment on plant biomass from the October harvest was evaluated with a linear model using a Chi Square test, followed by a Tukey test.

Separate analyses were undertaken for each of the three different sampling methods (aboveground invertebrate vacuum sampling, belowground macro fauna and soil nematodes). Groups of invertebrates (separated by Order or feeding guild identity) that were captured infrequently - i.e., found in <10% of samples or constituting <50 individuals in total - were excluded to permit statistical analysis (*sensu* Hillstrom and Lindroth, 2008).The effects of altered rainfall on invertebrate abundance were tested using generalized linear models (GLMs). Models contained a negative binomial error structure to account for overdispersion of the data [glm.nb(), *MASS*; Venables and Ripley, 2003] in all but three groups for which model dispersion parameters <1.7; these were analyzed using Poisson error structures. In order to determine the significance of the rainfall treatment, the full model described above was compared to a reduced model without rainfall treatment as a factor (likelihood ratio test). A concurrent experiment inoculated half of the ambient, reduced frequency and reduced amount plots with scarabs; therefore herbivore treatment

was included in models as a covariate to account for this. Additionally, due to the documented effect of vegetation complexity on suction sampling efficiency, plant biomass was also included as a covariate when analyzing the aboveground abundance data (Brook et al., 2008; Facey and Torode, 2016). The model fit was determined by inspecting residual plots.

The effect of altered rainfall regimes on the community composition of invertebrates was analyzed using permutational multivariate analysis of variance [PERMANOVA, adonis() in the *vegan* package; Oksanen et al., 2016], with rainfall treatment included as a fixed effect.

A Pearson's correlation matrix was used to explore linkages between above- and below-ground invertebrate groups for each rainfall treatment separately. In order to avoid type II errors, only highly statistically significant ($P < 0.01$) results from the matrix were investigated further. A mixed model was used to show which of the highly significant correlations appeared to have a linear relationship; those that did not appear to have a linear relationship are not shown. Models used the abundance of two invertebrate groups that were significantly correlated, with the abundance of the aboveground group explained as a function of the abundance of the belowground group, with plot as a random effect. The fit of the model was assessed using residual plots and significance was determined using an ANOVA. Plots treated with additional root herbivory may have altered the relationships between above-below-ground invertebrates, so were not included in the analysis of above-belowground associations.

RESULTS

Soil Moisture and Plant Responses

Irrigation regime significantly affected soil moisture which varied by month, as shown by the interaction ($F_{48,8700} = 35.6$, $P < 0.001$) (**Figure 1**). Plant biomass also varied between treatments ($\chi^2_4 = 10.069$, $P = 0.039$), with a greater plant biomass in the summer drought plots at the time of sampling, 5 months after the drought period ended (**Figure 2**). It was also in these plots that soil moisture decreased throughout the drought period, but returned to ambient levels in May, 1 month after the drought period ended (**Figure 1**; **Table 1**).

Invertebrate Population Responses

In total, 6,604 aboveground invertebrates and 3,736 belowground macro invertebrates were counted and identified. A number of aboveground invertebrate taxa varied significantly in abundance between rainfall treatments (**Table 2**). Specifically, summer drought increased the abundance of invertebrates in the Orders Hemiptera, Orthoptera, Diptera, and Acari (**Table 2**; **Figures 3A–D**). In terms of functional guilds, summer drought positively affected the abundance of sucking herbivores, parasitoids, chewing herbivores, and detritivores (**Figures 4A–D**). The abundance of Collembola also varied between rainfall regimes, with a greater number found under the increased amount treatment (**Table 2**; **Figure 3E**). A number of taxonomic groups and guilds did not vary in abundance significantly between treatments (**Table 2**). The community composition of aboveground invertebrates varied significantly between rainfall treatments (**Table 3**). Despite a number of groups varying in their abundance, sucking herbivores and their associated order Hemiptera largely accounted for the change in community composition because of their very large increase in abundance under summer drought (**Figures 3** and **4**).

Belowground Hymenoptera (Formicidae) and their associated guild, scavengers, were the only belowground groups for which abundance was significantly affected by altered rainfall regimes (**Table 2**, **Figures 5A,B**). The community composition of belowground fauna was not detectably affected by the rainfall treatments (**Table 3**).

Exploring Above- and Below-Ground Linkages

The abundances of aboveground Acari and belowground Coleoptera were positively correlated under ambient and increased amount rainfall scenarios (**Figures 6A,B**). The same relationship was observed for their associated guilds, scavengers and root chewers, under ambient rainfall regimes only (**Figure 7A**). A number of other relationships were only observed under ambient rainfall, including positive correlations between aboveground Collembola and belowground Megadrilacea (**Figure 6C**), and aboveground parasitoids and belowground fungal feeding nematodes (**Figure 7B**). Conversely, a negative relationship was found between the abundance of belowground fungal feeding nematodes and root chewers (**Figure 7C**). Additionally, the abundances of aboveground Collembola and belowground Hemiptera were positively correlated under the reduced amount treatment (**Figure 6D**), while a positive relationship was found between the abundance of aboveground detritivores and belowground sucking herbivores under summer drought conditions (**Figure 7D**). The abundance of a number of other aboveground and belowground invertebrates where found to have a strong correlation, however they did not appear to have a linear relationship (see Supplementary Table 3).

DISCUSSION

To our knowledge, this is the first report of a community-level field investigation of above- and below-ground invertebrate abundances across a range of potential future rainfall scenarios. In addition to reporting changes in the abundances of some invertebrates under altered rainfall, we showed that some above- and below-ground populations were tightly correlated, suggesting strong, yet precipitation-sensitive, linkages between these spatially separated organisms.

Aboveground Invertebrate Responses

Of the four rainfall regimes, summer drought had the greatest effect on aboveground invertebrates, increasing the abundance of a number of invertebrate groups at both a taxonomic and guild level. These plots also had the greatest plant biomass, which is

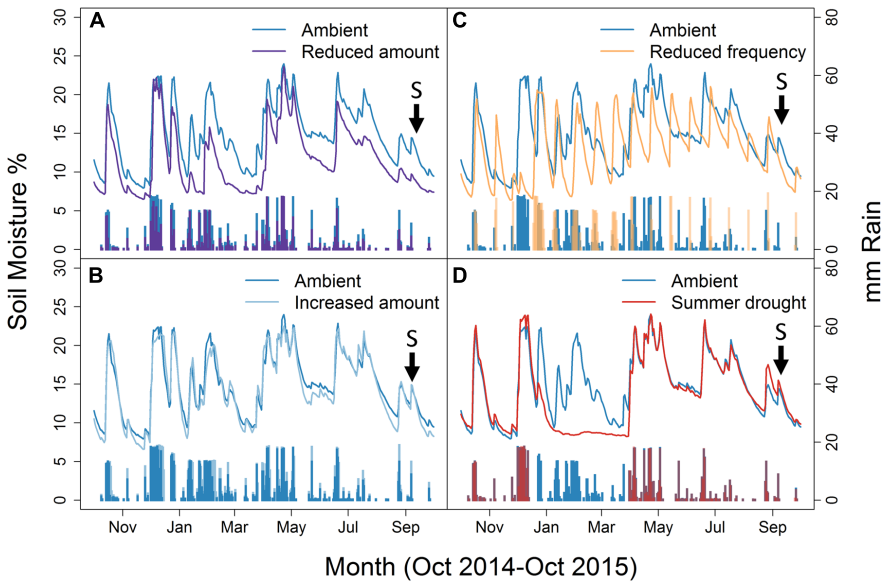


FIGURE 1 | Soil moisture and mm rain applied to ambient plots relative to; (A) Reduced amount, (B) Increased amount, (C) Reduced frequency, and (D) Summer drought. Lines show the Soil moisture, while the bars represent mm of rain applied. Arrows and 'S' indicates the time sampling took place. Weekly data shown from October 2014 to October 2015.

indicative of vigorous plant growth in the 5 months since the end of the summer drought. It is probable that there was more dieback in the 13 weeks without water during the growing season (January–March 2015). When precipitation returned to ambient levels, it is likely that resilient plants could take advantage of reduced competition and display vigorous growth. We propose that these results support the plant vigor hypothesis (Price, 1991). In particular, sucking herbivores (and Hemiptera) frequently show population spikes in spring because amino acids are being translocated for new growth (Dixon, 1998), so the more vigorous plant growth observed in summer drought plots benefited this group most.

Our observations of increased abundances of sucking herbivores in plots experiencing summer drought also has compatibilities with the pulsed-stress hypothesis (Huberty and Denno, 2004). Their meta-analysis demonstrated that sucking herbivores often benefit from plants subjected to intermittent water stress. In particular, reduced soil moisture often increases plant foliar nitrogen because pre-existing proteins can be hydrolysed, resulting in higher concentrations of free amino acids (Brodbeck and Strong, 1987). When rainfall resumes, phloem turgor pressure increases, allowing sucking herbivores (reliant on positive turgor pressure in the plant) to utilize the plant's improved nutritional quality (Huberty and Denno, 2004). However, 5 months had elapsed since irrigation was applied at ambient levels, so we consider that increases in foliar nitrogen wouldn't persist for so long. While it is possible that an increase in sucking herbivore abundance due to pulsed-drought in April underpinned larger populations observed in September, we find the plant vigor hypothesis more convincing, particularly since we also saw increases in other groups (e.g., chewing herbivores and detritivores). Moreover, our reduced

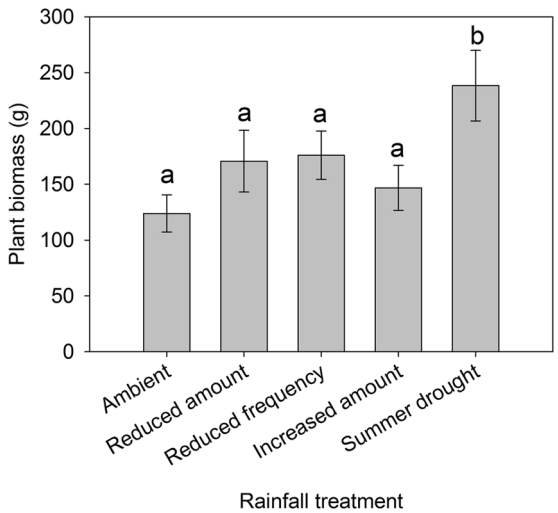


FIGURE 2 | Total aboveground plant biomass from grassland plots subjected to different rainfall regimes. Bars with different letters ("a" and "b") are significantly different from one another. Mean values ± SE shown ($N = 12$ for Ambient, Reduced amount, and reduced frequency, $N = 6$ for Increased amount and summer drought).

frequency treatment (intermittent periods of drought, followed by larger rainfall events) is probably more similar to the pulsed-drought described by Huberty and Denno (2004), and we saw little impact on sap-suckers and Hemiptera in these plots.

The abundance of parasitoids also increased under summer drought, suggesting that parasitoids are tracking their

TABLE 1 | Results from linear model *post hoc* test showing differences in soil moisture between ambient and summer drought plots.

Month	Estimate	Standard error	*Z* value	*P*-value
January (Summer drought started)	0.441	0.179	4.088	<0.001
February	0.589	0.108	5.442	<0.001
March (Summer drought terminated)	0.240	0.108	2.313	0.021
April	−1.365	0.1527	−8.937	<0.001
May	0.031	0.10787	0.291	0.771

Soil moisture is shown for months during the summer drought and 2 months after the summer drought period.

TABLE 2 | Results from general linear model, showing how absolute abundance varied between rainfall treatments.

Community	Classification level	Figure reference	Community/Group	Likelihood ratio	*P*-value
Aboveground invertebrate macro fauna	Taxonomic classification		Community	16.466	**0.002**
		Figure 3A	Hemiptera	18.521	**<0.001**
		Figure 3B	Orthoptera (Poisson)	−10.510	**0.032**
		Figure 3C	Diptera	14.313	**0.006**
		Figure 3D	Collembola*	18.979	**<0.001**
		Figure 3E	Acari*	12.155	**0.016**
			Araneae	2.860	0.581
			Hymenoptera	6.806	0.147
			Coleoptera	1.564	0.815
	Feeding guild		Community	16.464	**0.002**
		Figure 4A	Sucking herbivore	18.094	**0.001**
		Figure 4B	Parasatoid	14.131	**0.006**
		Figure 4C	Chewing herbivore (Poisson)	−12.637	**0.013**
		Figure 4D	Detritivore	18.405	**0.001**
			Predator	1.694	0.791
			Scavenger	9.222	0.055
			Omnivore	2.367	0.669
Belowground invertebrate	Taxonomic classification		Community	5.991	0.199
		Figure 5A	Hymenoptera	12.242	**0.015**
			Diptera	6.438	0.168
			Hemiptera (Poisson)	−2.444	0.654
			Coleoptera	5.973	0.201
			Araneae	6.275	0.179
			Megadrilacea	3.845	0.427
	Feeding guild		Community	5.855	0.210
		Figure 5B	Scavenger	12.519	**0.014**
			Chewing herbivore	9.071	0.059
			Detritivore	3.647	0.455
			Omnivore	3.496	0.478
			Sucking herbivore	6.774	0.133
			Predator	5.044	0.28
Nematodes	Feeding guild		Community	3.462	0.177
			Fungal feeders	3.829	0.147
			Bacterial feeders	2.300	0.317
			Omnivore	0.734	0.693
			Plant parasites	3.774	0.152

*All groups have been classified to an order level with the exception of these groups marked with * which have been classified to subclass. Statistically significant differences indicated in bold.*

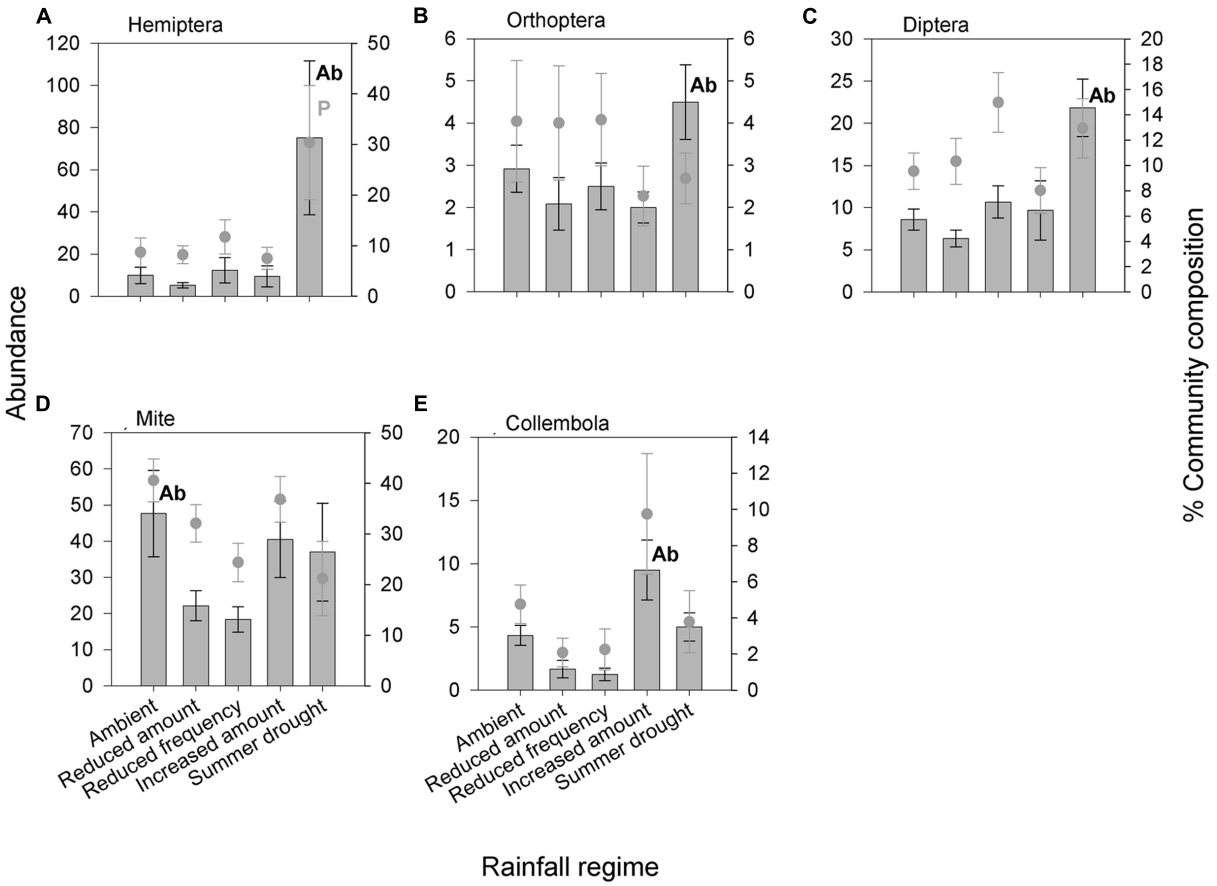


FIGURE 3 | Aboveground invertebrate taxonomic groups (A) Hemiptera, (B) Orthoptera, (C) Diptera, (D) Mite and (E) Collembola were significantly affected by rainfall regime in terms of absolute abundance and/or change in proportion of the community. Bars show absolute abundance and points indicate the proportion of the overall community represented by the group. Statistically significant changes in absolute abundance indicated 'Ab' (see **Table 2** for corresponding analysis). Taxonomic groups most influencing the reported change in community composition indicated 'P'. Mean values ± SE shown ($N = 6$).

TABLE 3 | Results from multivariate permutational analysis (PERMANOVA) showing the effect of rainfall treatment on the community composition of above- and below-ground invertebrates at both order and guild level.

Group	Classification	d.f	Sum of squares	Mean of squares	Psuedo-F	*P*-value
Aboveground invertebrates	Order-level	4	0.885	0.221	2.265	**0.005**
	Guild- level	4	0.765	0.191	2.117	**0.016**
Belowground macro- invertebrates	Order-level	4	0.722	0.181	1.191	0.255
	Guild- level	4	0.718	0.179	1.205	0.268
Belowground nematodes	Order-level					
	Guild- level	2	0.060	0.030	0.397	0.892

Belowground macro invertebrates and nematodes were analyzed separately due to differences in sampling techniques.

Hemipteran hosts' population dynamics, as found in Zhu et al. (2014). Indeed, the abundance of parasitoids was positively correlated with sucking herbivores in summer drought plots, providing further evidence that these insects are tracking the abundance of their hosts.

The general increase in herbivores under summer drought associated with the higher levels of cool season plant growth may explain the increased abundance of detritivores as well. In particular, the greater quantity of insect cadavers, frass and plant detritus is likely to have increased resources for this group. Increases in fungus gnats (Diptera), rather than Collembola, underpinned the increase in detritivores. Instead, the abundance of Collembola was more sensitive to increases in rainfall as evidenced by their greater numbers under the increased amount treatment compared with reduced frequency and amount plots. The abundance of Acari was reduced within the reduced frequency plots, indicating that this group may be sensitive to reductions in water availability. Both Collembola and

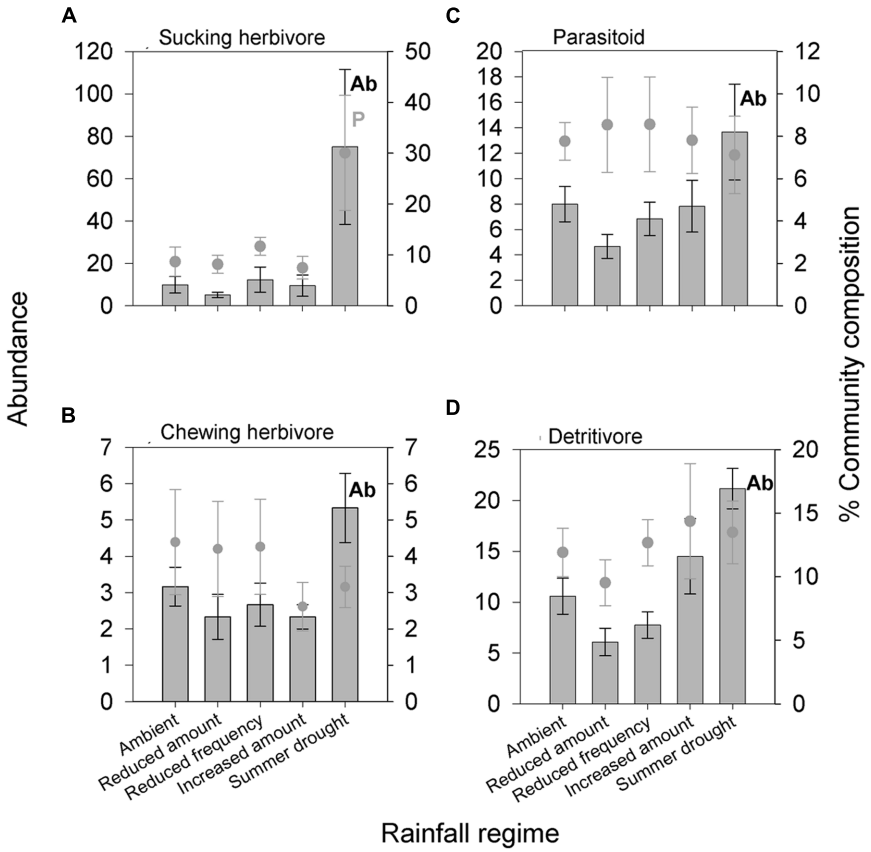


FIGURE 4 | Aboveground invertebrate feeding guilds (A) sucking herbivore, (B) parasitoid, (C) chewing herbivore, and (D) detritivores significantly affected by different rainfall regimes in terms of absolute abundance and/or change in proportion of the community. Bars show absolute abundance and points indicate the proportion of the overall community represented by the group. Statistically significant changes in absolute abundance indicated 'Ab' (see **Table 2** for corresponding analysis). Feeding guilds most influencing the reported change in community composition indicated 'P'. Mean values ± SE shown ($N = 6$).

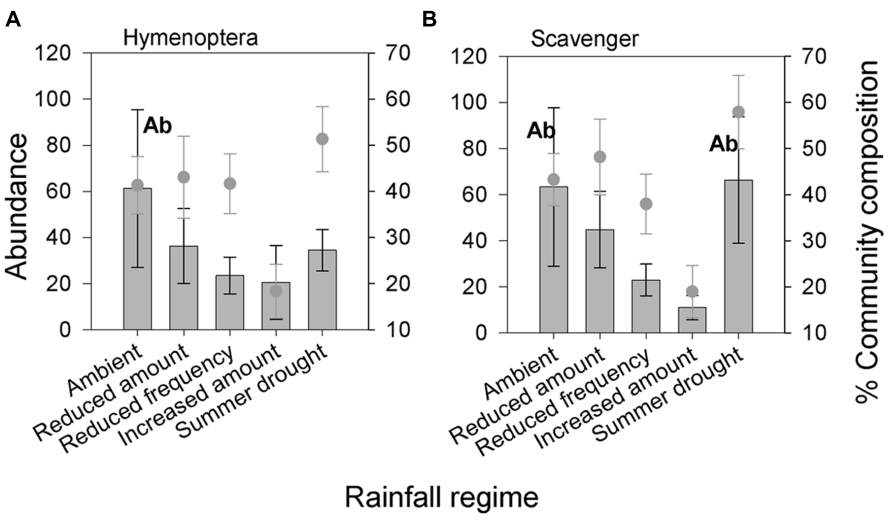


FIGURE 5 | Belowground invertebrate taxonomic groups and feeding guilds (A) Hymenoptera and (B) Scavengers significantly affected by different rainfall regimes in terms of absolute abundance. Bars show absolute abundance and points indicate the proportion of the overall community represented by the group. Statistically significant changes in absolute abundance indicated 'Ab' (see **Table 2** for corresponding group. Mean values ± SE shown ($N = 6$).

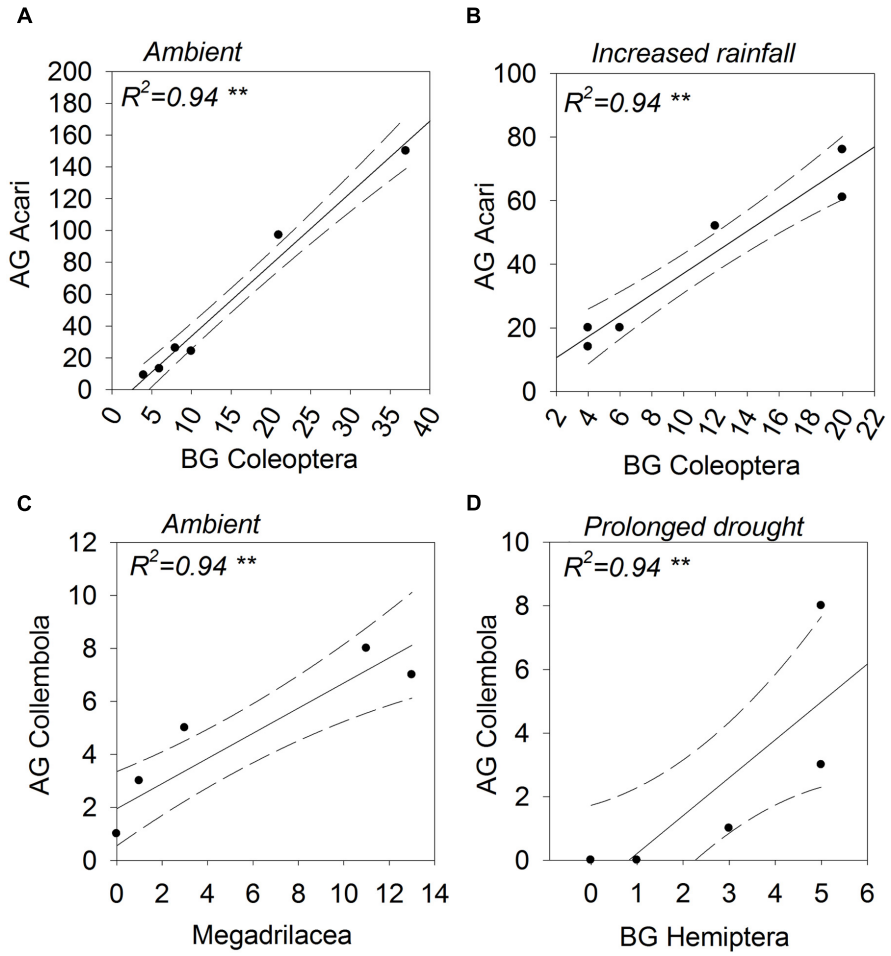


FIGURE 6 | Above- (shown on the *y*-axis) and below-ground (shown on the *x*-axis) taxonomic groups showing statistically significant correlations in abundance; (A) Acari (aboveground) and Coleoptera (belowground), (B) Acari (aboveground) and Coleoptera (belowground), (C) Collembola (aboveground) and Megadrilacea (belowground), and (D) Collembola (aboveground) and Hemiptera (belowground). The rainfall regime under which the correlation occurred indicated for each panel. Solid lines indicate model predicted values; points represent actual values and dashed lines represent 95% confidence intervals.

Acari could have been negatively affected directly by the low soil moisture in the reduced rainfall plots (Convey et al., 2002; Lindberg et al., 2002; A'Bear et al., 2014).

In addition to changes in absolute abundance, the community composition of invertebrates also varied between rainfall treatments. Despite a number of aboveground invertebrates increasing in absolute abundance under summer drought plots, the observed changes in community composition aboveground were driven primarily by increases in the relative abundance of sucking herbivores (Hemiptera).

Below-Ground Invertebrate Responses

Altered rainfall regimes had little measurable effect on the belowground community, with no change in community composition found between treatments. Altered precipitation patterns may have affected belowground invertebrates less because they have a host of adaptations to mitigate changes in their microclimate, such as utilizing metabolic water, moving through the soil profile and constructing earthen chambers (Barnett and Johnson, 2013). Only Hymenoptera (Formicidae) and their associated scavenger guild were affected by altered rainfall, showing reduced abundance in plots receiving increased amounts of rainfall. This could be the result of negative effects of increased soil humidity on ants, as suggested by Seal and Tschinkel (2010).

Above- and Below-Ground Linkages

A number of above- and below-ground linkages were observed under ambient rainfall, but dissipated under altered rainfall regimes. The reasons for such linkages occurring and their decoupling under altered rainfall patterns remain unknown, and we stress that explanations postulated below are highly speculative. They do, however, provide the basis for formulating hypotheses to direct future work.

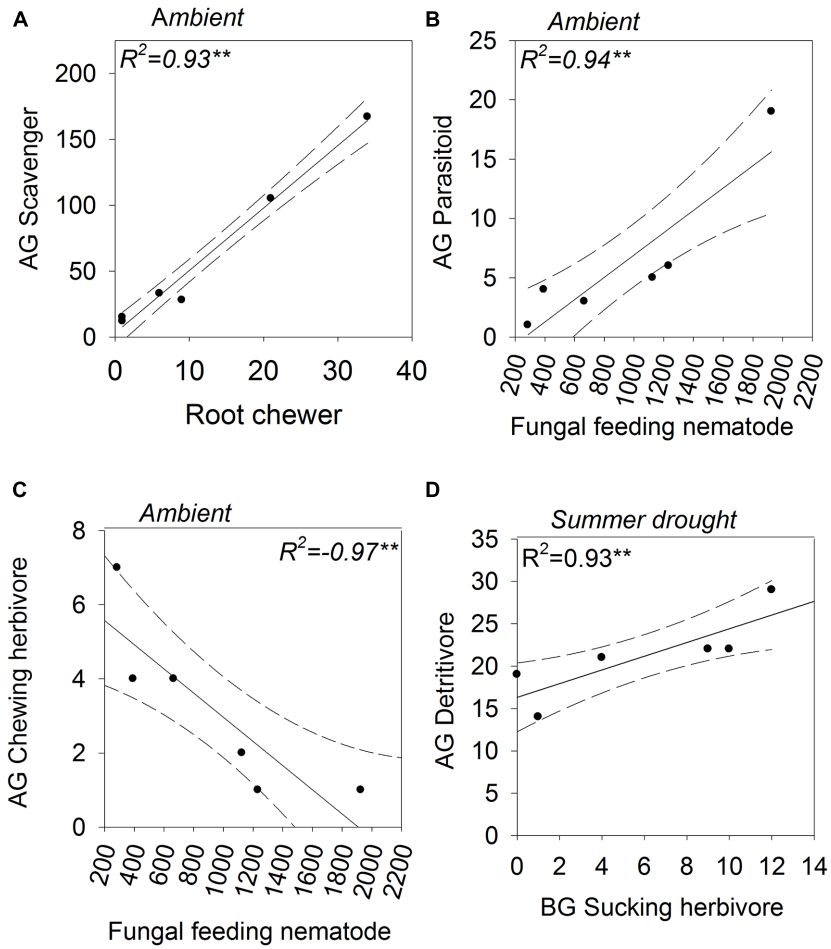


FIGURE 7 | Above- (shown on the *y*-axis) and below-ground (shown on the *x*-axis) feeding guilds showing statistically significant correlations in abundance; (A) scavengers (aboveground) and root chewers (belowground), (B) parasitoids (aboveground) and fungal feeding nematodes (belowground), (C) chewers (aboveground) and fungal feeding nematodes (belowground), and (D) detritivores (aboveground) and suckers (belowground). The rainfall regime under which the correlation occurred indicated for each panel. Solid lines indicate model predicted values; points represent actual values and dashed lines represent 95% confidence intervals.

The positive relationship between the abundance of Acari (aboveground) and Coleoptera (belowground), for example, was only observed under ambient and increased amount plots. Root chewing herbivores like Coleopteran larvae can induce plants to reallocate resources aboveground, moving resources away from the site of attack, termed 'resource sequestration' (Orians et al., 2011; van Dam and Heil, 2011). Improved nutritional quality of plant detritus aboveground could then benefit scavengers, like Acari. The breakdown of these relationships could be a result of changes in the composition of species within these groups under altered rainfall regimes, even if the groups' abundance didn't change. Similarly, changes in plant community traits under drought (e.g., greater root:shoot ratios, accelerated senescence and litter production, and shifts in nutrient stoichiometry [Dijkstra et al., 2012]) could influence the plant-mediated aboveground-belowground interactions.

Collembola (aboveground) and Megadrilacea (belowground) were positively correlated under only ambient rainfall. Both groups are detritivores therefore their abundances may simply reflect similar responses to variations in the detritus inputs in ambient plots, resulting in a correlation. Supplementing detrital material has, for example, been shown to increase detritivore abundance (Halaj and Wise, 2002). Altered rainfall may have negatively affected aboveground detritivores to a greater extent than belowground detritivores, decoupling the association between the two groups.

The negative relationship between fungal feeding nematodes (belowground) and chewing herbivores (aboveground) was also only present under ambient rainfall; this finding may possibly be mediated via mycorrhizal communities. Fungal feeding nematodes can indicate the presence of mycorrhizal fungi in the soil (Landesman et al., 2011). Mycorrhizal infection frequency increases plant resistance to herbivory aboveground (Fontana et al., 2009), thereby potentially reducing the abundance of generalist chewing herbivores (Gange et al., 2002).

More information on treatment effects on mycorrhizal root colonization and community composition would be required to support this theory. Altered precipitation regimes didn't result in the association between herbivores and fungal feeding nematodes. Variation in the composition of mycorrhiza species, as well as colonization rates, under different rainfall regimes could have altered their effect on plant chemistry and the aboveground invertebrate community.

CONCLUSION

This study indicates that changes in precipitation, specifically changes in the seasonality of rainfall, are likely to cause alterations in the abundance and composition of aboveground, and to a lesser extent belowground, invertebrate communities. In particular, summer drought resulted in outbreaks of sucking herbivores, which probably underpinned the concurrent increase in the abundance of parasitoids. However, this study represents a 'snapshot' of impacts on invertebrate communities in spring only and patterns may be different during the growing season. It remains to be determined whether these changes persist over the longer term or whether communities return to a state of equilibrium. These findings, together with the precipitation-sensitive linkages outlined in this study, do, however, lend further empirical support to the idea that climate change will modify grassland invertebrate communities with potential cascading effects. Moreover, linkages between above- and below-ground communities may be modified by climate change (McKenzie et al., 2013), which we propose can be termed 'climatic decoupling.'

AUTHOR CONTRIBUTIONS

SJ conceived the experimental design. MT, KB, UN, SP, and SF collected field data. MT and SF analyzed the data with help from KB and SJ. MT wrote the paper with the assistance of KB, SF, UN, SP, and SJ.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01468

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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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frontiers in Plant Science

ORIGINAL RESEARCH
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Above–Belowground Herbivore Interactions in Mixed Plant Communities Are Influenced by Altered Precipitation Patterns

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Root- and shoot-feeding herbivores have the capacity to influence one another by modifying the chemistry of the shared host plant. This can alter rates of nutrient mineralization and uptake by neighboring plants and influence plant–plant competition, particularly in mixtures combining grasses and legumes. Root herbivory-induced exudation of nitrogen (N) from legume roots, for example, may increase N acquisition by co-occurring grasses, with knock-on effects on grassland community composition. Little is known about how climate change may affect these interactions, but an important and timely question is how will grass–legume mixtures respond in a future with an increasing reliance on legume N mineralization in terrestrial ecosystems. Using a model grass–legume mixture, this study investigated how simultaneous attack on lucerne (*Medicago sativa*) by belowground weevils (*Sitona discoideus*) and aboveground aphids (*Acyrthosiphon pisum*) affected a neighboring grass (*Phalaris aquatica*) when subjected to drought, ambient, and elevated precipitation. Feeding on rhizobial nodules by weevil larvae enhanced soil water retention under ambient and elevated precipitation, but only when aphids were absent. While drought decreased nodulation and root N content in lucerne, grass root and shoot chemistry were unaffected by changes in precipitation. However, plant communities containing weevils but not aphids showed increased grass height and N concentrations, most likely associated with the transfer of N from weevil-attacked lucerne plants containing more nodules and higher root N concentrations compared with insect-free plants. Drought decreased aphid abundance by 54% but increased total and some specific amino acid concentrations (glycine, lysine, methionine, tyrosine, cysteine, histidine, arginine, aspartate, and glutamate), suggesting that aphid declines were being driven by other facets of drought (e.g., reduced phloem hydraulics). The presence of weevil larvae belowground decreased aphid numbers by 30%, likely associated with a significant reduction in proline in weevil-treated lucerne plants. This study demonstrates how predicted changes to precipitation patterns and indirect interactions between herbivores can alter the outcome of competition between N-fixing legumes and non-N-fixing grasses, with important implications for plant community structure and productivity.

Keywords: aboveground–belowground interactions, alfalfa, amino acids, aphids, climate change, grass–legume mixture, herbivores

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INTRODUCTION

Species Interactions and Climate Change

Ecological communities form a network of directly and indirectly interacting species (Wootton, 1994; Polis, 1998). These networks are often categorized into tractable ecosystem components (e.g., food chains, aboveground–belowground interactions, plant–insect interactions, competitive interactions) used to elucidate the causal mechanisms that underpin species interactions, and community dynamics (Holt, 1997; Rudolf, 2012). Notably, plants are exploited by multiple root and shoot herbivores, which can interact indirectly through plant-mediated mechanisms. They have the capacity to influence one another by modifying the chemistry of the shared host plant (Blossey and Hunt-Joshi, 2003; Johnson et al., 2012). Changes in host plant nutrients (e.g., amino acid concentrations) often underpin interactions between aboveground and belowground herbivores, with knock-on effects on plant community structure and productivity (Johnson et al., 2013). In particular, altered rates of root exudation in response to herbivory can influence rates of nutrient mineralization and acquisition by neighboring plant species (Bardgett et al., 1999; Ayres et al., 2007). However, all trophic interactions, both direct and indirect, are specific to the species combinations involved (Kos et al., 2015) and may be altered by climate change (McKenzie et al., 2013). Such changes may propagate through communities and sway competitive advantages between species (Johnson et al., 2011; Barton and Ives, 2014; Jing et al., 2015). The incidence of extreme precipitation events, including droughts and floods, is expected to increase in many regions throughout the world, with a generally drier future predicted for south-eastern Australia (Chiew et al., 2011; Dai, 2011). Drought is one of the most important environmental stressors for plants, often altering root to shoot biomass ratios (Gargallo-Garriga et al., 2014) and sometimes altering plant N and amino acid concentrations (Girousse et al., 1996; Garten et al., 2009; Johnson et al., 2011). Excess water can also create anaerobic conditions around plant roots, affecting growth, and nutrient status, although in areas with sufficient drainage, increased water availability is likely to positively affect plant productivity. Nonetheless, effects of drought are of longer duration and are considered to have far more disruptive effects on plant–insect interactions (Pritchard et al., 2007).

Grass–Legume Ecosystems

Ecosystem processes, including primary productivity and nutrient cycling, tend to increase in biologically diverse plant communities, especially in mixtures containing leguminous plant species (Hooper et al., 2005; Hatch et al., 2007). Most legumes have the ability to fix atmospheric N_2 via their symbiotic relationship with rhizobial bacteria (Long, 1989). Rhizobial bacteria are accommodated by nodules on legume roots, the development of which is strongly affected by soil conditions including pH, nutrient availability, temperature, and moisture (Ramos et al., 2003; Ferguson et al., 2013). Drought, in particular, can negatively impact nitrogenase activity in legume nodules (Sprent, 1972), although the mechanisms remain unclear (Streeter, 2003; Nasr Esfahani et al., 2014). Benefits of mixed plant communities (e.g., enhanced resource acquisition) arise from species complementarity (i.e., resource niche differentiation) or from selection effects (i.e., mixed communities are more likely to harbor a dominant productive species; Loreau and Hector, 2001; Turnbull et al., 2013). Legumes, in particular, fix more atmospheric N_2 when mixed with grasses than when planted in monocultures, allowing companion grass species to utilize plant-available N for production (Chmelíková et al., 2015). Hence, diverse communities can enhance primary production and increase soil fertility (Hatch et al., 2007). Compared with legume monocultures, grass–legume mixtures also show more efficient water utilization and improved ground cover, which reduces runoff and erosion (Ta and Faris, 1987; Humphries, 2012). Moreover, mixed plant communities in general are often more productive (Tilman et al., 2001) and experience reduced herbivore damage by diluting the availability of specific host plants (Root, 1973; Haddad et al., 2009; Fabian et al., 2012). Water stress may have important consequences for grass–legume ecosystems. In particular, grasses, with their shallow root systems, tend to be more sensitive to drought than legumes (Hayes et al., 2012) but less sensitive to waterlogging (Neal et al., 2009). Hence, changes in rainfall patterns may disrupt the ability of plant species to coexist in a mixture (Tow, 1993; Tow et al., 1997).

Belowground Herbivory

Root-feeding herbivores of legumes, such as *Sitona* weevils, have the potential to disrupt the interactions between grasses and legumes by altering the flow of N between plant species (Ta et al., 1986; Murray and Hatch, 1994). *Sitona* adults feed on the foliage of legumes while the larvae feed on and in the N-fixing root nodules and on roots as they develop (Aeschlimann, 1979; Arbab and McNeill, 2014). Larval feeding can enhance legume root exudation and soil N availability and result in the increased availability of N for neighboring grasses (Murray and Clements, 1998). Previous studies, for example, have demonstrated that root pruning and herbivory by *Sitona* spp. enhanced the direct transfer of N from white clover (*Trifolium repens* L.) to a neighboring grass species (*Lolium perenne* L.; Hatch and Murray, 1994; Murray and Hatch, 1994). *Sitona* larvae are consistently more damaging than foliar-feeding adults and peak larval densities are often driven by prevailing moisture conditions (Cantôt, 1979; Goldson et al., 1986; Goldson S. et al., 1988). While root nodule herbivory can be highly damaging to the legume, reducing nodulation and impairing N-fixation, legume root recovery can also be over-compensatory, producing higher numbers of nodules, and increasing N-fixation in response to herbivory (Quinn and Hall, 1992; Ryalls et al., 2013b).

Aboveground Herbivory

Root damage by weevil larvae is likely to indirectly affect aboveground phloem-feeding herbivores by altering phloem turgor pressure and/or through changes in phloem amino acid concentrations. Aphids, in particular, tend to perform better on plants with higher N and amino acid concentrations (Ponder et al., 2000; Karley et al., 2002; Nowak and Komor, 2010; Ryalls et al., 2015). Moreover, aphid performance on plants could be

affected not only by the overall amino acid concentration, but also by the proportional composition of different amino acids (Mittler, 1967; Srivastava and Auclair, 1975; Pritchard et al., 2007). Masters et al. (1993) argued that root herbivory promotes aphid performance by impairing soil water and nutrient uptake, which reduces the relative water content of foliage and increases soluble N (e.g., amino acids) and carbohydrate concentrations in the phloem. In contrast, Ryalls et al. (2013b) suggested that negative effects of *Sitona discoideus* Gyllenhal on pea aphids (*Acyrthosiphon pisum* Harris) could arise through lower quality phloem sap from nodule damage specifically, or reduced phloem turgor, and increased sap viscosity (via impaired root function), which would make the phloem more difficult to access.

The majority of sap-sucking invertebrates, including aphids, respond negatively to host plant water stress, which may relate similarly to a decrease in turgor pressure and an increase in phloem sap viscosity (Raven, 1983; Huberty and Denno, 2004). Moreover, increases in plant N (Johnson et al., 2011) and amino acids (Hale et al., 2003) under drought do not necessarily benefit aphids due to these accompanying changes in phloem physiology (Aslam et al., 2012). Effects are likely to be contingent on a range of factors including plant functional group or whether species are growing alone or in competition with others (van der Putten et al., 2004; Johnson et al., 2011).

Study System, Objectives, and Hypotheses

Using a model grass–legume mixture of Harding grass (*Phalaris aquatica* L.) and lucerne (otherwise known as alfalfa, *Medicago sativa* L.), this study addressed the effects of water stress (simulated drought and elevated precipitation) and root nodule herbivory by *S. discoideus* on both the plant–plant interactions between lucerne and Harding grass and on foliar-feeding pea aphids (*A. pisum*), one of the most damaging pests of lucerne [see Ryalls et al. (2013a) for review]. The long-term co-existence of lucerne and grass can be perilous, with one often failing to persist under competition with the other (Bishop and Gramshaw, 1977; Dear et al., 1999). Harding grass, one of the most persistent sown temperate perennial grasses in south-eastern Australia, is one species that complements lucerne and achieves a dynamic balance in a mixture with lucerne (Sherrell, 1984; McKenzie et al., 1990; Culvenor et al., 2007).

This study aimed to determine whether (i) water stress and insect herbivory on lucerne influenced plant growth and N dynamics in both lucerne and Harding grass and (ii) whether water stress and insect herbivory affected total and individual foliar amino acid concentrations in lucerne and, subsequently, the population growth of *A. pisum*. We hypothesized that: (i) drought and elevated precipitation would decrease and increase plant growth, respectively, although both would negatively impact plant N concentrations in general. Lucerne nodule herbivory by *S. discoideus* was predicted to cause N leakage into the soil, which would positively affect the productivity of the co-occurring grass species; (ii) the negative impacts of drought and weevil herbivory on nodulation and N acquisition would decrease foliar amino acid concentrations and/or phloem turgor pressure in lucerne, which would reduce aphid populations. Moreover, specific decreases in individual amino acid concentrations would cause aphid populations to decline.

MATERIALS AND METHODS

Rain-Exclusion Shelters

Rain-exclusion shelters (249 × 188 cm) located at the Hawkesbury campus of Western Sydney University (latitude −33.609396, longitude 150.737800), as described by Johnson et al. (2015), were used to exclude 100% of ambient rainfall from four mesocosms beneath each of 18 shelters. Mesocosm pots (41 × 41 × 31 cm) were arranged in a 2 × 2 formation and dug into the ground so that the rim of the pot was flush with the soil surface. Each of the 72 mesocosms was filled with the excavated soil, which was air-dried and sieved to <4 mm. Removable mesh cages (34 × 34 × 36 cm) were fitted to each mesocosm to prevent escape of experimental insects or entry to non-experimental (free-living) insects. The cages were designed to maximize air movement and allow good light transmittance (Johnson et al., 2015).

Insect Cultures

Acyrthosiphon pisum cultures, originating from a single parthenogenetic adult female collected from a local lucerne field in Richmond, NSW in August 2014, were maintained on propagated lucerne plants at 26/18°C day/night on a 15L:9D cycle until required. Eggs produced from 30 sexually mature *S. discoideus* adults collected from the same field and reared under the same conditions were collected every 24 h and stored on damp filter paper at 4°C until required. Hatching success of 2-month old eggs was confirmed (>97% hatched within 1 week) by placing 200 eggs on 10 Petri dishes at 25°C.

Experimental Procedure

Lucerne (cv. Sequel) seeds were inoculated with *Rhizobium* bacteria 1 h prior to sowing by submerging in a solution containing 250 g Nodule N lucerne seed inoculant (New Edge Microbials, Albury, NSW) and 800 mL distilled water. Lucerne seeds ($N = 144$) were sown and grown in seed cells (38 × 57 mm), with one lucerne plant per cell, under field conditions. Six grass seeds were sown in each of 72 seed cells and grown under the same conditions. After 4 weeks (1 October 2014), grass from one cell was transplanted into the center of each mesocosm and two lucerne plants were transplanted either side of the grass (see **Figure 1**). The root systems of the individual lucerne plants were contained within porous organza bags. This was done to confine weevil larvae to the soil around each lucerne plant, prevent their movement between plants and simplify inoculation with weevil eggs, but at the same time to not restrict transfer of water and nutrients within each mesocosm. Seed numbers within each mesocosm were based on a 1:1 lucerne:grass seed weight ratio (Boschma et al., 2010; Sandral, 2013).

Shelters were assigned at random to one of three rainfall treatments giving six shelters per rainfall regime. The ambient treatment was set at the 65.4 mm average precipitation in Richmond between September–November from 1881 to 2014 (Bureau of Meteorology, Australia). The drought treatment was

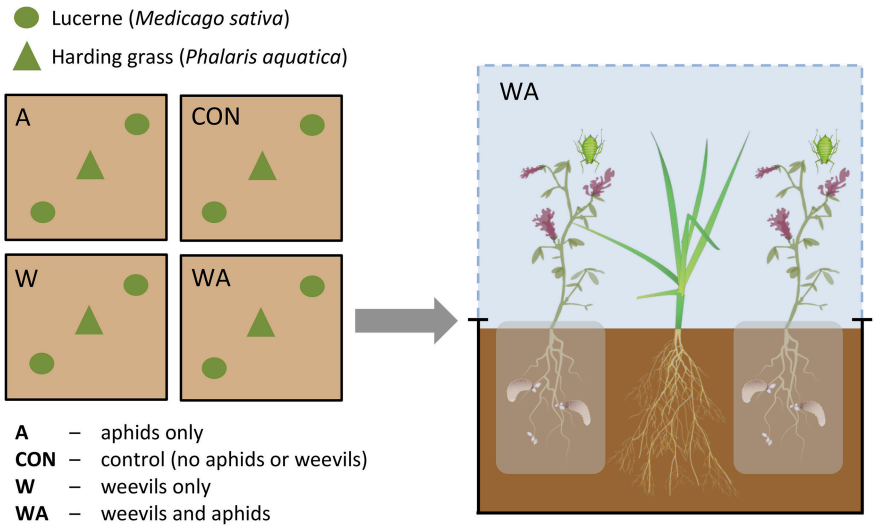


FIGURE 1 | Schematic diagram of experimental plots beneath one rain-exclusion shelter.

50%, and elevated precipitation treatment was 150% of the ambient rainfall amounts. This translated to an application of 586 mL (ambient precipitation), 293 mL (drought), and 878 mL (elevated precipitation) of rainfall water collected at the site three times per week. Soil moisture measurements were taken weekly using a 12 cm Hydrosense II probe (Campbell Scientific, Queensland, Australia).

Two weeks after transplantation, both lucerne plants in two of the mesocosms under each shelter were inoculated with 50 *S. discoideus* eggs, which were placed on top of the soil beside the stem of each plant. Egg numbers (i.e., 50 eggs per plant) were based on average densities of 4000 eggs and 80 lucerne plants per m^2 (Aeschlimann, 1983; Goldson and French, 1983). After a further 2 weeks, three teneral *A. pisum* adults were applied to each of the two lucerne plants in one mesocosm containing *S. discoideus* and one mesocosm without *S. discoideus*. The factorial insect treatment design under each shelter therefore comprised one mesocosm with *S. discoideus* alone (W), one mesocosm with *A. pisum* alone (A), one mesocosm with both insects (WA), and one mesocosm with neither (CON; **Figure 1**).

Acyrthosiphon pisum individuals were counted and removed 4 weeks after being applied, whereupon the experiment was harvested. The number of plants that were colonized by aphids (i.e., aphid colonization success) were recorded. Grass tiller numbers and plant heights of both lucerne (from ground level to the base of the highest leaf) and Harding grass were measured six (weevil inoculation period), eight (aphid inoculation period), and 12 (harvest period) weeks after sowing. Roots were separated from the soil and shoots and the number of lucerne nodules were counted. Roots and shoots were frozen at −20°C, freeze-dried, and weighed prior to chemical analyses.

C, N, and Amino Acid Analyses

Freeze-dried root and leaf material was ball-milled to a fine powder and carbon (C) and nitrogen (N) concentrations of 4–6 mg of milled samples were determined using an elemental analyser (LECO TruSpec Micro, LECO Corp., St. Joseph, MI, USA). The N content of lucerne roots was calculated by multiplying root dry mass by the concentration of N in roots. Soluble amino acids were extracted and analyzed from milled lucerne foliage samples (15–20 mg) following the protocol set out by Ryalls et al. (2015). Foliar amino acids were used as a reliable and quantifiable proxy for the composition of phloem amino acids, as proposed by Riens et al. (1991) and Winter et al. (1992) for spinach and barley, respectively. Amino acid standards within the AA-S-18 (Fluka, Sigma-Aldrich) reference amino acid mixture were supplemented with asparagine and glutamine (G3126 and A0884, Sigma, Sigma-Aldrich). Nine essential amino acids (i.e., those that cannot be synthesized by insects *de novo*), including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine (Morris, 1991) and 10 non-essential amino acids (alanine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine, and tyrosine) were detected using this method.

Statistical Analyses

Precipitation and herbivore treatment effects on plant growth (height, mass, grass tiller numbers, and lucerne nodulation), chemistry (carbon and nitrogen), and aphids (population numbers and colonization success) were analyzed using mixed models in the *nlme* (Pinheiro et al., 2014) and *lme4* (Bates et al., 2014) statistical packages within the R statistical interface v3.1.1. The fixed effects included herbivore treatment (CON, A, W, and WA) and precipitation (drought, ambient, and elevated precipitation) as well as the interactions between these terms. The random terms included shelter (1–18) as a block term, with plot number (i.e., positioning under each shelter) as a nested factor to account for shelter and plot effects that could confound any treatment effects. Plant heights measured at the three time-points (i.e., weeks 6, 8, and 12) were combined into

TABLE 1 | Soil, plant, and aphid responses to precipitation and herbivore treatments from linear mixed models.

Response variable	Figures	Herbivore treatment			Precipitation			Herbivore treatment × Precipitation		
		F	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*
SOIL CHARACTERISTICS										
Soil water (%)[#]	2	16.10	**0.001**	3,45	223.00	**<0.001**	2,15	2.44	**0.039**	6,45
PLANT CHARACTERISTICS										
Phalaris aquatica										
Height (week 6)	3	1.91	0.141	3,45	0.35	0.708	2,15	0.58	0.741	6,45
Height (week 8)	3	2.41	0.080	3,45	28.09	**<0.001**	2,15	1.85	0.111	6,45
Height (week 12)	3	6.05	**0.002**	3,45	48.61	**<0.001**	2,15	1.38	0.245	6,45
Number of tillers	S1	3.12	**0.035**	3,45	84.22	**<0.001**	2,15	0.53	0.786	6,45
Shoot mass[#]	4	0.91	0.445	3,45	91.58	**<0.001**	2,15	1.67	0.149	6,45
Root mass[#]	4	2.24	0.097	3,45	21.15	**<0.001**	2,15	0.22	0.968	6,45
Shoot %N*	5	2.92	**0.044**	3,45	2.57	0.110	2,15	0.76	0.607	6,45
Root %N*	5	3.10	**0.036**	3,45	2.07	0.161	2,15	2.21	0.059	6,45
Shoot %C	S2	0.37	0.775	3,45	3.82	**0.046**	2,15	0.77	0.600	6,45
Root %C	S2	1.84	0.154	3,45	1.65	0.226	2,15	0.75	0.616	6,45
Medicago sativa										
Height (week 6)	3	0.47	0.702	3,45	0.08	0.926	2,15	0.52	0.793	6,45
Height (week 8)	3	0.69	0.565	3,45	3.90	**0.043**	2,15	0.31	0.926	6,45
Height (week 12)	3	1.67	0.187	3,45	70.57	**<0.001**	2,15	0.85	0.538	6,45
Shoot mass*	4	0.70	0.558	3,45	19.99	**<0.001**	2,15	0.47	0.827	6,45
Root mass*	4	1.51	0.226	3,45	9.44	**0.002**	2,15	0.50	0.801	6,45
Nodulation	6	4.38	**0.009**	3,45	3.84	**0.045**	2,15	0.26	0.952	6,45
Root %N	6	3.48	**0.024**	3,43	1.40	0.277	2,15	0.67	0.672	6,43
Root N content*	6	2.29	**0.092**	3,43	18.25	**<0.001**	2,15	1.02	0.423	6,43
Root %C	7	0.43	0.731	3,43	5.39	0.017	2,15	2.63	**0.029**	6,43
APHID RESPONSES										
Population[#]	9	4.56	**0.040**	1,33	5.38	**0.017**	2,15	2.14	0.134	2,33
Colonization	S5	3.35	0.076	1,33	3.73	0.048	2,15	3.32	**0.049**	2,33

*P-values highlighted in bold indicate significance ($P < 0.05$). Where appropriate, response variables were transformed (*Log, [#]Square root) before analysis.*

an individual model incorporating time as a fixed effect to account for repeated measures. *Post-hoc* Tukey's tests using the *glht* function in the R package *multcomp* (Hothorn et al., 2008) and the R package *LMERConvenienceFunctions* (Tremblay and Ransijn, 2015) were used for pairwise comparisons of means for treatment and interaction effects. The effects of precipitation and herbivore treatment on all individual amino acid concentrations were determined using principal components analysis (PCA) and groupings of co-varying individual amino acids were determined using a correlation matrix and hierarchical clustering within R. Permutational analysis of variance (PERMANOVA) was used to determine the effects of precipitation and herbivore treatment on concentrations of correlated amino acid groups.

RESULTS

Soil Water

Soil water content was significantly affected by both precipitation and herbivore treatment, and their interaction (**Table 1**). Specifically, under ambient and elevated precipitation, soil water increased in plots containing weevils alone (**Figure 2**), although soil water did not increase significantly when both weevils and aphids were present.

Plant Growth

Precipitation affected both grass and lucerne height at week 8 and 12 (**Table 1**). Specifically, grasses and lucerne plants subjected to drought at week 8 were shorter than those grown under ambient and elevated precipitation, although height did not vary significantly between plants grown under ambient and those grown under elevated precipitation. By harvest (week 12), the height of both grasses and lucerne plants had increased with increasing precipitation (**Figures 3A,B**). Additionally, grasses were significantly taller in plots where lucerne plants had been inoculated with weevils (i.e., treatments W and WA; **Figure 3C**). For lucerne, herbivore treatment had no significant effect on height at any point (**Table 1**). Combining plant heights into individual models (accounting for repeated measures) revealed similar results (Table S1).

Grass tiller numbers increased with increasing precipitation (Figure S1A), with grasses grown under drought having, on average, 51 and 65% fewer tillers than those grown under ambient

and elevated precipitation, respectively (Figure S1A). Moreover, grasses within plots inoculated with weevils alone contained 23% more tillers than those in control plots (Figure S1B). Grass and lucerne shoot mass increased with increasing precipitation (i.e., the biomass of grasses increased and decreased under elevated precipitation and drought, respectively, compared with those subjected to ambient precipitation), although root biomass did not vary significantly between those grown under ambient and elevated precipitation (**Figures 4A,B**). Root to shoot ratios of both plants decreased as water availability increased. Herbivore treatment had no effect on lucerne or grass biomass in general, individually or interactively (**Table 1**).

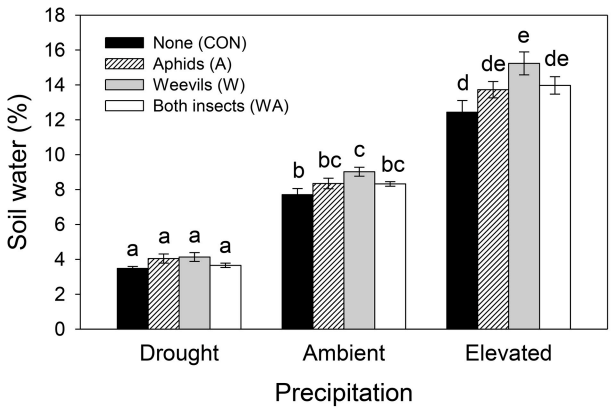


FIGURE 2 | The interactive effects of precipitation and herbivore treatment on soil water content. Mean values (±SE) are shown. Bars with the same letters were not significantly different ($P < 0.05$).

Carbon, Nitrogen, and Nodulation

Precipitation had no effect on grass root or shoot N concentrations, although shoot C concentrations decreased in grasses subjected to elevated precipitation compared with drought-stressed plants (**Table 1**; Figure S2A). Herbivore treatment significantly affected grass N concentrations, whereby plots inoculated with weevils alone contained grasses with higher root and shoot N concentrations compared with grasses in control plots (**Figure 5**).

Precipitation significantly affected the number of lucerne root nodules, with plants grown under drought containing, on average, 20 and 30% fewer nodules than those grown under ambient and elevated precipitation, respectively (**Figure 6A**). Herbivore treatment also significantly affected nodulation, whereby lucerne plants inoculated with weevils alone contained, on average, 48, 77, and 42% more root nodules than those containing aphids alone, those containing both insects and control plants, respectively (**Figure 6B**).

Roots of lucerne plants subjected to drought contained 52 and 60% less N than those under ambient and elevated precipitation, respectively (**Figure 6C**). Precipitation did not significantly impact the concentration of N in lucerne roots, although concentrations significantly increased in plants inoculated

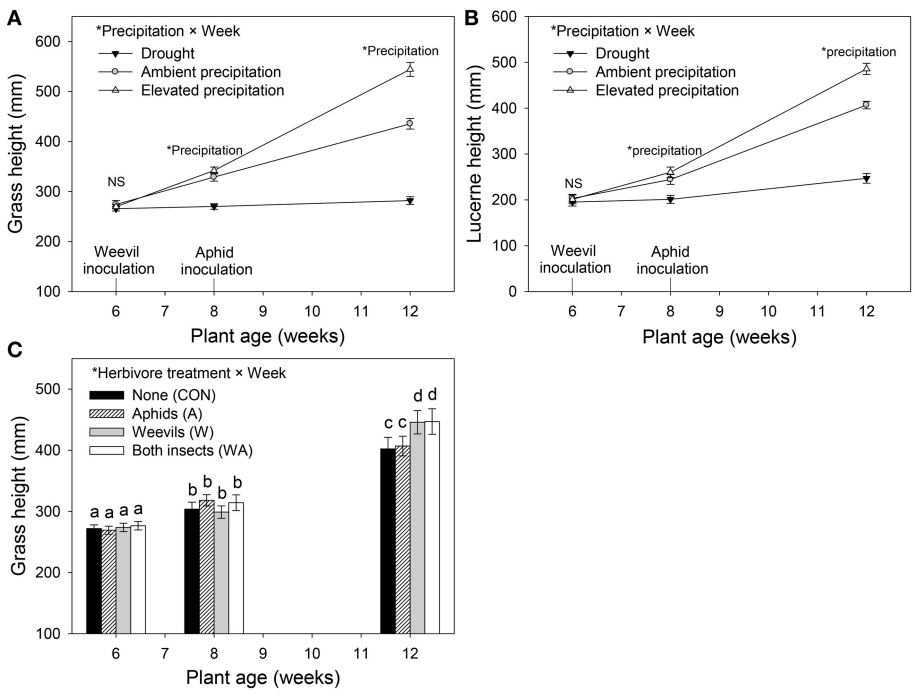


FIGURE 3 | The impacts of precipitation and herbivore treatment on the height of Harding grass (A,C) and lucerne (B) at week 6 (plant transplant period), week 8 (weevil inoculation period), and week 12 (harvest period). Herbivore treatment had no significant effect on lucerne height. Mean values (±SE) are shown. Statistically significant effects are indicated by *($P < 0.05$) and bars with the same letters were not significantly different ($P < 0.05$).

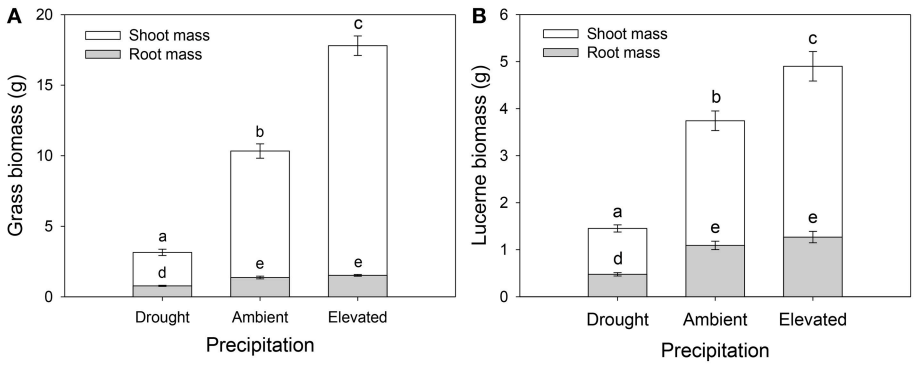


FIGURE 4 | The impacts of precipitation on the root and shoot biomass of Harding grass (A) and lucerne (B). Herbivore treatment had no significant effect on biomass. Mean values (±SE) are shown. Bars with the same letters were not significantly different ($P < 0.05$).

with weevils alone compared with control plants (**Figure 6D**). Precipitation interacted with herbivore treatment to significantly affect lucerne root C concentrations, with roots of plants inoculated with weevils alone containing a higher concentration of C than control plants, but only under drought conditions (Figure S2B).

Amino Acids

Precipitation and herbivore treatment had no significant effect on total amino acid concentrations (Table S2) and PCA combining all amino acid concentrations revealed no separation between precipitation or herbivore treatments (Figure S3). Correlated amino acid groups (Figure S4) consisted of group one (glycine, lysine, methionine, tyrosine, cysteine, histidine, and arginine), group two (isoleucine, phenylalanine, leucine, threonine, and valine), group three (aspartate and glutamate), group four (glutamine and serine), and three independently varying amino acids (alanine, asparagine, and proline). PERMANOVA revealed a significant effect of precipitation on amino acid groups one, three, and four (Table S2). Specifically, concentrations of amino acid groups one and three increased in lucerne plants subjected to drought and concentrations of amino acid group four increased in those under elevated precipitation (**Figure 7A**). Herbivore treatment only affected one amino acid, with concentrations of proline significantly decreasing in lucerne plants that were inoculated with weevils (i.e., treatments W and WA; **Figure 7B**).

Aphid Responses

Precipitation and herbivore treatment independently affected the number of aphids (**Table 1**). Lucerne plants subjected to drought supported 54 and 61% fewer aphids than those under ambient and elevated precipitation, respectively (**Figure 8A**). Additionally, aphid numbers decreased by 30% on lucerne plants inoculated with weevils compared to those with aphids alone (**Figure 8B**). Precipitation interacted with herbivore treatment to affect aphid colonization success, with weevils reducing aphid colonization success on plants under elevated precipitation. Drought generally reduced aphid colonization success, especially on plants with aphids alone (Figure S5).

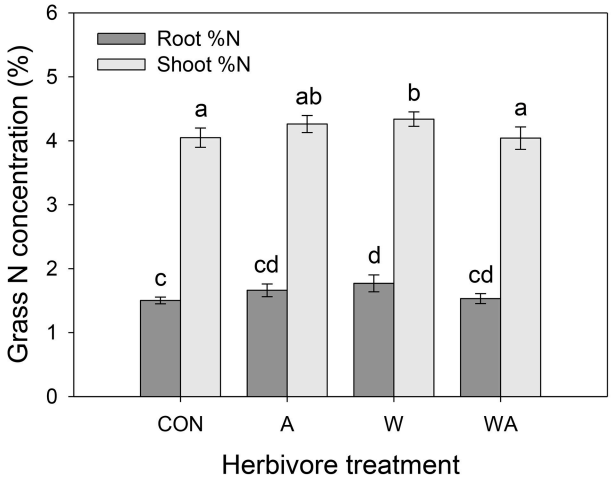


FIGURE 5 | The impacts of herbivore treatment on the root and shoot nitrogen concentrations of Harding grass. CON were the control plants (no insects), A had aphids alone, W had weevils alone, and WA had both insects. Mean values (±SE) are shown. Bars with the same letters were not significantly different ($P < 0.05$).

The main findings are summarized in **Figure 9**, with numbers referring to relevant figures associated with each effect.

DISCUSSION

To our knowledge, this study is the first to investigate the combined effects of herbivory (both above- and below-ground) and climate change (i.e., drought and elevated precipitation) in a grass–legume system. The main results demonstrate that drought and weevil root herbivory have contrasting effects on plant growth and lucerne nodulation, whereas both factors reduced aphid population growth. These effects may scale-up to play important roles in governing ecosystem function.

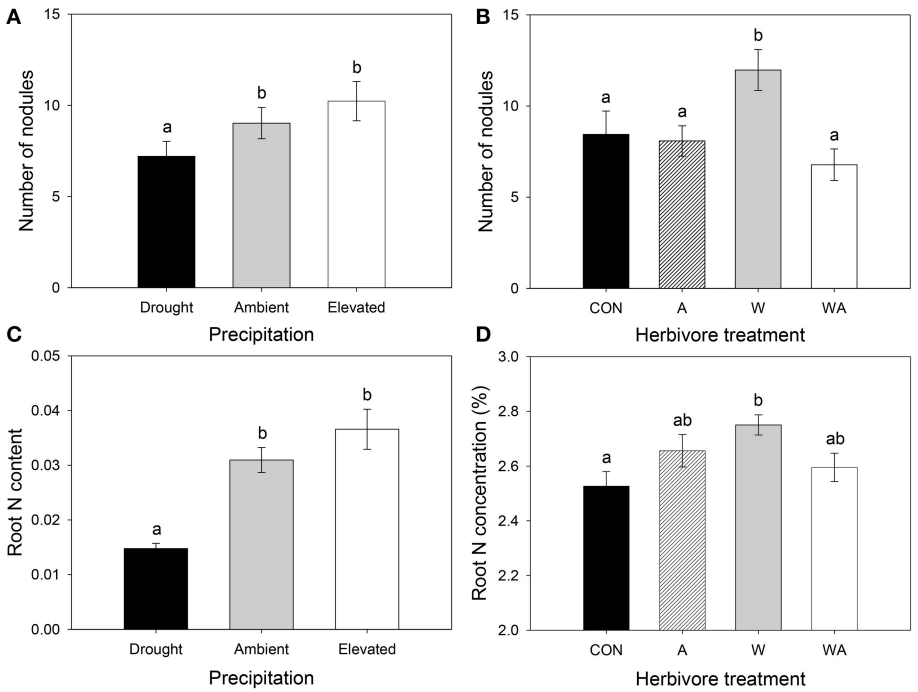


FIGURE 6 | The impacts of precipitation and herbivore treatment on lucerne nodulation (A,B) and the impacts of precipitation and herbivore treatment on root N content (C) and root N concentration (D), respectively. CON were the control plants (no insects), A had aphids alone, W had weevils alone, and WA had both insects. Mean values (± SE) are shown. Bars with the same letters were not significantly different ($P < 0.05$).

Soil Water Availability

Overall, drought reduced soil water content by 55% and elevated precipitation increased soil water content by 65% compared with ambient precipitation. Plots inoculated with weevils alone had increased soil water availability, suggesting that damage to nodules and roots by weevil larvae likely reduced water uptake by lucerne roots. Alternatively, burrowing activity of the weevils may have increased water penetration and reduced run-off, as observed in Johnson et al. (2015) with a soil conditioning ecosystem engineer (dung beetle; *Bubas bison* L.). The lack of an effect when aphids were feeding on lucerne simultaneously suggests that aphids counteracted the effects of weevils on soil water availability by either promoting soil water uptake or reducing weevil damage. In fact, aphid presence appeared to nullify the effects of weevils on multiple plant characteristics throughout this study, demonstrating the strong interactions between above- and below-ground stressors.

Plant Growth under Water Stress and Herbivory

Drought clearly inhibited growth of both lucerne and Harding grass. Once water availability reaches an optimum level, more resources may be allocated to shoot growth, which likely explains why root biomass of both plant species did not vary significantly between ambient and elevated precipitation. Similarly, the ratio of roots to shoots decreased as water availability increased. This is consistent with the resource optimization hypothesis, which posits that plants will allocate fewer resources to their roots when water and nutrient availabilities are high (Gargallo-Garriga et al., 2014). Under drought, the biomass of lucerne and grass shoots decreased by 63 and 70%, respectively, relative to those under ambient precipitation. Grasses tend to be more sensitive to drought due to their shallower roots compared with the deeper taproots of lucerne (Hayes et al., 2012). While elevated precipitation increased plant growth in general, grasses dominated lucerne in plots subjected to elevated precipitation (i.e., shoot biomass increased by 37 and 72% in lucerne and grass, respectively, under elevated precipitation relative to ambient precipitation). Increased precipitation may therefore significantly disrupt the long-term ability of these plant species to co-exist (Tow, 1993; Tow et al., 1997). By harvest, weevil presence significantly increased grass height, most likely associated with the fertilizing effect of increased N leached from lacerated lucerne nodules. Alternatively, the increase in soil water availability for grasses in plots with weevils present alone may have been responsible for increasing the height of grasses, although that would not explain the concurrent increase in grass height when both insects were present. The significant effect of herbivory on grass height and tiller numbers, however, was not reflected in grass biomass.

Impacts of Water Stress on C and N Dynamics

When water is a limiting factor, metabolites involved with energy production and growth (especially carbohydrates) are often shifted from shoots to roots (Gargallo-Garriga et al.,

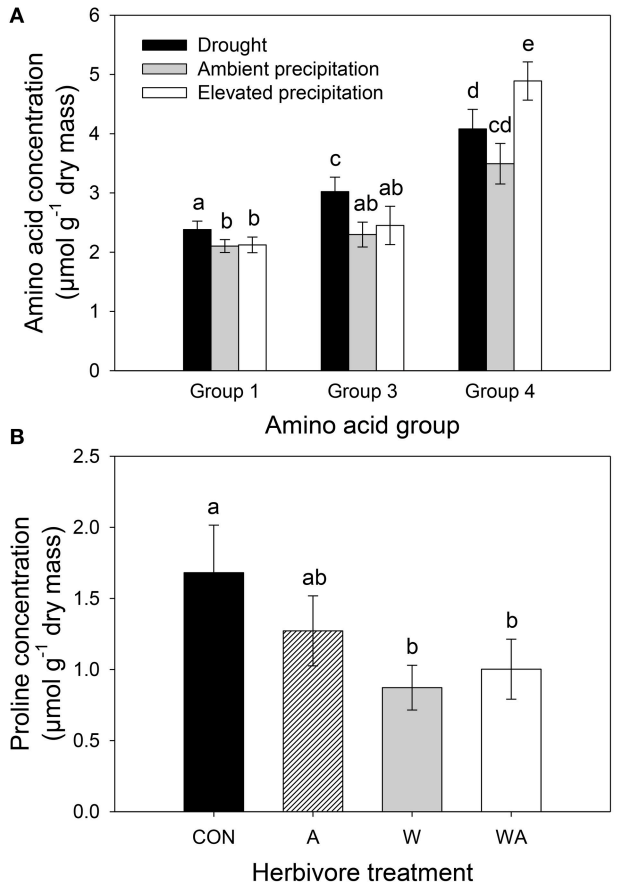


FIGURE 7 | The impacts of precipitation on individual groups of amino acids (A), namely group one (glycine, lysine, methionine, tyrosine, cysteine, histidine, arginine), group three (aspartate and glutamate), and group four (glutamine and serine) and the impacts of herbivore treatment on proline concentrations (B). CON were the control plants (no insects), A had aphids alone, W had weevils alone, and WA had both insects. Mean values (±SE) are shown. Bars with the same letters were not significantly different ($P < 0.05$).

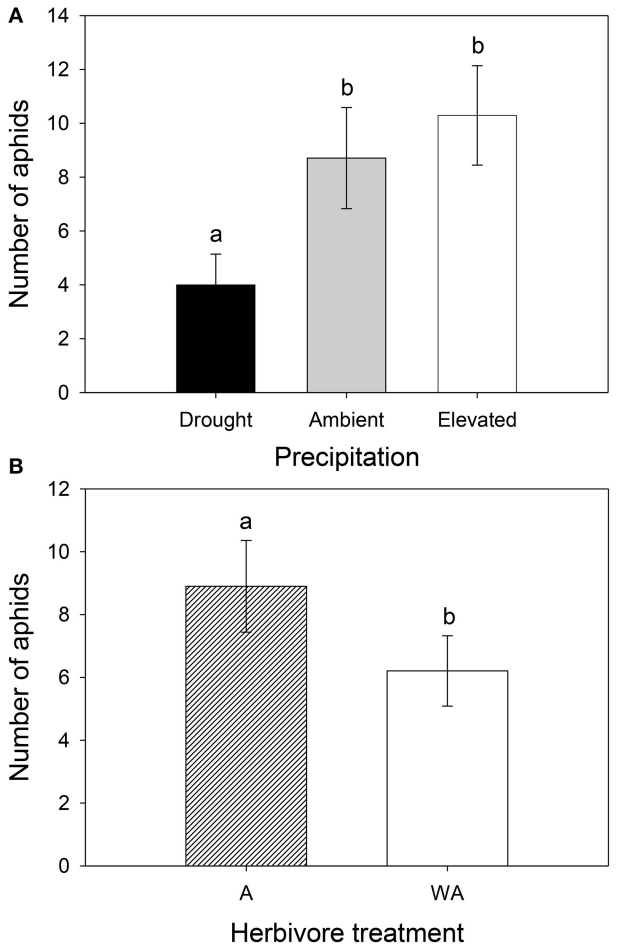


FIGURE 8 | The impacts of precipitation (A) and weevil presence (B) on the number of aphids. A had aphids alone and WA had both weevils and aphids. Mean values (±SE) are shown. Bars with the same letters were not significantly different ($P < 0.05$).

2014), although drought may also lead to a decrease in the belowground C demand (Hasibeder et al., 2015). Drought effects on C allocation have also been found to be dependent on community composition. For example, Sanaullah et al. (2012) noted an increased allocation of C from shoots to roots under drought in monocultures of *Festuca arundinacea* (Schreb.), *L. perenne*, and *M. sativa*. When these three species were combined into a grass–legume mixture, however, C allocation to shoots increased when exposed to drought. The current study identified an increase in grass shoot C concentrations under drought compared with elevated precipitation, suggesting that Harding grass allocates more C to anti-stress mechanisms under drought (Peñuelas and Estiarte, 1998; Jentsch et al., 2011), although concentrations in drought-stressed grasses were not significantly higher than in those under ambient precipitation.

Drought reduced root N content and nodulation in lucerne plants, likely associated with decreased root N-fixation activity. The negative impact of drought stress on nitrogenase activity in leguminous plants is well-known, although the exact mechanisms remain unclear (Gil-Quintana et al., 2013). A decline in the concentration of root C suggests that the supply of reduced C to bacteria may limit nitrogenase activity and nodule development. Root N concentrations, however, did not decrease under drought, suggesting that drought-stressed lucerne plants were not N-deficient. Under drought conditions, root nodulation, and subsequently, nitrogen fixation activity may decline due to a lower demand for N to support growth (Streeter, 2003). Predicting the responses of legumes to environmental change will be particularly important to maintain the N dynamics within the many terrestrial systems that are currently dominated by inputs of fixed N by legumes (Whitehead, 2000; Lilley et al., 2001; Peoples and Baldock, 2001; Angus and Peoples, 2012).

Impacts of Weevils on C and N Dynamics

Lucerne nodulation and root N concentrations increased in response to weevil herbivory, suggesting overcompensatory growth in response to nodule damage. Other studies have also

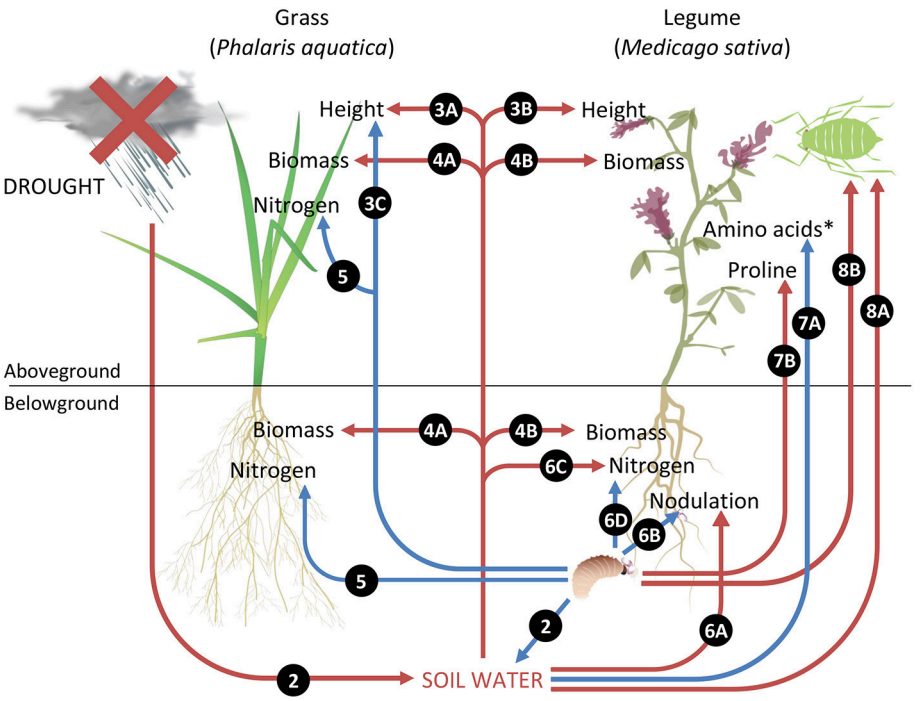


FIGURE 9 | Summary of key results illustrating plant–herbivore interactions under drought. Red arrows indicate negative impacts and blue arrows represent positive impacts. Numbers refer to relevant figures associated with each effect. *Amino acids represent concentrations of groups one and three amino acids, namely glycine, lysine, methionine, tyrosine, cysteine, histidine, arginine, aspartate, and glutamate.

shown nodule overcompensation in lucerne after root feeding by *Sitona hispidulus* Fabricius (Quinn and Hall, 1992) and *S. discoideus* (Ryalls et al., 2013b). No significant increase, however, was observed when plants were inoculated with both insects. When both weevil larvae and aphids are feeding on the plant, root recovery, and stress-related increases in plant nutrients or the diversion of resources above- or below-ground may be less likely because the plant remains in a constant state of stress (Ryalls et al., 2015). Weevils mitigated the effects of drought on lucerne root C concentrations by increasing root C concentrations. This is surprising considering that drought would most likely decrease weevil damage associated with declines in larval numbers (Goldson et al., 1986; Johnson et al., 2010). The difficulty in extracting *S. discoideus* larvae from the soil (Wightman, 1986) made it impossible to corroborate this assumption but additional studies using a more conspicuous species (e.g., whitefringed weevil, *Naupactus leucoloma* Boh.) may benefit from counting and weighing larvae.

Grass root and shoot N concentrations increased in plots that were inoculated with weevils alone, suggesting that larval damage to lucerne roots and nodules caused N leakage into the soil, which subsequently increased the uptake of N by Harding grass (Bardgett et al., 1999; Murray et al., 2013). This information may be particularly useful for ecosystems that suffer from legume-feeding pests or processes that damage legume roots, with consequences for productivity, nutrient balance, and species richness within ecological communities. Murray and Clements (1998) similarly identified an increase in N in wheat (*Triticum arvense* L.) from weevil-infested white clover. Other studies have identified a transfer of N from white clover to perennial ryegrass in response to herbivory by root-feeding nematodes (Bardgett et al., 1999; Dromph et al., 2006). In contrast, Ayres et al. (2007) noted a 13% reduction in N transfer from white clover to perennial ryegrass when clover roots were damaged by nematodes. They also identified a significant increase in grass root N when clover was subjected to simulated defoliation (through clipping). In this case, *S. discoideus* adults are defoliating insects so simultaneous feeding of larvae and adults may lead to even greater increases in N transferred to grasses. Increasing community complexity can alter the nutrient balance of plants differently, even if one plant (in this case, Harding grass) is not directly impacted. Given the prevalence of N-limitation in terrestrial ecosystems (Vitousek and Howarth, 1991), increased N supply has the potential to influence plant productivity, especially in grassland ecosystems (Ayres et al., 2007).

Amino Acid and Aphid Responses to Water Stress and Weevils

Sustained water stress in plants tends to affect aphids negatively (Huberty and Denno, 2004). *A. pisum* densities, in particular, have been closely linked to lucerne water content, with populations suffering lower growth rates when lucerne is drought stressed (Forbes et al., 2005). In the current study, drought negatively impacted aphids, although amino acid concentrations generally increased in plants under drought compared with those

under ambient precipitation. Hale et al. (2003) also reported a reduction in aphid (*Rhopalosiphum padi* L.) performance alongside an increase in amino acid concentrations in plants subjected to drought, suggesting that lower sap ingestion rates on drought-stressed plants are responsible for reducing aphid performance overall and override any positive effects on amino acid concentrations. From a community perspective, drought may somewhat alleviate the impacts of sap-feeding herbivores on plants.

Many studies have demonstrated how root feeders can influence aphid populations through plant-mediated mechanisms. Generally, aboveground aphids are positively affected by belowground root feeders (Johnson et al., 2012), although species that feed on legumes should be considered separately from those that feed on non-leguminous plants since legume root herbivores feed directly on the sites of N fixation and are more likely to negatively impact aphids by impairing root function and lowering the quality of phloem sap (Goldson S. L. et al., 1988; Ryalls et al., 2013b, 2015). Here, proline concentrations decreased when weevils were present, which may have contributed to the reduction in aphid numbers when weevils were feeding on the plant simultaneously. Proline concentrations in plants that were inoculated with aphids alone, however, were not significantly lower than concentrations in plants that were inoculated with both insects. Aphids may have mitigated the negative effects of weevil nodule feeding on foliar amino acid concentrations by promoting the metabolism of amino acids in lucerne (Guo et al., 2013) and masking the decrease in proline caused by weevils. While weevil larvae increased nodulation in lucerne, the effects on aphids may not have been apparent by the end of the experiment. Uncertainty exists as to whether the stimulation of nodulation over a longer timeframe would actually have a positive impact on aphids considering the dependency of aphids on plant nitrogen (Butler et al., 2012). Incorporating weevil larval performance data and quantifying root damage would provide useful insights into damage intensity and thresholds in lucerne, as described by Goldson et al. (1987; 1988) in New Zealand. Moreover, these data would allow us to examine aboveground–belowground interactions in the opposite direction (i.e., the impacts of shoot herbivory on root herbivores). No clear trend has yet emerged from studies analysing the effects of shoot herbivory on root herbivores (Johnson et al., in press and references therein), although it is uncertain whether patterns in lucerne or other legumes would emerge if these data were available. Understanding the complex interactions between above- and below-ground communities and how they fluctuate over time is essential for characterizing the fundamental mechanisms driving plant community structure (De Deyn and Van der Putten, 2005; De Deyn et al., 2007).

Conclusions

Global climate change and herbivory can modify the transfer of N and shape the competitive interactions between legumes and non-N-fixing grasses, with important implications for plant community structure. The results demonstrate how legume nodule herbivory by weevil larvae can increase N uptake and productivity of a companion grass species and decrease aphid populations via changes in individual amino acid concentrations. Moreover, drought decreased plant productivity in general and reduced aphid populations on lucerne, potentially via reduced phloem turgor pressure. With the frequency and length of droughts projected to increase under global climate change (Trenberth et al., 2003; IPCC, 2007; Gargallo-Garriga et al., 2014), understanding how these factors can shape plant susceptibility to insect pests and maintain the balance between an efficient grass–legume mixture is clearly a priority for achieving food security (Ayres et al., 2007; Gregory et al., 2009; Boschma et al., 2010). This will be especially important in terrestrial ecosystems that rely more on mineralization as a source of N for grasslands and other plant communities in the future (Newbould, 1989; Angus et al., 2006; Grace et al., 2015). Ultimately, these data inform adaptation strategies aimed at relieving the disruption caused by insect herbivore pests. With the threats of climate change on ecological communities apparent, combining multiple interacting species in environmental studies is key to maintaining ecosystem services and protecting our food resources, especially as human population levels climb toward 10 billion by the end of the century (Lal, 2006; Birch et al., 2011).

AUTHOR CONTRIBUTIONS

JR and SJ conceived and designed the experiment. JR collected the field data. JR conducted chemical analyses and analyzed the data. JR wrote the paper with assistance from SJ, BM, and MR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00345

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REVIEW
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Belowground Ecology of Scarabs Feeding on Grass Roots: Current Knowledge and Future Directions for Management in Australasia

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Many scarab beetles spend the majority of their lives belowground as larvae, feeding on grass roots. Many of these larvae are significant pests, causing damage to crops and grasslands. Damage by larvae of the greyback cane beetle (*Dermolepida albohirtum*), for example, can cause financial losses of up to AU$40 million annually to the Australian sugarcane industry. We review the ecology of some scarab larvae in Australasia, focusing on three subfamilies; Dynastinae, Rutelinae, and Melolonthinae, containing key pest species. Although considerable research on the control of some scarab pests has been carried out in Australasia, for some species, the basic biology and ecology remains largely unexplored. We synthesize what is known about these scarab larvae and outline key knowledge gaps to highlight future research directions with a view to improve pest management. We do this by presenting an overview of the scarab larval host plants and feeding behavior; the impacts of abiotic (temperature, moisture, and fertilization) and biotic (pathogens, natural enemies, and microbial symbionts) factors on scarab larvae and conclude with how abiotic and biotic factors can be applied in agriculture for improved pest management, suggesting future research directions. Several host plant microbial symbionts, such as arbuscular mycorrhizal fungi and endophytes, can improve plant tolerance to scarabs and reduce larval performance, which have shown promise for use in pest management. In addition to this, several microbial scarab pathogens have been isolated for commercial use in pest management with particularly promising results. The entomopathogenic fungus *Metarhizium anisopliae* caused a 50% reduction in cane beetle larvae while natural enemies such as entomopathogenic nematodes have also shown potential as a biocontrol. Key abiotic factors, such as soil water, play an important role in affecting both scarab larvae and these control agents and should therefore feature in future multi-factorial experiments. Continued research should focus on filling knowledge gaps including host plant preferences, attractive trap crops, and naturally occurring pathogens that are locally adapted, to achieve high efficacy in the field.

Keywords: ***Anoplognathus*, belowground herbivory, *Cyclocephala signaticollis*, *Dermolepida albohirtum*, *Heteronychus arator*, pasture, pest management, *Sericesthis nigrolineata***

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INTRODUCTION

Worldwide there are over 31,000 species of scarab beetles (Coleoptera: Scarabaeidae; Jameson, 2015) and within Australia alone there are well over 2,200 described species (Hangay and Zborowski, 2010). These scarabs can be found across tropical, subtropical and temperate regions of Australia and New Zealand in a broad range of ecosystem types including agroecosystems (Allsopp, 1999). Many scarabs have become destructive pests of grasslands as root-feeders (Potter and Braman, 1991). There are also instances where introduced plant species have become the preferred host to a number of native scarabs such as greyback cane beetle larvae (*Dermolepida albohirtum* Waterhouse, subfamily: Melolonthinae) feeding on sugarcane (*Saccharum* sp.). Moreover, the problem of such species becoming pests has been exacerbated by agriculture (Robertson et al., 1995), such as large-scale transition of grassland into arable crop production, or of forests and woodlands into pastures. Crop losses due to scarab larval damage for sugarcane in Australia alone can result in losses up to AU$40 million annually (Chandler, 2002). Historically, this problem has been addressed by using chemical pesticides, which can have serious collateral effects on non-target organisms and the environment (Jackson and Klein, 2006). As such, alternative management strategies are being continually investigated (Goldson et al., 2015).

Understanding the biology and behavior of scarab larvae, including their interactions with host plants and the soil environment (or rhizosphere) is an essential component to enabling effective management and control, both in Australia and at a global scale. There are numerous studies on these larvae within Australasia, some of which have elucidated core biology, behavior and even responses to future environment such as climate change (Johnson et al., 2014). However, for many scarab species this work was carried out some time ago, while for others the majority of their ecology has yet to be described. This is partly due to their soil-dwelling habit which has made culturing and experimentation particularly challenging. It is therefore timely to synthesize the fragmented information available on this group of root-feeding pests in Australasia. In this review we identify where knowledge is lacking, highlight promising research avenues into pest management, to suggest where continued research should be focused. In particular, this review focuses on belowground influences which impact larval development and survival. Edaphic variables such as soil moisture and temperature alongside biotic interactions with microbiota both in the soil and with host plants show most promise for improved pest management.

We concentrate on three subfamilies belonging to the family Scarabaeidae: Dynastinae (e.g., African black beetle *Heteronychus arator* Fabricius and Argentine scarab *Cyclocephala signaticollis* Burmeister), Rutelinae (e.g., Christmas beetles *Anoplognathus* sp. Leach) and Melolonthinae (e.g., dusky pasture scarab *Sericesthis nigrolineata* Boisduval and greyback cane beetle *D. albohirtum*). Within these subfamilies we focus on the key pest species/genera examples mentioned, while including any relevant information from other species within the subfamilies. The redheaded cockchafer, *Adoryphorus couloni* Burmeister (subfamily: Dynastinae) is also a significant pasture pest within Australia and was comprehensively reviewed recently (Berg et al., 2014). Hence, we do not include this species within the review. Within the three subfamilies we specifically focus on:

1. Host plants and feeding behavior
2. Abiotic soil factors (temperature, moisture, and fertilization)
3. Biotic soil factors (pathogens, natural enemies, and symbionts)
4. Applied perspectives
5. Directions for future research

HOST PLANTS AND FEEDING BEHAVIOR

While the majority of scarabs are grass root-feeders in their larval stages (**Figures 1** and **2**; Goodyer and Nicholas, 2007), some larvae feed on organic matter in the soil litter (Jackson and Klein, 2006). For some pest scarab species, feeding ecology has been documented relatively well. Across the subfamilies discussed here the most damaging and voracious feeding occurs during the third instar, therefore the timing of development of pest scarab larvae is important to consider from a pest management perspective (**Figure 3**). Indeed, the ability of all scarab larvae to locate suitable hosts is equally as important as the nutritional value of the host plant. Carbon dioxide emissions by the host plant is an important

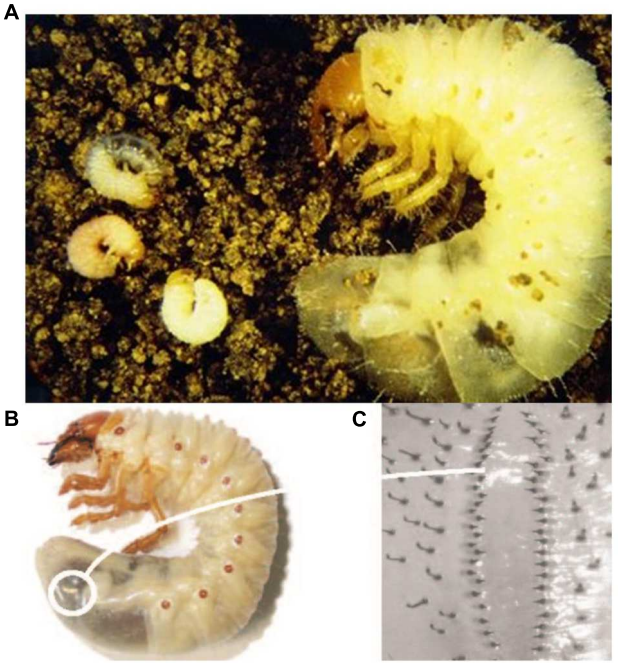


FIGURE 1 | Scarab larvae: (A) African black beetle larvae *Heteronychus arator*, (B) greyback cane beetle larva *Dermolepida albohirtum*, (C) close-up of hair pattern (raster) used to identify greyback cane beetle larvae. Images supplied by Western Australian Department of Agriculture and Food (African black beetle) and Sugar Research Australia (greyback cane beetle larva).



FIGURE 2 | Third instar larva of the greyback cane beetle (*D. albohirtum*). Image supplied by Adam Frew.

root exudate that plays a role in host plant location by root herbivores (Johnson and Gregory, 2006); however, other volatile root exudates are clearly critical in host plant location by scarab larvae (Eilers et al., 2012). The topic of host plant location by root-feeders was reviewed by Johnson and Gregory (2006) and revised by Johnson and Nielsen (2012), and we will not discuss this in detail here. Here we will present what is known regarding the feeding behavior of some of the key species from within Dynastinae, Rutelinae, and Melolonthinae.

Dynastinae

The African black beetle has been described as a sporadic pest of pastures and crops across New Zealand and Australia (Matthiessen and Ridsdill-Smith, 1991). Plant species composition influences the distribution of the African black beetle across the landscape (King and Kain, 1974; King et al., 1982). The larvae seem to have reduced performance on species such as *Medicago sativa* (King et al., 1975) and tend to avoid feeding on *Trifolium repens* (Sutherland and Greenfield, 1978), which is due, at least in part, to the feeding deterrents medicarpin and vestitol present in the roots (Russell et al., 1982). That said, larvae will eat *T. repens* roots if given no other choice (King et al., 1981c). Despite this, *T. repens* is a common food source for other scarab larvae such as *Costelytra zealandica* White (subfamily: Melolonthinae; King et al., 1981a; Russell et al., 1982; Prestidge et al., 1985).

By contrast, the grasses *Lolium perenne* and *Paspalum dilatatum* have been shown to be a preferred food choice of pasture grass species (King, 1977; King et al., 1981a). King (1977) found that African black beetle larval mass gain was greater on *L. perenne* when compared with *T. repens* and *Lotus pedunculatus*, but also that organic matter in the soil stimulated this feeding and increased weight gain. The organic content of the soil acting as a feeding stimulant has therefore been suggested as having implications for damage in soil with high peat content (Bell et al., 2011). Indeed the African black beetle is a significant pest of *L. perenne* pastures, both as larvae and adults, feeding on below- and aboveground portions of the plant, respectively (Popay and Bonos, 2008). The endophytic fungus *Neotyphodium lolii*, forms a mutualistic relationship with *L. perenne* (Raman et al., 2012). Feeding by adult African black beetles is well documented to be deterred by *N. lolii* infected *L. perenne* (Popay and Baltus, 2001), which has been attributed to the presence of alkaloids (Thom et al., 2014). More recently, Qawasmeh et al. (2015) found that different strains of *N. lolii* had an impact on the aboveground volatile profile of *L. perenne* and the attractiveness of this host plant to adult African black beetles.

The majority of research into endophyte (**Table 1**) induced protection has focused on aboveground herbivores (Popay and Baltus, 2001). One study on a specific *N. lolii* strain noted that the African black beetle larvae were observed to have a reduced occurrence in *N. lolii* infected grasses (Hume et al., 2007). More recently, another study has found changes in the root volatile profile in response to *Neotyphodium uncinatum* infection and found decreased attraction to *C. zealandica* larvae belowground (Rostás et al., 2015).

Considering damage can be significant, more research focusing on the efficacy of *N. lolii* strains in deterring African black beetle larvae would be the logical next steps. In the field, replacing turfgrass or pasture with *N. lolii* infected *L. perenne* could convey protection against African black beetle adults at the very least, perhaps reducing oviposition, and indeed may deter all alkaloid sensitive insect herbivores (see 'Applied perspectives' section).

The feeding behavior of Argentine scarab larvae has not received significant attention in the literature despite its pest status on turf and pastures (Carne, 1957a). Within Argentina, the larvae are known as pests particularly of potato crops (Berón and Diaz, 2005), but are known to feed on roots of flax, lucerne, sunflower, and carrot crops as well (Mondito et al., 1997). In Australia, however, the larvae feed mainly on grass roots. Carne (1957a) noted that the larvae were found in the greatest numbers in grasslands with *Cynodon dactylon* and *P. dilatatum*. It was also noted that this scarab could successfully develop on a diet composed solely of decomposing organic matter; however, the abundance found in pastures indicates some of their nutrient requirements are derived from grass roots. It is evident the Argentine scarab larvae feed on both organic matter and actively on grass roots but other than a few studies no other feeding behavior investigation has been carried out on the Argentine scarab in Australian grasslands. The lack of context specific studies on the larval feeding preferences of this scarab species, alongside the efficacy of management practices, calls for initial host preference studies to be conducted before any control initiatives can effectively be researched and applied.

Rutelinae

The feeding behavior of adult *Anoplognathus* spp., which consume the leaves of eucalypts, is addressed well within the literature (Carne et al., 1974; Edwards et al., 1993; Steinbauer and Wanjura, 2002; Johns et al., 2004; Steinbauer and Weir, 2007), in

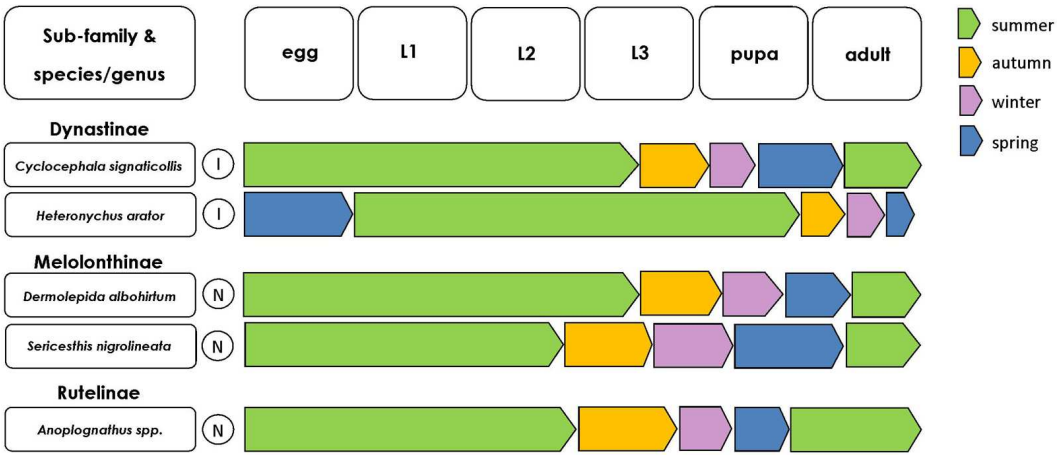


FIGURE 3 | Seasonal occurrence of scarab life stages for each of the key scarab pest species. Such information can help to design life-stage specific, targeted pest control programs. Color of arrows indicates the season in which each scarab life stage typically occurs (within Australia and New Zealand). Circles with I indicate species invasive to Australasia, circles with N indicate species native to Australasia.

TABLE 1 | Glossary of terms.

Term	Explanation
Endophyte	Bacterium or fungus which lives within a plant; endophyte infected ryegrass deter feeding by African black beetles.
Entomopathogenic fungus	Fungus which is a parasite to insects, often killing them; particular fungi have been used as part of pest management of scarab larvae.
Entomopathogenic nematode	Nematodes (thread worms) which kill insects via the bacteria they harbor inside them; some species have been used as part of pest management of scarab larvae.
Endosymbiotic bacteria	Bacteria living within another organism; found in the hindguts of scarab larvae, aiding digestion of plant material.

contrast to the information on larval feeding behavior, which is relatively scarce.

Anoplognathus larvae are known to feed on organic matter in the soil, grass roots, and crop roots (Carne, 1957b; Sallam et al., 2011). Some species within the genus, such as *Anoplognathus montanus,* will commonly feed on rotting organic material such as timber, but will also feed on the finer roots of eucalypts (Carne, 1957b). Carne et al. (1974) stated that larvae of *Anoplognathus* feed primarily on organic matter in the soil and tend not to seek out plant roots. While Davidson and Roberts (1968a) confirmed this, they nonetheless stated that the organic matter they feed on is composed mainly of plant roots. Here, they also found that when Christmas beetle larvae fed on the grass *Phalaris tuberosa* and *T. repens*, larvae often failed to reach pupation, which could be due to secondary metabolites in the plant. In a further study that year, it was found that Christmas beetle larvae avoided feeding on *T. repens* altogether (Davidson and Roberts, 1968b), a behavior also exhibited by African black beetle larvae.

The larvae of *Anoplognathus* spp. have been reported as pests of sugarcane, although only when numbers are high (Samson et al., 2013). Significant damage to pastures by Christmas beetle larvae is well known, particularly by the third instar (Urquhart, 1995). Feeding populations of larvae can be influenced by aboveground herbivores. A study by Roberts and Morton (1985) investigated the effects of grazing pressure on the biomass of *Anoplognathus* sp. larvae, and found that larval abundance peaked under low to intermediate grazing pressure. Therefore, low pasture damage by larvae may be exacerbated by moderate grazing of livestock aboveground.

Melolonthinae

The greyback cane beetle is a long standing pest within sugarcane and the larvae can cause devastating damage to crops (Chandler, 2002). Initial uncertainties regarding the feeding of mainly organic material in the soil (Illingworth and Dodd, 1921) have been resolved as there is compelling evidence for grass roots as the main resource (Sallam, 2011). Root feeding was shown by Logan and Kettle (2002) who investigated the effect of food type on the survival and development of first instar greyback cane beetle larvae. Larval survival and development was highest in treatments with grass seedlings and lowest in soil alone. This result was confirmed by a second experiment using sugarcane, Guinea grass (*Panicum maximum*), cane trash (mulch), and a soil only environment, where larval survival and mass was lowest in the soil only treatment and highest when cane or grass were available (**Figure 4**).

In Australia, cane beetles are the major pests to the sugar industry (McLeod et al., 1999; Horsfield et al., 2008) and as a result there have been several studies into pest management and environmental conditions that may impact on larval induced damage to sugarcane (Robertson et al., 1995; Robertson and Walker, 2001; Chandler, 2002). Coupled with the development of pest management strategies, Allsopp (1991) investigated feeding stimulants of greyback cane beetle larvae, which could be used

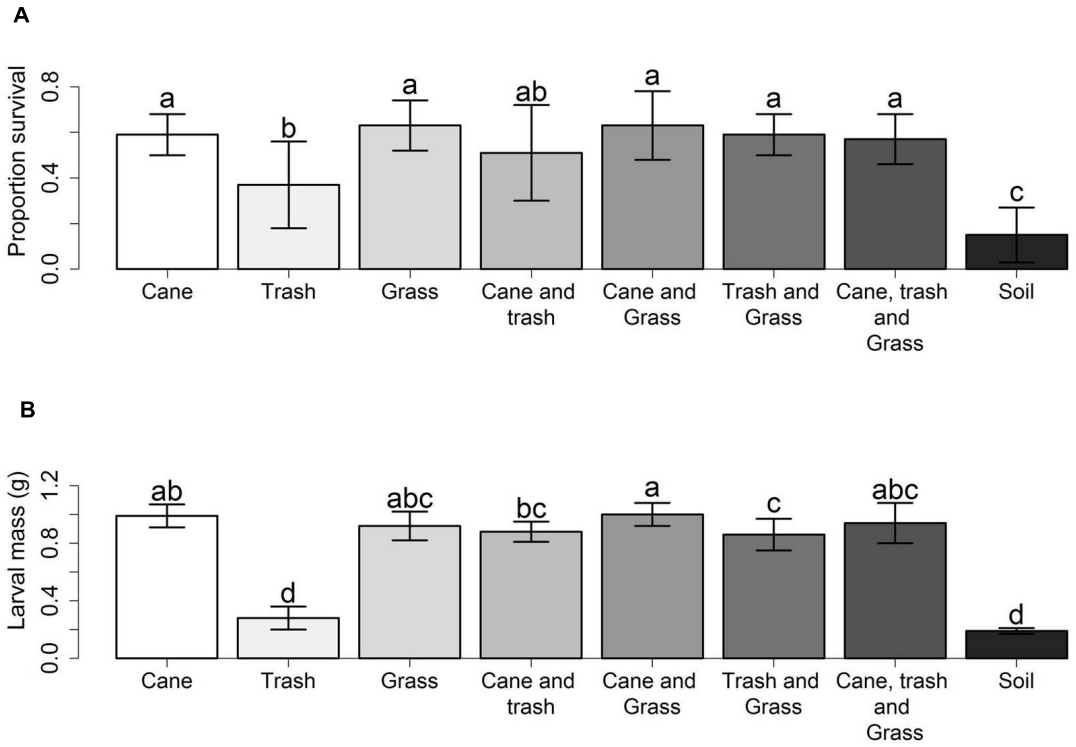


FIGURE 4 | Survivorship and mass of early instar larvae of *D. albohirtum*. (A) Mean proportion survival (±SD), and **(B)** mean larval mass in grams (±SD), of larvae after 4 weeks in bins with food of either sugarcane, Guinea grass, cane trash, combinations of two or three of these, or none of these. Different letters indicate significant effects of treatments. Adapted from Logan and Kettle (2002).

to enhance the efficacy of larval baits. Larvae showed a strong feeding response to fructose and sucrose. Both sucrose and fructose, along with glucose, are the most abundant sugars found in sugarcane, and are both at higher concentrations in the lower stem of sugarcane compared with the roots (Irvine, 1977).

Estimates of population size and density within sugarcane fields vary from three or four larvae per cane plant (Ward and Robertson, 1999) to numbers of 15 per plant, or more (Jarvis, 1933; Sallam, 2011). Some Melolonthinae larvae have shown specific soil type preferences. A study by Cherry and Allsopp (1991) found distinct soil type preferences between different species, with some larval populations of some species positively correlated with clay and silt, and negatively with sand content, while other species showed opposing correlations. For yet other species, such as the greyback cane beetle, soil type has little influence on the distribution (Robertson and Walker, 2001). Overall there is no 'one soil type fits all' for scarab species as studies have shown species specific preferences (Gordon and Anderson, 1981; Cherry and Hall, 1986).

Studies conducted into the feeding behavior of dusky pasture scarab larvae have focused on climatic and abiotic influences rather than host preference. The larvae can feed and survive in soil in the absence of plant roots (Ridsdill-Smith, 1975; Smith and Porter, 1980), however, it is not clear if they are able to develop into adults on soil organic matter alone. The feeding behavior, and relative consumption of food is largely influenced by temperature (Davidson et al., 1972b; Ridsdill-Smith et al., 1975; Cairns, 1978) and under field conditions there is often a seasonal pattern of larval feeding as a result of local temperatures. Ridsdill-Smith (1975) carried out an investigation into the feeding behavior of dusky pasture scarab larvae using slices of carrot under different temperatures. It was found that the larval consumption of food peaked at 30°C while, interestingly, the efficiency of conversion of ingested food (which accounts for larval growth and the mass of food consumed) peaked at a temperature of 14°C. To build upon this, a follow-up study utilizing larvae that had been fed on living roots and a variety of food sources was conducted by Ridsdill-Smith (1977). It found that the feeding of dusky pasture scarab larvae declined when the population densities were high, although this was likely a result of a lack of young living roots. This was confirmed by Ridsdill-Smith and Roberts (1976), who also showed that larval growth reduced as density increased, which was also likely to be due to a limited food supply. The study also suggested that the larvae preferred to feed on younger roots.

One recent study by Johnson et al. (2014) provided evidence of compensatory feeding by the dusky pasture scarab larvae under elevated atmospheric CO_2 (eCO_2) on *Microlaena*

stipoides, a C_3 grass. Despite this increased feeding, the performance of the dusky pasture scarab was much lower under these eCO_2 conditions, which was likely due to a reduction in the root nitrogen concentrations. Interestingly, under ambient CO_2, larvae consumed 48% more material from *M. stipoides* than from *Cymbopogon refractus*, a C_4 grass. Generally, C_3 grasses are thought to be more susceptible to herbivory than C_4 grasses (Caswell et al., 1973). More studies of this type are necessary to elucidate the relationship between scarabs and their host plants, particularly when considering changes in feeding behaviors as a result of climate change. It can be concluded from these studies that the feeding behavior of the dusky pasture scarab larvae is strongly influenced by abiotic factors such as temperature and, indirectly, atmospheric CO_2. As such, future research should investigate host plant preferences alongside abiotic and biotic interactions, including changes in atmospheric CO_2 concentrations.

ABIOTIC SOIL FACTORS

Abiotic factors have been seen to have a strong influence on insect pests of Australasia (Powell et al., 2003). All root-feeding insects respond directly to their immediate physical and chemical environment (Barnett and Johnson, 2013). Here, we review some significant abiotic factors impacting on scarab larvae: temperature, moisture, and fertilization. We focus on species within Dynastinae, Rutelinae, and Melolonthinae found in Australasia. We also draw on studies of other species within these subfamilies outside Australasia to indicate the general impact of abiotic rhizospheric factors on scarab larvae. These factors are considered with a view to highlight where agricultural practices could be modified to reduce damage by scarab larvae (discussed in more detail in 'Applied perspectives' section).

Temperature

The temperature of the soil can impact significantly on scarabs, particularly in the egg and early larval stages. For example, temperature has been seen to have an impact on population fluctuations of the African black beetle (East et al., 1981; King et al., 1981b). Despite this importance, few studies have focused on the temperature preferences for oviposition by scarab females.

Regarding larval stages, a single exposure of 35°C for 24 h has been shown to kill 100% of first instar larvae of *Anoplognathus* spp. and the dusky pasture scarab, while around 62% survive when exposure to such temperatures is only for 12 h (Hassan and Hilditch, 1976). Within the same study, second instar larvae showed a higher tolerance for high temperatures, for example at 37.5°C, 73% of first instar larvae died while only 40% of second instar died. Regarding the lower temperature threshold it is generally understood that at low temperatures (below 16°C) scarab eggs will take longer to hatch and larvae will take longer to develop (Davidson et al., 1972b). This relationship between temperature and development was investigated in greyback cane beetle pupae (Logan and Kettle, 2007), where the minimum and maximum time for pupal development was found to be 26 days at 30°C and 75 days at 18°C, respectively. The low temperature threshold, at and below which no development occurs was 12°C. There have several studies showing the influence of temperature on the growth and development of the dusky pasture scarab (Davidson et al., 1972b; Ridsdill-Smith, 1975; Ridsdill-Smith et al., 1975; Cairns, 1978). The relative growth rate of these larvae was found to have lower and upper temperature limits of 5°C and 32°C, respectively, with optimum growth occurring around 17.5°C (Ridsdill-Smith et al., 1975). One study on *Rhizotragus majalis* Razoumowsky (subfamily: Melolonthinae), indicated that later instar larvae have much greater mobility and therefore older scarab larvae are likely to be less susceptible to temperature stress through avoidance behavior (Villani and Nyrop, 1991). This was confirmed by Zhang et al. (2003) who confirmed higher mobility in second and third instars by monitoring their acoustic sounds, which also increased with soil temperature, while below 9°C sound production fell to a minimum. Overall, temperature plays an important role in the survival, and the rate of development of scarab larvae. Generally, larval growth rate increases with temperature, where upper limits tend to be between 35 and 40°C, and as temperatures drop to 16°C or below, development is significantly reduced. First instar larvae tend to be the most sensitive to temperatures stress, while scarab eggs and later instar larvae are more tolerant.

These larval responses to temperature indicate how significant climate can be to larval populations. Indeed, high temperatures at a particular time of development can have particularly large impacts on greyback cane beetle populations. Horsfield et al. (2008) analyzed larval damage records and climatic averages from 1989 to 2003 and showed that prolonged hot and dry conditions during the late spring can limit population numbers by impacting on emergence, as well as synchrony of emergence with feeding, mating and egg laying. Conversely, milder and wetter spring season can promote adult emergence and the ability of the adults to successfully feed, mate and lay eggs. This would directly impact on successive larval populations and therefore damage to cane the following year.

Moisture

Soil moisture is often referred to as the most important property that affects the development and survival of scarab larvae belowground (Brown and Gange, 1990; Barnett and Johnson, 2013). Indeed, eggs of many scarab species must absorb water before hatching (Potter, 1983), and hence the availability of water in the soil can be critical to scarab population dynamics. Soil moisture is also the factor best examined in the literature with regards to female oviposition in scarabs (Potter, 1983; Cherry et al., 1990; Allsopp et al., 1992; Logan, 2007). Several studies have shown different optimal soil moisture conditions for maximum oviposition. Some Melolonthinae scarabs are known to oviposit in soils around field capacity (−74 kPa; Logan, 2007), while others within the same subfamily prefer a range between field capacity and dry soil near wilting point (−1500 kPa; Logan, 2007). Ward and Rogers (2007) carried out a study on soil moisture ovipositional preferences in four Melolonthinae scarabs found in

Australia, including the greyback cane beetle. It was concluded that those species adapted to the semi-arid tropics, where rainfall is unreliable, have little or no preferences observed beyond a reduction in oviposition in very dry soil (−1500 kPa). However, in subtropical and temperate (with less seasonal rainfall) adapted species there were clear preferences for drier soils (−1000 kPa). This suggests that the climates in which key/target pest species have originated and are adapted to, must be considered in attempts to manage populations. It also indicates that for those tropically adapted species, moisture control as a form of pest management may not be the way forward, as their ovipositional preferences are likely to be driven by factors other than soil moisture.

Moisture content of the soil can directly impact on scarab larvae populations. African black beetle populations, for example, have been shown to be suppressed in regions with early summer rainfall (Matthiessen and Ridsdill-Smith, 1991), as first instar larvae are more moisture sensitive than egg stage or later instars (King, 1979; King et al., 1981b). In periods of seasonal drought, the larval populations are no longer suppressed by the normally high moisture content, resulting in damaging outbreaks (Matthiessen and Ridsdill-Smith, 1991). Whether these population responses would be the same in different soils is uncertain. Matthiessen (1999) showed that soil type had a significant impact on African black beetle larval survival, and that this factor interacted with soil moisture, where larval survival was higher under regular watering treatments compared with no watering, but only in some soil types. With these studies in mind, investigations are necessary to elucidate the interaction between soil moisture and soil texture, where larval populations are monitored under different common soil types in the field, under a range of soil moisture treatments. Future work should also include extreme climate events, such as drought and flooding, as the frequency of such events are predicted to increase in the future (Pachauri et al., 2014). This way, we can gain a better picture of how belowground scarab pest status will change in the future.

Several studies have reported responses from other scarabs to soil moisture. For example, within the genus *Cyclocephala*, larvae are significantly more abundant and also have higher mass in irrigated, compared to non-irrigated plots (Potter et al., 1996). Survival of dusky pasture scarab larvae have been shown to be optimal between −100 and −150 kPa, while in saturated soils, larval survival is negatively proportional to the length of exposure (Davidson et al., 1972b). While studies involving *R. majalis*, have shown that larvae move quickly toward the surface when the moisture content of the soil is increased, yet little movement is exhibited in response to drought conditions (Villani and Wright, 1988).

Changes in soil moisture will also impact the host plants of scarab larvae. In addition to this, the diffusion of plant root volatiles is reduced in high soil moisture, however, some moisture is required to prevent total vertical diffusion (Hiltpold and Turlings, 2008). Indeed, natural enemies of scarab larvae, such as entomopathogenic nematodes (**Table 1**) (EPNs), are more effectively recruited by plant volatiles and have higher virulence in soils with high moisture content (Grant and Villani, 2003). Therefore future studies into the effects of different soil moisture contents within a variety of soil types, would also benefit to consider how the natural enemies and pathogens respond under these conditions. This way a more holistic and ecologically relevant picture can be constructed.

Fertilization

The response of soil dwelling root-feeders to fertilization has received some attention within the literature. Frew et al. (2013) found that the application of nitrogen, phosphorus, and potassium (NPK) fertilizers promoted more nutritionally superior grass species, which in turn increased abundance of dusky pasture scarab larvae. However, Potter et al. (1996) who investigated the effects of different agricultural practices on scarab populations over 3 years and found no significant effect of NPK fertilizer on *Cyclocephala* spp. density or growth. On the other hand, Radcliffe (1970) added organic (cow dung) fertilizer to the soil and found that this lessened the damage to grass roots by *C. zealandica.* This may have been where the larvae switched from feeding on the grass roots to the increased provision of organic matter in the soil, or the addition of excess organic matter may have contributed to better compensatory root growth in response to damage, or a combination of both. In the same study it was found that larvae development was more advanced when treated with nitrogen fertilizer (Radcliffe, 1970). It has also been shown that the addition of organic fertilizer increases the mass gain of *C. zealandica* larvae (Wightman, 1974). In contrast to these findings, other studies on *C. zealandica* have shown the addition of nitrogen fertilizers has had no effect on larval feeding and survival (Prestidge et al., 1985) or population density (Prestidge and East, 1984), with similar responses found with *Popillia japonica* Newman (subfamily: Rutelinae) to the application of NPK fertilizer (Crutchfield et al., 1995). Other root feeding insects have been shown to respond positively to the addition of nitrogen fertilizer, such as the rice weevil larvae [*Lissorhoptrus oryzophilus* Kuschel (Curculionidae, Erirhininae)] and the western corn rootworm larvae [*Diabrotica virgifera virgifera* LeConte (Chrysomelidae, Galerucinae)] (Spike and Tollefson, 1988). In the comprehensive review of belowground herbivores by Brown and Gange (1990), it was suggested that the timing of fertilization is important to the effect on the root feeding larvae. They suggested that if nitrogen fertilizer is applied before larvae are present then this promotes root growth, which in turn gives a greater food supply to larvae, while if fertilizer is added after larval establishment then the damage to grasses is less (Spike and Tollefson, 1988).

It is known in some plants that when nitrogen is limiting in the soil, plant defense investment increases in the leaves (Schmelz et al., 2003; Chen et al., 2008). Low soil nitrogen content could similarly affect root defense investment allocation, thereby impacting the root-feeding scarab beetle larvae populations. It has been suggested that fertilization may cause a reduction in the defensive root compounds (Hol, 2011; Erb and Lu, 2013). These may be direct secondary defenses affecting scarab feeding or performance, or indirect defenses involving recruitment of natural enemies such as EPNs (see section on 'Pathogens, natural enemies and symbionts' below). Such plant

responses to fertilization addition could be linked to arbuscular mycorrhizal fungal (AMF) associations. AMF associations have been shown to increase induced plant defense responses (Pozo and Azcón-Aguilar, 2007), but root colonization by AMF is known to be reduced when soil nutrients (particularly nitrogen and phosphorus) are high (Vannette and Hunter, 2009; Smith and Read, 2010). Therefore any decrease in plant defenses in response to high nitrogen, could be mediated by limited AMF colonization.

Overall, the literature is not consistent regarding the impact of fertilization on scarab larvae and similar species, although both positive and null effects seem to be the most common responses reported. Any positive effect is likely to be due to an increase in organic matter for younger instar scarabs to ingest and an increase in the nutritional value of host plant species. An increase in nutrient availability may also result in an increase in the tolerance of the host plant to herbivory, although this is likely to be dependent on the nutrient and specific herbivore in question (Wise and Abrahamson, 2007). This may also impact on important microbial plant associations in the soil (Smith and Read, 2010), which can indirectly impact on herbivores (Bennett and Bever, 2007; Biere and Bennett, 2013). Therefore soil fertility may promote root-feeding scarabs, but also may increase plant tolerance to herbivory as well as benefit the natural enemies of scarabs belowground. Continued research should aim to include as many contributing factors to plant–insect interactions within the soil (such as AMF and EPNs) as possible, as these are likely to produce outcomes more relevant in the field.

BIOTIC SOIL FACTORS

Pathogens, Natural Enemies and Symbionts

Scarabs have a number of natural enemies and insect pathogens that threaten their survival. Scarab larvae have evolved within the soil environment, which naturally brings them in close contact with numerous soil organisms and microbiota, some of which are pathogens (Jackson and Klein, 2006). Here we discuss some pathogens and natural enemies that have been identified to hold potential as biocontrol agents against scarab larval pests in the field.

Entomopathogenic fungi are ubiquitous in soils, particularly those within the genera *Metarhizium* and *Beauveria.* Greyback cane beetle larvae are easily infected by the entomopathogenic fungus (**Table 1**) *M. anisopliae.* The impact of this naturally occurring fungus on the larval populations is not density dependent and as such has been shown to account for a fixed mortality rate, regardless of the population density, while the spores are known to be resistant to many agricultural practices (Sallam et al., 2003, 2007). This fungus has been isolated and commercialized as BioCane^{TM} and used as a fungal biocontrol that in trials has shown more than 50% control of the canegrub after 6 months of a single application (Logan et al., 2000). Interestingly Berón and Diaz (2005) carried out susceptibility trials of the Argentine scarab larvae to different strains of *M. anisopliae*. All strains showed low virulence against the larvae, possibly due to the lack of host specificity to the Argentine scarab. However, a particular strain of the entomopathogenic fungus *Beauveria bassiana*, did show up to 70% mortality in Argentine scarab larvae. The differences in virulence of *M. anisopliae* toward different scarab larvae species shows how the insect response to microbial pathogens can often be species specific, and can vary significantly. Another *Beauveria* sp. that has shown success as a biocontrol is *B. brongniartii*, which has been successful acting against a broad range of hosts. Some native strains have been isolated from *Melolontha melolontha* Linneaus (subfamily: Melolonthinae) and used as pest controls across Europe with good success (Dolci et al., 2006). Similar work with *Beauveria* strains isolated from Madagascar and Turkey have also seen success (Maurer et al., 1997; Sevim et al., 2010). These are further examples of successful isolation and application of naturally occurring scarab pathogens.

A significant pathogenic microorganism, particularly noted in efficacy against the greyback cane beetle larvae, is the protozoan *Adelina* sp. which is a density dependent pathogen (Robertson et al., 1998). High *Adelina* incidence causes a drop in the larval population which in turn impacts on the *Adelina* incidence in the soil. Interestingly Sallam et al. (2003) found that *Adelina* incidence was higher in soil with grass cover compared to bare soil areas, which could be due to higher moisture retention and cooler temperatures. Responses such as these should be taken into account when managing larval populations in agriculture to optimize natural pathogen efficacy.

Within New Zealand, the bacteria *Serratia entomophila* and *S. proteamaculans* were isolated from *C. zealandica* as the cause of amber disease, which leads to the cessation of feeding of the scarab grub resulting in eventual death (Hurst et al., 2004). These bacteria were developed as biopesticides against scarabs and have been used for almost 20 years as biocontrol agents. These are further examples of microbial pathogens adapted to their host, and their host range which were used to great success as a control method of scarabs (Hurst et al., 2000).

There are a number of viruses that infect scarabs, such as pox viruses and iridescent viruses; however, little research has been done on their potential as biocontrols, and their presence and effect on scarab populations under natural conditions has not yet been documented (Jackson and Glare, 1992). Damage by the Dynastinae scarab larvae within the genus *Oryctes* has been successfully mitigated via the *Oryctes* virus (Huger, 2005), which is a unique virus, in that it was identified as the first rod-shaped, non-occluded insect virus, and is highly infectious. It has been isolated, purified and used in pest control for over 10 years, but it has low success on any species outside of the target scarab genus *Oryctes* (Huger, 2005). Current research is focused on selecting strains of the virus for greatest persistence in the environment.

One of the major natural enemies of scarabs are EPNs, which are internal parasites of scarabs. They do not act alone, but rather it is their association with entomopathogenic bacteria that kill the scarab hosts. *Steinernema* and *Heterorhabditis* are the two genera of EPNs and there are a number of species within both genera that infect scarabs (Klein, 1993). The EPNs kill the larvae via their symbiotic bacteria *Xenorhabdus* sp. Several species have been isolated from scarab grubs, such as *Steinernema glaseri*, *S. anomaly, Heterorhabditis megidis,* and

several different strains of *S. carpocapsae* and *H. bacteriophora* (Klein, 1993), their potential to control scarab larvae populations is being investigated. Some nematodes have shown success in laboratory and field trials against scarab larvae, with particular interest in *S. scarabaei* as an effective control against a range of scarabs dominant in North America and Asia (Stock and Koppenhöfer, 2003). However, other efforts to use EPNs in the field have not been successful, which have been attributed to a lack of understanding of the nematode–bacterium complex and differences in target species susceptibility, biology or behavior (Klein, 1993; Georgis et al., 2006). Recently Wu et al. (2014) tested and compared the virulence of four EPN species and their interactive effects with entomopathogenic fungi against the scarab larvae of *Cyclocephala lurida* Bland (subfamily: Dynastinae). They concluded that the impact of *H. bacteriophora* alone or in combination with the fungal pathogens was comparable to that of an imidacloprid insecticide against the larvae. This indicates the potential EPNs have as biocontrols and that further work is warranted to fully elucidate the interaction between natural enemies, pathogens, and host. Plants can recruit EPNs via attractive volatile signals as a natural defense strategy (Grewal et al., 1994; Rasmann et al., 2005). It has been shown that EPNs can be selectively bred for enhanced responsiveness to these volatile cues (Hiltpold et al., 2010), meaning that improved efficacy of the commercial EPN use is still ongoing and holds great potential as a biological control method of scarabs in agriculture and industry.

Finally, diverse communities of endosymbiotic bacteria (**Table 1**) that assist with the digestion of plant material, particularly cellulose and hemicelluloses, live within the hindguts of scarab larvae (Cazemier et al., 2003; Huang et al., 2010). Pittman et al. (2008b) found that there were species within the bacterial community of the greyback cane beetle larvae hindgut that were consistently found within the larvae across their geographical distribution. These bacteria were successfully transformed and reintroduced into the hindgut of the larvae, which indicates they are strong candidates to control the populations of greyback cane beetle larvae through the expression of anti-feeding compounds within the larval gut (Pittman et al., 2008a). Non-resident bacteria are normally not useful in such paratransgenic control methods because they are unable to remain established within the gut (Chapco and Kellin, 1994). Therefore the discovery, successful transformation and establishment of these candidate bacteria within the greyback cane beetle larval gut provides good grounding for the future development of paratransgenic control methods of the larvae.

APPLIED PERSPECTIVES

We have discussed the impacts of some abiotic and biotic factors within the soil environment that impact on scarab larval populations. Many agricultural practices interact with these factors within the soil, and could potentially mitigate or exacerbate scarab damage to grasses and crops (Barnett and Johnson, 2013; see **Figure 5** for a summary of key interactions within an applied context).

Scarab larvae have been shown to respond to the application of fertilizers (Wightman, 1974; Frew et al., 2013). However, it is important to note that AMF plant associations can be negatively impacted by fertilization (Smith and Read, 2010). Therefore, the application of NPK fertilizer, particularly to newly establishing crops or pastures should be kept to a minimum, to minimize any positive impacts on scarab populations and to ensure effective AMF colonization to enhance grass productivity and defenses. The addition of mulch, is commonly used to conserve moisture and generally improve soil fertility, and therefore could reduce the priming of plant defenses to herbivores by reducing AMF colonization (Grant et al., 2005; Smith and Read, 2010).

Mulch also affects temperature, which in turn may influence scarab beetle larvae. Different types of mulch have been shown to have different effects on the temperature of the soil (Ramakrishna et al., 2006). For example, polythene mulch has been shown to increase soil temperature by 6°C, while straw mulch also increased soil temperature, but to a lesser extent (Ramakrishna et al., 2006). Contrastingly, a study by Lal (1974) found that mulch consistently decreased the maximum soil temperature across a range of depths (5, 10, and 20 cm), with the biggest difference of 8°C, seen at 5 cm below the soil surface. Tillage is another agricultural practice which has been shown to affect soil temperature (Griffith et al., 1973; Malhi and O'Sullivan, 1990; Licht and Al-Kaisi, 2005). Conventional tillage increases top soil temperatures by 2.8°C compared with no tillage (Malhi and O'Sullivan, 1990), although smaller increases in temperature of 1.9°C have also been reported (Licht and Al-Kaisi, 2005). Higher soil temperatures (depending on climatic conditions) reduce greyback cane beetle populations (Horsfield et al., 2008), and first instar larvae of the dusky pasture scarab have been found to be the most temperature sensitive (Davidson et al., 1972a). However, other common practices such as irrigation are known to lower soil temperatures by up to 3.8°C (Wang et al., 2000).

Taking these effects into account, the timely refrain from irrigation alongside the application of polythene or straw mulch coupled with tillage, for example, could raise soil temperature sufficiently to impact on larval populations. However, limiting soil moisture could decrease the efficacy of EPN populations within the soil at controlling scarab populations. The effects of raising temperatures in this manner on crop health and yield, however, should also be investigated.

The effects of other land management practices on scarab larvae populations have been reported such as the study by Potter et al. (1996) who found that intense mowing of grasses and the addition of aluminum sulfate treatments significantly decreased populations of *Cyclocephala* spp., as well as the average larval mass. This study, however, only was done within one soil type, which is a critical factor (Cherry and Allsopp, 1991; Matthiessen, 1999), and scarab responses may differ under different soils.

Many crops have irrigation systems in place to ensure sufficient water is supplied, which can lead to very different soil conditions compared to natural systems. Mulch, as discussed, is commonly used in agriculture to conserve moisture and increase fertility of soil, and so it naturally follows that in mulched systems, moisture retention of the soil will be higher (Moody et al., 1963; Lal, 1974; Ramakrishna et al., 2006). Host plant location by

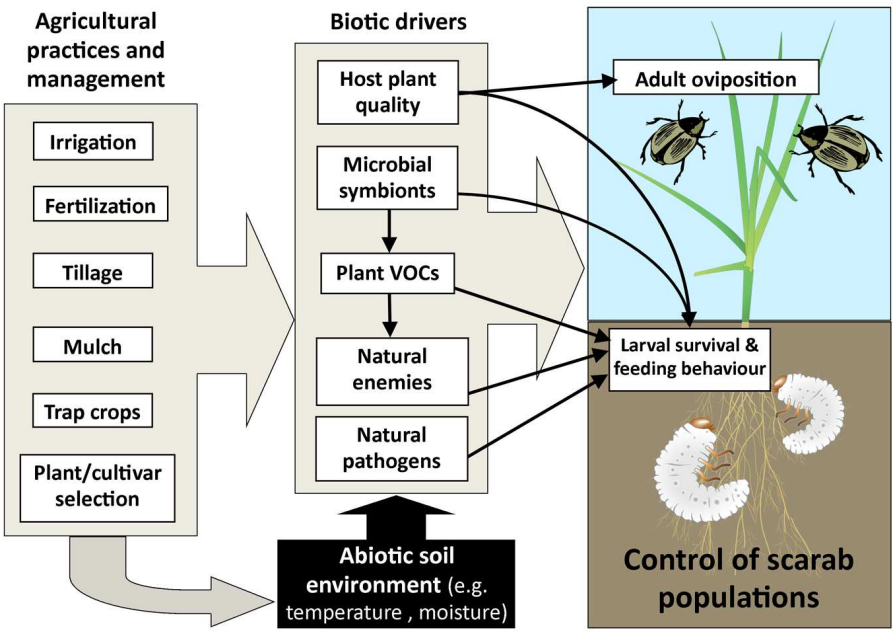


FIGURE 5 | Diagram of agricultural practices and management factors that impact on plant and soil factors (abiotic and biotic), which in turn can influence oviposition by adults together with larval survival and feeding behavior. Arrows indicate key linkages between interacting factors.

larvae beneath the soil surface could be improved under these moist soil conditions due to the fluid dynamics of root exudates (Gouinguené and Turlings, 2002; Hiltpold and Turlings, 2008). However, at the same time, natural enemies such as EPNs will also benefit from this phenomenon as it has been shown across several species that EPN virulence increases with soil moisture content (Kung et al., 1991; Grant and Villani, 2003; Frew et al., 2013). Therefore, as practices such as fertilization may decrease EPN attracting volatiles while irrigation enhances EPN mobility and survival, effective strains of host specific EPNs should be applied to pastures or crops requiring little fertilization alongside ample irrigation to effectively repress scarab larval populations.

Other soil antagonists can be impacted by land use practices. For example, larvae of the scarab *Ataenius spretulus* Haldeman (subfamily: Aphodiinae) were found, within a golf course environment, to be in greater abundance where the turf had been mowed to fairway height (1.6 cm), compared with turf mowed to rough height (5.1 cm). This correlated with the number of larvae found to be infected with a bacterial pathogen, *Bacillus* sp., where 68% of larvae were infected in the turf mowed to rough height, compared to 34% of larvae infected in turf mowed to fairway height. In addition to this, *Anoplognathus* spp. and *Sericesthis* spp. larval populations have been shown to peak under moderate grazing pressure, yet were lowest under high intensity grazing (Roberts and Morton, 1985). These findings alone are unlikely to have a direct applied significance to all scarab larval pest management. However, they may provide critical information for other managed grassland systems, where decreasing regular mowing or allowing high intensity grazing may mitigate larval infestations in future years. Common practices as mowing should be investigated for their impacts on critical soil abiotic factors such as moisture alongside scarab larval populations and their interactions with natural pathogens.

In direct attempts to mitigate damage caused by insect herbivores, the 'push–pull' system is a method which aims to utilize repellant or unattractive plants while simultaneously using attractive yet less valuable plants to attract pests away from valuable crops or pastures (Pickett et al., 2014). A similar system could be utilized against scarab larval pests. For example, where African black beetle populations are problematic, the use of *T. repens* and *N. lolii* infected *L. perenne* could be used as a repellant [the former of which may also be effective against Christmas beetle larvae (Davidson and Roberts, 1968a)], while *L. perenne* and *P. dilatatum* could be utilized within 'trap crops,' particularly as areas with *P. dilatatum* are also preferred sites for oviposition. Indeed, *P. dilatatum* could also be useful, alongside *C. dactylon* in 'trap cultures' for other Dynastinae species such as the Argentine scarab (Carne, 1957a). It has been suggested, however, that the efficacy of 'push–pull' systems would be improved if a better understanding of the mechanisms were obtained, for example the specificity and distance ranges of plant volatile cues (Eigenbrode et al., 2016).

In the end, where effective biocontrol methods are commercially available, these should be employed in conjunction with the use of agricultural and land-use practices, such as irrigation and mowing (where applicable) to create optimal conditions for efficacy and infectivity. Where scarab plant host preferences are known (for feeding or oviposition), these can be employed in 'push–pull' strategies, to limit larval populations in areas of interest. Where either of these are unavailable or remain unknown, such is the case for some of our focal species, timely utilization of certain land-use practices can be applied to create

poor conditions for the scarab populations (e.g., during the first instar, when larvae are most vulnerable to temperature stress). Indeed, in either situation, encouragement of natural beneficial soil microbes (such as AMF) should also be applied. However, as there are gaps in the knowledge for ecology of many scarab species, the direction of future research is of primary importance in improving strategies to limit pest scarab larvae in grasses across Australasia.

DIRECTIONS FOR FUTURE RESEARCH

Basic Ecology

Some of the work on the basic ecology of scarab larval pests to grasses was carried out over 20 years ago (Carne, 1957a; Carne and Chinnick, 1957; Ridsdill-Smith, 1975), with little research on particular species since. It is our belief that for those species where there remains some paucity of knowledge in their basic ecology, feeding trials looking at host preference alongside population monitoring under different conditions (this includes monitoring of abiotic factors and microbial sampling) should be prioritized. With this knowledge, more effective implementation of strategies such as 'push–pull' systems or other agricultural practices that suppress scarab beetle populations can be applied within context. This means management systems could take into account species specific responses, accounting for local abiotic and biotic interactions.

Volatile Cues

The effectiveness of classic pest management strategies such as 'push–pull' systems have recently been criticized, particularly for focusing too much on long-range effects, and should consider all cues that can work synergistically (Eigenbrode et al., 2016). Indeed we would concur with this framework for application to belowground pests, but such behavioral cues would first require investigation. We recommend that future research should investigate olfactory cues of pest larvae and their natural enemies belowground to plant roots, and how these may interact with common agricultural and land-use practices. Experiments such as those carried out by Rasmann et al. (2005) using six-arm olfactometers are an ideal starting point to determine attractiveness of plant species to scarab larval pests and/or their natural enemies.

Pathogens and Microbes

Biocontrol of scarab pests has been particularly successful where a naturally occurring pathogen is identified, isolated and then applied within its naturally occurring range (Maurer et al., 1997; Hurst et al., 2000; Sallam et al., 2003, 2007; Dolci et al., 2006; Sevim et al., 2010). Hence, knowledge of belowground community composition is important if native microbes or EPNs are to be utilized in the control of insect pests in the soil. Using methods similar to that of Sevim et al. (2010), the presence of naturally occurring scarab pathogens could be identified using a baiting method (Zimmermann, 1986). The pathogen can then be isolated from infected larvae and the DNA sequenced; effective isolates can then be used in bioassays to test pathogenicity against the target pest species. We recommend the isolation, identification and ultimately the application of natural pathogens, where possible. The persistence of scarab pathogens in the soil indicates some level of evolutionary success, which should be exploited in efforts to control problematic species.

CONCLUDING REMARKS

Here, we have presented information on several key scarab larval species within three subfamilies, known to cause significant damage to grasslands and crops within Australia and New Zealand. While the ecology of some species has been well researched, information on others, including the Argentine scarab, has not been described in any detail. The feeding behavior and general ecology has been investigated for species such as African black beetle larvae and greyback cane beetle larvae. These pests have had significant attention as a result of their impact on agriculture, and control methods such as the application of natural pathogens, or the application of host plant endophytes have shown noteworthy promise. Although our knowledge is somewhat limited for some species, there is good evidence that changes in management can potentially have a large impact in limiting damage to crops and grasslands. Overall it seems clear that, in terms of improved pest management of scarab larvae, it does not make sense to run before we can walk. Immediate research concerns should lie with filling knowledge gaps in the ecology of scarab species within Australasia. This should include assessing population dynamics, interactions and influences with abiotic factors within the local environment. In addition to this, successful biocontrol strategies, both within and outside Australasia, have utilized naturally occurring pathogens and natural enemies, which are adapted to their host and local environment. Therefore, similar strategies need to be central to future biocontrol research on Australasian scarab pests. This will necessitate multi-factorial studies to investigate how best to integrate these antagonists under different abiotic conditions. Overall, pest management strategies that are applied within context would be more effective with an improved fundamental ecological understanding of key scarab pests.

AUTHOR CONTRIBUTIONS

AF wrote the main body of the review, contributing the majority of the intellectual content and concept of the review. KB read the review in detail, giving advice and contributing important intellectual content. KB was responsible for the production of **Figure 3**. MR read the review in detail, giving advice and contributing intellectual content. UN read the review in detail, giving advice and contributing intellectual content. SJ aided in the concept of the review, contributing important intellectual content.

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Dispersal of the Invasive Pasture Pest *Heteronychus arator* into Areas of Low Population Density: Effects of Sex and Season, and Implications for Pest Management

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African black beetle, *Heteronychus arator* (Scarabaeidae), is an exotic pest of pastures in northern New Zealand. Both adults and larvae feed on pasture grasses. Adults disperse by walking (short range) or flying (long range). Dispersal flights are triggered by warm night temperatures in spring and autumn. Short range adult dispersal in search of mates, food or oviposition sites is poorly understood. This study investigated walking activity of *H. arator* adults over three seasons in New Zealand pastures. Adult walking activity was monitored using pitfall traps along fence lines and in pasture plots on a dairy farm in Waikato, New Zealand, in spring 2013, spring 2014, and autumn 2015. Beetle populations were reduced by application of a biopesticide bait to compare walking activity between treated and control plots for up to 26 days post-treatment. Marked beetles were released into the pasture plots to measure the distance traveled by recaptured individuals. Trap catches along the fence lines were correlated with air temperatures in 2013. Trap catches were male biased in spring 2014 compared with autumn 2015. Trap numbers in the control plots were nearly double that of treated plots in both seasons. More beetles were caught in the pitfall traps at the edges of the treated plots than in the center. Trap catches were consistent throughout the control plot in spring 2014, but in autumn 2015 more beetles were caught in the center of the control plot than at the edges. Few marked beetles were recaptured with dispersal rates estimated as <0.5 m per day. Warmer temperatures encouraged short range dispersal in *H. arator*. Males were more active than females during the spring mating season. Edge effects were strong and should be considered in the design of field experiments.

Keywords: black beetle, insect dispersal, mark–release–recapture, pitfall traps, ryegrass

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INTRODUCTION

The African black beetle, *Heteronychus arator* (Scarabaeidae), was first discovered in New Zealand in 1937 (Chapman, 1984). The distribution of this subtropical species in New Zealand is limited by climate (Watson, 1979) to Northland, Waikato, Bay of Plenty and coastal areas of the northern North Island (Bell et al., 2011). It has become a major pest of pastures and maize crops in northern

New Zealand and is also a pasture pest in parts of Australia (Bulinski et al., 2006). In both countries, *H. arator* overwinters as an adult with mating and oviposition taking place in spring. Larvae are present during summer followed by pupation and adult emergence in autumn (Chapman, 1984; Matthiessen and Ridsdill-Smith, 1991). Adults feed on plant stems and roots just below the soil surface, while larvae feed below ground on detritus and plant roots, with third instar larvae the most damaging life stage (Ball et al., 1997). *H. arator* also damages horticultural crops such as potato (Matthiessen and Learmonth, 1995) and even eucalypt seedlings (Bulinski et al., 2006).

The primary strategy for controlling *H. arator* in New Zealand pastures is sowing ryegrass varieties with associated endophytes that confer resistance to herbivory (Thom et al., 2014). These varieties limit *H. arator* populations, but outbreaks still occur, particularly during warmer La Niña years (Bell et al., 2011; Gerard et al., 2013). No insecticides are registered for use against *H. arator* in New Zealand pastures, and results of insecticide experiments in Australia and New Zealand have been mixed (Matthiessen and Learmonth, 1995; Bulinski and Matthiessen, 2002; Bulinski et al., 2006; Eden et al., 2011). A common outcome for experiments conducted in open field plots has been short-term *H. arator* mortality immediately after insecticide treatment, but no associated reduction in damage and/or no reduction in subsequent populations. Two possible explanations have been proposed: The first is short insecticide persistence and difficulty achieving contact between the insecticide and beetle adults or larvae. The second is *H. arator* re-colonization of treated plots from surrounding areas (Matthiessen and Learmonth, 1995; Bulinski and Matthiessen, 2002; Eden et al., 2011). This study focused on *H. arator* dispersal.

Adult *H. arator* disperse by flying and walking. Dispersal flights occur primarily in autumn when the beetles are reproductively immature (Watson, 1979; Matthiessen and Learmonth, 1998; Hardwick, 2004). Newly sown or renovated pastures are colonized by dispersal flights (Hardwick, 2004) and flights are probably most important for dispersal between paddocks and farms. Walking is, however, more relevant for field plot experiments, which are usually on a smaller spatial scale than whole paddocks. For example, adults walk to aggregate around patches of favored food plants within paddocks (King et al., 1981).

To understand short range dispersal of *H. arator* requires measurement of population density and adult activity. Population density can be measured by sampling either cores or spade squares of soil to count the number of individuals and converting them to numbers/m^2 (Watson et al., 1980). When *H. arator* densities are high (>50 adults/m^2), this method is sufficiently sensitive to measure population responses to treatments, but when populations are low (typically 0–25 adults/m^2; Gerard et al., 2013), impractically high sample numbers are required to assess treatment effects within field plot experiments. Pitfall traps are commonly used to measure dispersal and spatial patterns in populations of ground dwelling beetles (Matthiessen and Learmonth, 1998; Noronha and Cloutier, 1999; Negro et al., 2008; Elek et al., 2014). Pitfall traps also estimate relative density, but should be interpreted cautiously because the number of traps and their positions will affect capture rates (Winder, 2004). Mark–release–recapture (MRR) is another technique used to study dispersal in ground dwelling beetles (Klingenberg et al., 2010; Elek et al., 2014) that has not been tried for *H. arator* in New Zealand. Here, we use pitfall traps to investigate (1) the relationship between adult *H. arator* walking activity and temperature, (2) movement of adults into field plots after treatment with a biopesticide, and (3) distances traveled by individuals using MRR. A greater understanding of dispersal behavior in *H. arator* will assist with design and interpretation of field plot experiments so that treatment effects can be detected reliably.

MATERIALS AND METHODS

Study Site and Trap Design

All experiments were conducted near Gordonton, New Zealand (−37.58, 175.28) using nine paddocks at a dairy farm with permanent pastures and consistently high populations of *H. arator*. The paddocks comprised mixtures of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) and were generally similar in terrain except that a runway for small aircraft was located in one paddock. Pitfall traps for all experiments consisted of plastic pots (approximately 100 mm diameter and depth), dug into the soil so the top of the cup was flush with the surface. Drainage holes (5 mm diameter) were drilled in the bottom of each pot and a small quantity of soil was put in the base of each pot to provide a refuge for trapped beetles.

Temperature and African Black Beetle Activity (Spring 2013)

On August 30, 2013, five fence lines were selected adjacent to eight paddocks known to be infested with *H. arator*, and 20 pitfall traps were placed along each of the fence lines at 3–5 m intervals (trap lines 1–5). Placement of traps adjacent to the fences minimized the risk of damage from grazing livestock. Beetles were removed and counted from traps twice each week until November 13, 2013 (total of 21 sampling occasions). The mean number of beetles caught per trap for each fence line was then calculated for each sampling interval. Trap catch was $\log_e$-transformed prior to analysis. Daily temperature data collected at the nearest weather station (Ruakura Research Centre, approximately 14 km away from the study site) was obtained from the National Climate Database via the NIWA CliFlo website (NIWA, 2015). Daily temperatures were averaged across each sampling interval before analysis. The relationship between trap catches and maximum (Tmax), minimum (Tmin), and mean (Tmean) air temperatures, and minimum grass temperature (Tgrass), was then investigated using separate non-linear regressions (Minitab v.16). This avoided problems with collinearity between the temperature variables.

African Black Beetle Dispersal into Areas of Low Density (Spring 2014 and Autumn 2015)

The spring trial ran from October 24 to November 17, 2014 and the autumn trial from April 15 to May 11, 2015 and the paddocks were grazed just before treatment application. Different paddocks were selected in spring and autumn to ensure sufficiently high African black beetle densities for detection of differences between the control and treated plots. In spring both plots were distanced from the paddock edges on all sides so that each plot had a similar area of surrounding untreated paddock. In autumn the control plot was approximately 5 m away from the paddock boundary on two sides, and was 10 m away from the treated plot. This plot placement was to avoid a microlight aircraft runway through the center of the paddock.

In both trials, two 40 × 40 m open field plots were established side by side and 10 m apart. In each plot, a 10 × 10 m grid of pitfall traps was set up and traps in each grid were classified as corner, edge, or center (**Figure 1A**). Traps were protected by wire crates (45 × 36 × 23 cm) to stop birds raiding them (**Figure 1B**), and were emptied twice weekly for 24 days (spring) or 26 days (autumn) after treatment. One plot was the control and the other was treated with a prototype biopesticide bait to reduce beetle numbers (Hurst et al., 2011a,b; Hurst and Swaminathan, 2016) at a rate of 70 kg bait per ha, which equated to 11.2 kg bait per plot. Baits were distributed by hand evenly throughout the treated plot in each season.

All trapped beetles were taken back to the Ruakura Research Centre to be counted, sexed and checked for markers (see section 2.4). The sex ratio of the trapped beetles was compared using a χ^2 test (Sigmaplot v.13). The number of beetles trapped was compared between treated and control plots, and between the three trap locations, using a generalised estimating equations (GEE) approach with a factor-specific negative binomial distribution and a log link function. The three factors were: treatment (treated or control), days after treatment (for seven sample dates) and trap location (corner, edge, or center). Two-way interactions between factors were included in the model: treatment*day, treatment*trap location, and day*trap location. The GEE analysis used a first order autoregressive covariance structure to account for correlation among the seven sample dates (i.e., repeated measurements). This analysis was conducted using SAS v.9.3.

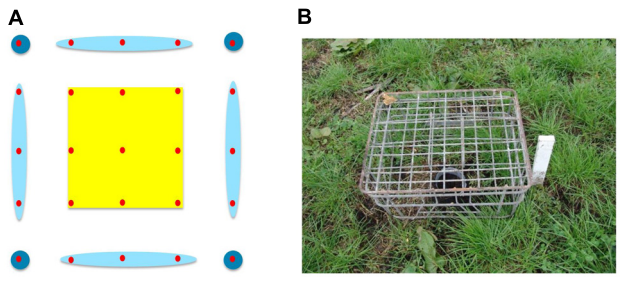


FIGURE 1 | (A) Spatial grouping of the 25 pitfall traps (red circles) within each plot. The center (yellow) includes nine traps, the edges (light blue) had 12 traps, and there were four corner traps (dark blue); **(B)** A pitfall trap in the paddock protected from damage by birds with a wire crate.

Mark–Release–Recapture (Spring 2014 and Autumn 2015)

In addition to the grid of pitfall traps, a MRR study was carried out. The intention was to measure the distances traveled by individual beetles to help interpret the general activity measured by trap captures across the plots. In spring live beetles ($n = 768$) were collected from pitfall traps placed along the fence lines. The day before the trial was set up, the beetles were divided into eight equal groups ($n = 96$); each group marked with a unique color (a patch of nail varnish on top of the prothorax). Each color group was assigned to one of four 20 × 20 m quadrants within each of the two 40 × 40 m plots (**Figure 2A**), and 24 beetles were released at each of four locations within each quadrant. Marked beetles were released by hand after the bait was applied to the treated plot. Beetles collected from the 10 × 10 m grid of traps were checked for color marks and their locations recorded so that the minimum distance traveled by individual beetles could be calculated.

The MRR study was repeated in autumn with some modifications, again using beetles collected from pitfall traps placed along paddock fence lines. We had observed deterioration of the nail varnish marks over time so there was concern that the lower than expected spring recapture rate [see Mark–Release–Recapture (Spring 2014 and Autumn 2015)] was due to mark loss as the beetles burrowed through soil. In autumn nail varnish was replaced with queen bee markers (small disks of colored plastic, Australian Entomological Supplies) that were glued to the beetles. Marked beetles were also released only in the control plot and in the buffer zone between plots, not in the treated plot ($n = 96$ beetles for each color group, **Figure 2B**) as there was limited availability of beetles.

The minimum distance traveled by each recaptured beetle in spring and autumn was calculated based on the distance between the trap where the marked beetle was captured and the nearest release point for beetles with that color mark. The sex ratio of marked beetles was 50:50 in both spring and autumn.

RESULTS

Temperature and African Black Beetle Activity (Spring 2013)

Average trap catches varied noticeably during spring (**Figure 3**), ranging from <0.5 beetles per trap at the earliest sample dates to >3 beetles per trap on some dates in October. Peak catches were generally seen on October 24, although trap line 5 had been destroyed by calves on this date (the only mishap of note). The relationship between trap catch and temperature took the form:

$$\log_e \text{catch} = a - \exp(-b * \text{temperature} + c)$$

for each temperature variable examined (**Table 1**). This meant that trap catches increased toward an asymptote with increasing

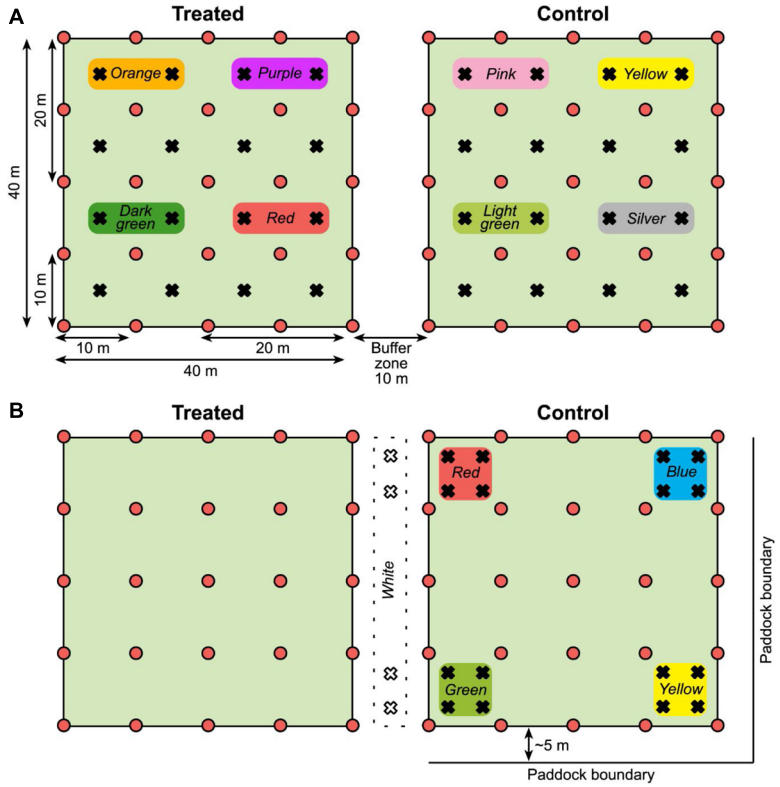


FIGURE 2 | (A) Plot layout for the spring 2014 MRR experiment (not to scale). Pitfall traps shown as red circles and release points for marked beetles as black crosses. Color markers used on beetles released in each quadrant are shown. Both plots were in the middle of the paddock, distanced from the boundaries. **(B)** Plot layout for the autumn 2015 MRR experiment. Symbols and colors as stated for the spring; in autumn the control plot was approximately 5 m from the paddock boundary on two sides. Note that marked beetles were released only in the control plot and the buffer zone between plots in autumn.

temperatures (**Figure 4**). Of the four temperature variables, the relationship with Tmax gave the closest fit to the data, although all four variables had a significant relationship with trap catch (**Table 1**).

African Black Beetle Dispersal into Areas of Low Density (Spring 2014 and Autumn 2015)

Spring 2014

More than 1100 beetles were caught in spring and the sex ratio was about 2:1 male:female ($P = 0.01$, **Table 2**). More beetles were caught in the control than the treated plots ($P < 0.0001$) and this treatment effect was consistent at all sample dates except the first (day 4 after treatment, $P = 0.76$; all other days, $P \leq 0.003$; **Figure 5A**). There were strong spatial effects on trap catches but these effects differed between treated and control plots (significant treatment*trap location interaction, $P < 0.0001$). The numbers of beetles caught in the treated plot declined from corner to edge to center but the control plot showed no significant spatial effects (**Figure 6A**). Corner trap catches were similar in treated and control plots ($P = 0.28$) but edge and center trap catches were lower in the treated plot than the control ($P < 0.0001$ for both comparisons). These spatial trends became apparent in the treated plot from the second sample date onward (day 7 after treatment, significant trap location*day interaction, $P < 0.0001$, **Figure 7**).

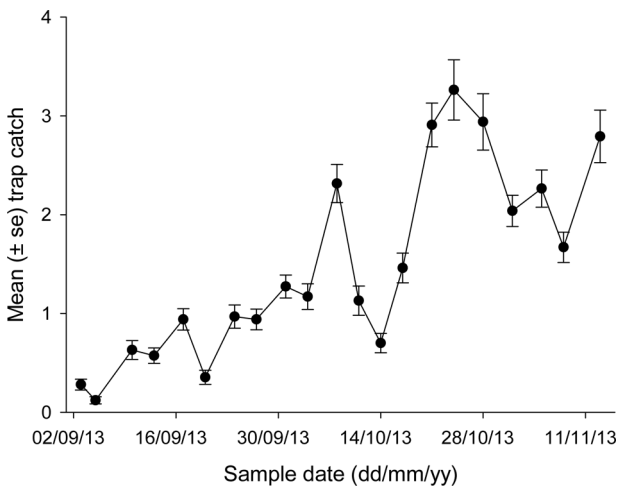


FIGURE 3 | Mean trap catch of African black beetles at each sample date for all five trap lines in spring 2013.

TABLE 1 | Non-linear regressions to estimate the relationships between mean trap catch and the four temperature variables.

Variable	Regression equation, $y = \log_e$catch, x = temperature	*P*	Variation (%) explained by model
Tmax	$y = 0.7 - \exp(-0.7^*x + 12.7)$	<0.001	60.1
Tmin	$y = 0.3 - \exp(-0.7^*x + 2.4)$	0.004	40.6
Tmean	$y = 0.5 - \exp(-0.4^*x + 6.0)$	0.01	40.2
Tgrass	$y = 0.3 - \exp(-0.4^*x - 0.1)$	0.01	18.4

All relationships were statistically significant ($P < 0.05$).

Autumn 2015

Fewer than 500 beetles were caught in autumn and the sex ratio was about 1:1 male:female (**Table 2**). More beetles were caught in the control than the treated plots ($P < 0.001$) but the treatment effect was less consistent across sample dates (days 9 and 16 after treatment, $P \leq 0.01$; days 2 and 13 after treatment, $P \leq 0.09$; all other days $P \geq 0.1$, **Figure 5B**). Again, there were strong spatial effects on trap catches that differed between treated and control plots (significant treatment*trap location interaction, $P = 0.0006$). The numbers of beetles caught in the treated plot tended to decline from corner to edge to center although only the corner traps captured significantly more beetles than the center (**Figure 6B**). There was an unexpectedly strong spatial effect in the control plot that was opposite to the pattern of the treated plot, i.e., trap catch increased from the corner to edge to center (**Figure 6B**). To compare spatial patterns between the two plots: more beetles were caught in the center of the control plot than the treated plot ($P < 0.0001$), similar numbers were caught in the edge traps of both plots ($P = 0.35$) and fewer beetles were caught in the corner traps of the control plot than the treated plot ($P = 0.015$). Spatial trends became apparent in the treated plot from the third sample date onward (day 9 after treatment,

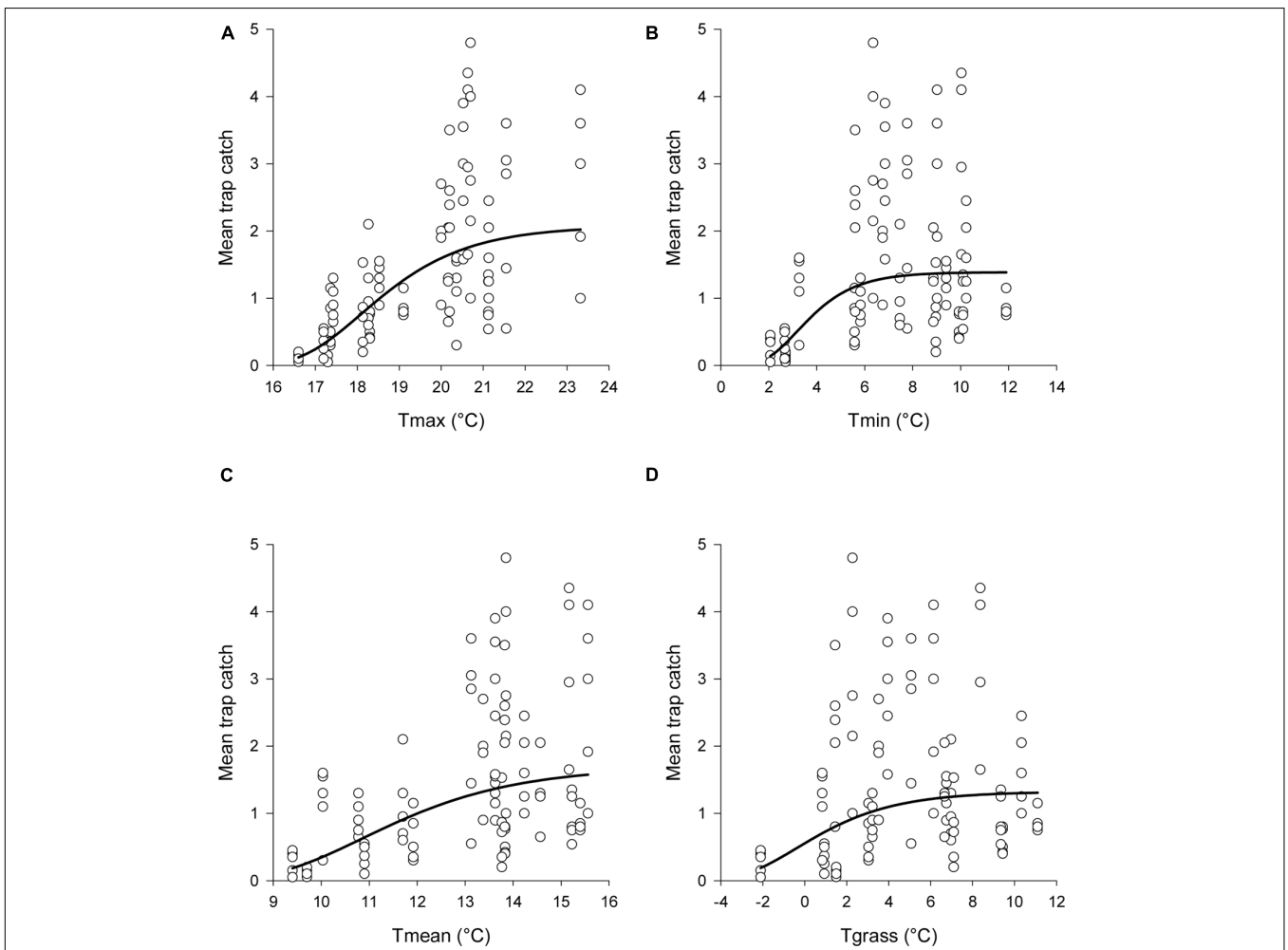


FIGURE 4 | Observed data and estimated relationships between mean trap catch and (A) Tmax, (B) Tmin, (C) Tmean, and (D) Tgrass. Daily temperatures were averaged across each sampling interval. The non-linear regression model was: $\log_e\text{catch} = a - \exp(-b^*\text{temperature} + c)$. Estimated catches from the regressions were back transformed before graphing. Note that the *x*-axis scale differs between graphs.

TABLE 2 | Total number of male and female beetles caught in the control and treated plots in spring 2014 and autumn 2015.

	Spring 2014			Autumn 2015		
Plot type	**Male**	**Female**	**Total**	**Male**	**Female**	**Total**
Treated	256	136	392	104	88	192
Control	470	292	762	166	136	302
Total	726	428	1154	270	224	494

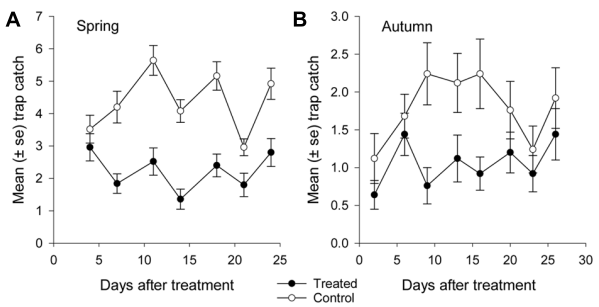


FIGURE 5 | Mean catch of African black beetles per pitfall trap in the treated and control plots in (A) spring 2014 (4–24 days after treatment) and (B) autumn 2015 (2–26 days after treatment).

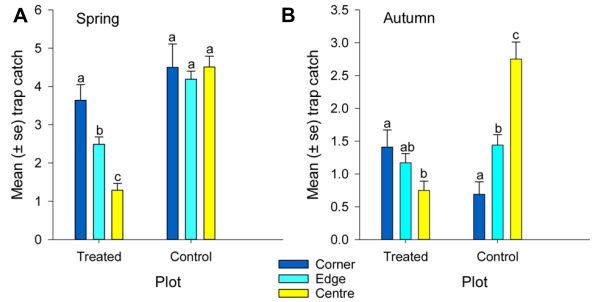


FIGURE 6 | Mean catch of African black beetles per pitfall trap from corner to edge to center of the treated and control plots in (A) spring 2014 and (B) autumn 2015. Bars from the same plot marked with the same letter are not significantly different ($P > 0.05$).

significant trap location*day interaction, $P < 0.0001$, **Figure 8A**) but were seen in the control plot throughout the sampling period (**Figure 8B**).

Mark–Release–Recapture (Spring 2014 and Autumn 2015)

Only marked males were recaptured in both spring and autumn (**Table 3**). In spring 11 marked beetles were recaptured (1.4% of marked beetles), including 1–2 individuals from each color group except silver, so recaptures were evenly spread across the two plots. The first marked beetle was collected 11 days after treatment and six marked beetles were collected on the last sample date, 24 days after treatment. The males were moving 0.45 ± 0.05 m/day (mean ± SE) in spring when the time elapsed between the release date and recapture dates is taken into account. Just one beetle with a blue marker was recaptured in autumn 23 days after treatment and approximately 12.8 m from its release point (= 0.55 m/day). No further analysis was attempted due to the low recapture rate in both seasons.

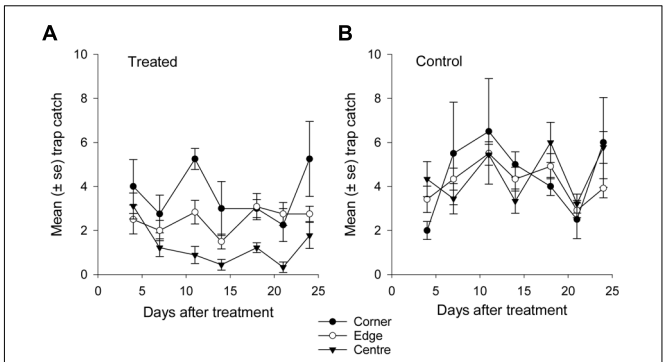


FIGURE 7 | Mean catch of African black beetles per pitfall trap in spring 2014 from corner to edge to center for 4–24 days after treatment in (A) treated and (B) control plots.

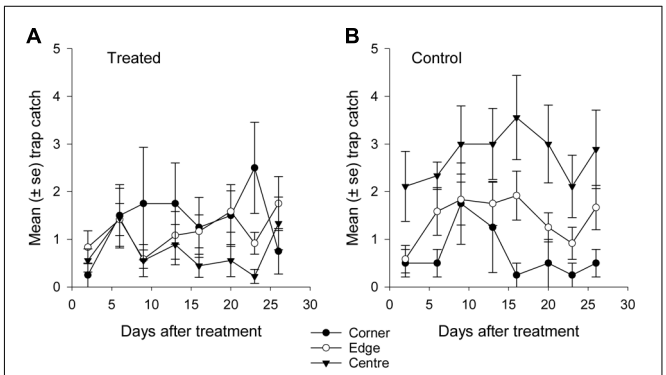


FIGURE 8 | Mean catch of African black beetles per pitfall trap in autumn 2015 from corner to edge to center for 2–26 days after treatment in (A) treated and (B) control plots.

TABLE 3 | Marked beetles recaptured in spring 2014.

Plot type	**Color mark**	**Days until recapture**	**Minimum distance traveled (m)**	**Daily distance (m)**
Treated	Dark green	14	7.07	0.51
	Purple	18	7.07	0.39
	Purple	21	15.81	0.75
	Orange	24	7.07	0.29
	Dark green	24	7.07	0.29
	Red	24	15.81	0.66
Control	Pink	11	7.07	0.64
	Yellow	14	7.07	0.51
	Yellow	24	7.07	0.29
	Light green	24	7.07	0.29
	Light green	24	7.07	0.29

The minimum distance traveled was calculated as the shortest route from release point to recapture point for each color mark.

DISCUSSION

Warmer temperatures increased walking by adult *H. arator* in Waikato and led to higher capture rates in pitfall traps, up to a maximum that presumably reflected the background population density and the catchment area of individual traps. Adult activity was also positively correlated with temperature in Tanzania (Abdallah et al., 2016). Higher surface activity relative to flight activity occurred in spring in Western Australia, but the reverse occurred in autumn (Matthiessen and Learmonth, 1998). Temperature variability will affect capture rates when monitoring field plot trials and probably accounts for some of the variation between sample dates seen here. While such variation is unlikely to change overall treatment effects, awareness of the prevailing weather conditions will assist interpretation of pitfall trap data for this pest.

Males were more active in spring, presumably while searching for mates, shifting to an approximately equal sex ratio in autumn when captured beetles are reproductively immature. Greater male activity was also seen in Australia during spring, but autumn catches were female-biased (Matthiessen and Learmonth, 1998). The reason for this difference in autumn captures is unclear. One possibility is the timing of the respective studies relative to the annual life cycle of *H. arator*. The Australian study continued trapping throughout most of the year. Perhaps trap catches in New Zealand shift to female dominated in late autumn, after this experiment ended.

The recapture rate for marked beetles was very low but did support the finding that males are more active than females in spring. A recent study used MRR on adult *H. arator* in Tanzanian maize crops with higher release numbers and achieved higher recapture rates, although recapture declined with increasing distance from the release point (Abdallah et al., 2016). The number of pitfall traps used was not reported, however, and the trap spacing did not follow a grid pattern so direct comparisons cannot be made easily between the two experiments. A longer trapping period after release may have increased recapture rates in this study, but the paddock was needed for grazing. A shorter distance between traps is also likely to increase recapture rates if MRR is used with *H. arator* again in New Zealand pastures.

The spatial analysis of trap catches suggested that adult *H. arator* were moving into the treated plot from the surrounding untreated area in both spring and autumn. Matthiessen (1999) inferred that adult beetles disperse in autumn from areas of high to low population density, leading to uniform spatial distributions for overwintering populations. Adult dispersal from untreated areas into treated field plots is a significant factor contributing to the failure of 'pulse' effect controls, e.g., insecticides (Eden et al., 2011). In contrast, open field plot experiments using endophyte varieties and similar plot sizes do report significant ongoing effects on *H. arator* populations (Thom et al., 2014; Barker et al., 2015). Endophytes provide (near) continuous expression of deterrent compounds, discouraging dispersal from untreated areas.

The most likely cause of spatial effects seen in the control plot in autumn was the placement of plots close to the paddock boundaries [as described in African Black Beetle Dispersal into Areas of Low Density (Spring 2014 and Autumn 2015)]. The control plot had the smallest area of surrounding paddock to act as a source of beetles compared to the treated plot in autumn and to both plots in spring. This demonstrates that placement of field plots relative to paddock boundaries and other landscape features can affect beetle movement.

Pitfall traps provide a measure of adult *H. arator* activity and therefore should be a useful tool to ensure optimal timing and greater efficacy of 'pulse' effect treatments used to protect pastures or crops. To prevent pasture damage from larvae over summer, treatments need to target African black beetle adults in spring before substantial oviposition occurs. For example, our results indicate late October would have been the optimum time for treatments to protect vulnerable new pastures in the study district. Pitfall traps also demonstrate spatial effects in field plots, and are an effective method for studying ground dispersal in this species, similar to other ground dwelling beetles (Noronha and Cloutier, 1999; Elek et al., 2014). For future insecticide experiments with *H. arator* larger plot sizes, more frequent monitoring after treatment, and consideration of spatial effects in the sampling design are all recommended so that treatment effects can be distinguished effectively from spatial effects due to beetle dispersal.

AUTHOR CONTRIBUTIONS

SM and PG designed the experiments; MH led the biopesticide development; SM, PG, RT, and DW set up the field experiments with DW and RT responsible for ongoing monitoring and data collection. CK analyzed the data and all authors contributed to data interpretation and writing.

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The reviewer AF 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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Novel *In vitro* Procedures for Rearing a Root-Feeding Pest (*Heteronychus arator*) of Grasslands

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Optimizing plant protection against insect herbivory relies on testing plant defense mechanisms and how the insect response to these defensive strategies. Such experiments benefit from using insects generated from standardized rearing protocols since this reduces stochastic variation. Such protocols can be challenging to devise, however, especially for root herbivores. These insects generally have complex and long life cycles, which are often only poorly described. Moreover, using field-captured root herbivores is often suboptimal because it involves extensive excavation from sites selected by chance (their location is not obvious) and larval stages are frequently indistinguishable beyond the family level. We investigated *in vitro* procedures to improve the availability of the African Black Beetle (ABB) *Heteronychus arator*, an invasive alien pest in both Australia and New Zealand. Native to Africa, this scarab beetle has established in Australian and New Zealand grasslands, pastures, and crops. Adults feed on the stem of young plants just beneath the soil surface. During the mating season, gravid females lay eggs in the soil, giving rise to larvae feeding on grass roots, causing severe damage, and impairing plant growth. Here, we propose laboratory approaches to collect eggs from field-captured adult beetles, to hatch eggs, and to rear neonate larvae to adults. We propose that these methods will provide plant scientists and entomologists with a better and more controlled supply of ABB larvae for laboratory and field assays. In turn, this will assist with the collection of important information for the management of this insect pest and enhanced protection of plants in crop and grassland ecosystems.

Keywords: grasses, insect rearing, plant pests, root herbivores, soil pests

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INTRODUCTION

Terrestrial plants can allocate up to 90% of their biomass to the production of belowground structures (root, rhizomes, and storage organs; Blossey and Hunt-Joshi, 2003). Insect herbivores, native or invasive, feeding on belowground plant organs not only affect net ecosystem primary productivity but also plant physiology, function, and growth (Brown and Gange, 1990; Blossey and Hunt-Joshi, 2003; Van Der Putten, 2003; Stein et al., 2010). Emerging evidence suggests that plants respond very differently to attacks above- and below-ground, since the nature of damage to the plant is very different (Johnson et al., 2016). Research has been hampered by the cryptic habitats of below-ground herbivores, but recent progress in various techniques such as X-ray tomography (Johnson et al., 2004), spectrometry (e.g., Rostas et al., 2015), and isotopic diet labeling (e.g., Traugott et al., 2008; Hiltpold et al., 2014) have allowed plant scientists and entomologists

to improve their knowledge and understanding of root herbivory and the ecological impact of soil-dwelling insects on ecosystems (Johnson et al., 2013).

Plants suffer excessively from belowground herbivory as root damage can result in (i) a decrease in nutrient and water uptake (e.g., Riedell, 1990; Hou et al., 1997), (ii) disproportionate resource losses (Johnson et al., 2016), (iii) diversion of assimilates away from shoot growth for the re-growth of below-ground structures (e.g., Soler et al., 2012; Zvereva and Kozlov, 2012), (iv) increased susceptibility to water stress (e.g., Gange and Brown, 1989), and (v) reduced mycorrhizal association (Bennett et al., 2013) and increased infection by root pathogens (van Dam, 2009). In grasslands, primary productivity losses to root herbivory can be up to 25% (Seastedt and Murray, 2008), and this is often due to scarab beetle larvae. For instance, it is estimated that the collective biomass of soil-dwelling scarab larvae pests is equivalent to or even exceeds that of sheep in some Australian pastures (Britton, 1978).

Research into plant defense of belowground structures has been hampered by the lack of a suitable model insect root herbivore for experimental work. An ideal model organism should (i) be representative of a broader group of organisms (in this case, insect root herbivores), (ii) be amenable to experimental manipulation, and (iii) be available at a reasonable cost. The greatest obstacle in developing an insect root herbivore model in grassland ecosystems at present is availability. Reliable, standardized methods for rearing grassland root herbivores in sufficient numbers are not available. Excavation in the field is very laborious as root herbivores generally exhibit patchy distributions (Frew et al., 2016) rendering the localisation of infested sites and collection of larvae in the field laborious and time consuming. Plant protection research would greatly benefit from a large, uniform, and predictable supply of insects of all life stages throughout the year (e.g., Fisher and Bruck, 2004).

Here, we investigate procedures for capturing, maintaining and rearing the African Black beetle (ABB) *Heteronychus arator* Fabricius (Coleoptera: Scarabaeidae). ABB is a scarab considered as a major pest of grasslands, pastures, turf, and agriculture in the Southern hemisphere. Known as the Black beetle in Africa, it was accidentally introduced to Australia [first record in 1938, Matthiessen and Ridsdill-Smith (1991) and New Zealand (Todd, 1959)] where it became a major, albeit sporadic, pest in pastures (Todd, 1959; King and Watson, 1982).

In Australasia, ABB is a univoltine pest spending most of its lifespan below-ground. Adults emerge in the last months of summer (February–March) and become sexually mature in spring (September–November). During this period, ABB flies above-ground to mate and select appropriate oviposition sites. Oviposition site selection is influenced by host preference for certain grass species (i.e., *Paspalum dilatatum* Poir., *Lolium perenne* L.; King et al., 1981a). Eggs laid in the ground start hatching in late spring–early summer (November–December; Jenkins, 1965; Mercer and King, 1976; King et al., 1981c; Matthiessen and Ridsdill-Smith, 1991). Larvae feed on decaying organic matter and roots of grasses (King, 1977), rendering them more susceptible to pathogens, drought events and pulling by grazing vertebrate herbivores. ABB adults also feed on plant tissues and can cause significant damage by feeding on the bases of grass tillers (Watson and Marsden, 1982), crop plants (Jenkins, 1965), and tree seedlings (Loch and Floyd, 2001). In Australia, ABB is known to feed on over 190 cultivated grass species in 33 genera (Hangay and Zborowski, 2010), potentially making it a good model for plant scientists. In addition, it is a medium size scarab thus representative of a large proportion of insect root herbivores. Finally, Australasian populations likely arose from limited introductions, resulting in a fairly genetically uniform meta-population. More details on the ecology of this insect are discussed in Frew et al. (2016).

Probably because beetles are easier to identify, collect and are available over a longer period of time, most studies on the impact of ABB on plant biology have been conducted with adults (e.g., Sutherland and Greenfield, 1978; Russell et al., 1982; Matthiessen and Learmonth, 1998; Popay and Baltus, 2001) in various agricultural ecosystems (e.g., Matthiessen and Learmonth, 1998; Loch and Floyd, 2001). However, only few studies have looked at larval ABB behavior and its impact on plants (e.g., Sutherland and Hillier, 1974; Sutherland and Greenfield, 1978; King et al., 1981a), probably because the hidden and patchy distribution of this pest. Here, we present a set of techniques to mass collect eggs from field-trapped ABB beetles and describe comprehensive rearing methods to obtain each developmental instar of the insect, from egg to adult.

MATERIALS AND METHODS

Field Trapping

In 2014 and 2015, two campaigns were undertaken to trap adult ABB in the field, to establish laboratory colonies. Light traps (Supplementary Figure S1, Australian Entomological Supplies Pty. Ltd., Coorabell, NSW, Australia) were placed in a pasture typical of those used for grazing in the Sydney region, located at the Hawkesbury Campus site (Western Sydney University, Hawkesbury Campus, Richmond, NSW, Australia). Each trap consisted of two sections. First, a white plastic container (230 mm height, 260 mm inner diameter), fitted with a funnel (160 mm height, 260 mm outer diameter, 30 mm funnel neck inner diameter), which was placed on the ground. Second, a vertically oriented 12 V 8 W black light fluorescent tube (Hitachi Group, Japan) attached to three clear plastic vanes (370 mm × 110 mm) cut to fit the funnel. The fluorescent tube was connected to a light-sensitive switch (Australian Entomological Supplies Pty. Ltd., Coorabell, NSW, Australia), which automatically activated the trap at dusk and deactivated it at dawn. The switch was connected to a rechargeable 12 V 7 Ah battery (CP1270EB battery, Vision Group, China).

In 2014, 2–3 traps were set up nightly from September 25th to December 12th. In 2015, insects were trapped from September 14th to December 23rd. During both campaigns, captured insects were collected every morning and transferred to the laboratory. Traps were not deployed on nights with heavy rainfall or strong wind.

Colony Maintenance

Adult ABB recovered from the field were first placed on a 500 μm sieve (Impact Test Equipment Ltd., UK), and rinsed with tap water to remove antagonists (e.g., mites, entomopathogenic nematodes, or fungal spores on the cuticle). They were then counted and released into microcosms (**Figure 1A**, Supplementary Figure S2). Each microcosm consisted of a first container (Maxi Pail 10 L Plastic Pail, 28 cm diameter, 25 cm height, Bunnings Warehouse, NSW, Australia) filled with 5 cm (King et al., 1981b) of 8:2 (w/w) autoclaved soil (Yarramundi Loam, from the site where the beetles were trapped) and water. The bottom of a second container (Maxi Pail 20 L Plastic Pail, 28 cm diameter, 41 cm height, Bunnings Warehouse, NSW, Australia) was removed in four pieces to form a 1 cm wide, 20 cm long cross, holding a wire net (5 cm × 1.0 mm mesh, Whites Group, Australia). This second container was inserted into the first one and filled with 8:2 (w/w) autoclaved potting mix (Oscmocote®, Scotts LLC, USA) and water. The central part of the top container lid was removed and replaced with insect mesh net (Cyclone Insect Screen, Bunnings Warehouse, NSW, Australia) to prevent beetles from escaping. Adult ABB were allowed to fly and move in the microcosm. Beetles were fed with carrots swapped every three days. Dead ABB on the surface of the potting mix were removed. Every other week, all the beetles were taken out the potting mix, sprayed with water to remove antagonists and placed back in the microcosm with new autoclaved substrate. The colonies were maintained in a greenhouse at 22°C, 65% RH, with natural photocycle.

Beetle Oviposition and Egg Collection

Every 3 days, the soil from the bottom container of the microcosm was removed and sieved through stacked 2 mm and 500 μm-sieves (Impact Test Equipment Ltd., UK) with low-pressure water. Eggs of the ABB were retrieved with soft entomological forceps and placed in a Petri dish (10 cm diameter, Greiner Bio-One GmbH, Germany) on moist filter paper (Grade 1 Whatmann™, GE Healthcare Australia, Parramatta, NSW, Australia). The Petri dish was then sealed with Parafilm® (Bemis Inc., USA) and stored in the dark at 6°C until used. Every 2 weeks, dishes were checked for mold and symptomatic eggs discarded. Filter papers were maintained moist.

Egg Hatching Protocol

Similar to the approach of Matthiessen and Learmonth (1998), eggs were hatched in 96-well plates (Greiner Bio-One GmbH, Germany; **Figure 1B**). A small piece of synthetic sponge was fitted into the bottom of the desired number of wells, up to one third of the well depth. Sponges were moistened with 3% sodium hypochlorite solution as a fungicide. The hatching plate was kept in the dark at 22°C. Eggs were monitored daily and kept moist until they hatched. Neonates were transferred to the rearing containers (details in the following section). Cumulative degree-days dd_D for egg hatching were calculated as

$$dd_D = (T_D - THR) + dd_{D-1}$$

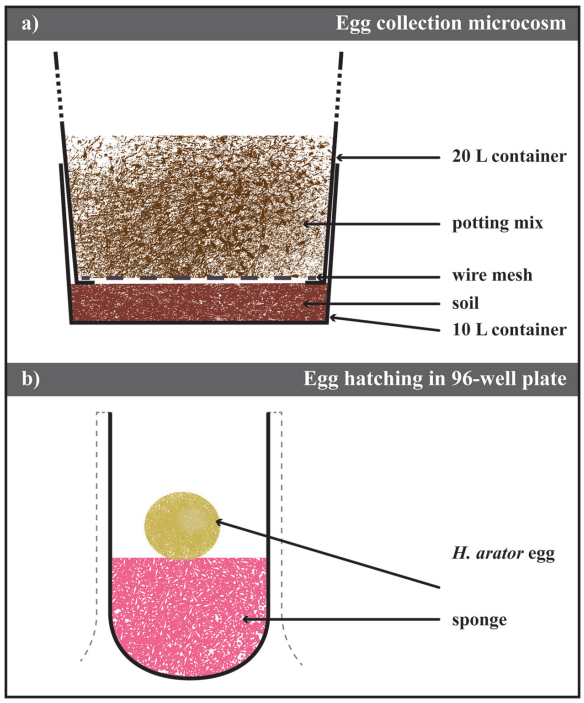


FIGURE 1 | Laboratory maintenance of *Heteronychus arator* population. (a) Schematic drawing of the microsm used to collect eggs. It consisted of two containers inserted one into the other. The bottom of the top container was replace with a metal mesh allowing the insect move to the bottom one containing soil from the field site of the insect collection. Eggs were recovered from this layer of soil after sieving. **(b)** Schematic drawing of the methodology used to hatch eggs. Eggs were laid on a moist piece of sponge and stored until hatching. Details of both protocols in the text.

where T_D was the temperature of the considered day, *THR* the estimated developmental threshold (10°C, King et al., 1981d), and dd_{D-1} the cumulative degree-day of the previous day.

Rearing Protocol

The first kind of rearing container consisted of a 70 ml flat-bottom specimen jar (Techno Plas Pty Ltd, Australia) filled with 8:2 (w/w) autoclaved potting mix (Oscmocote®, Scotts LLC, USA) and water. To avoid competition and larval cannibalism (King et al., 1981c), a maximum of two ABB neonate larvae were placed in each container. The lids of the rearing containers were perforated with four 3 mm holes to allow airflow. The containers were stored at 22°C and emptied onto a sheet of black plastic every week. The survival of the larvae was recorded and live immature insects were placed back in the containers, with new moist potting mix. Fine strips of carrot were provided on top of the potting mix (King et al., 1981d) and changed every other day.

To overcome the low larval survival rates recorded with the technique described above, an alternate, less intrusive approach was tested in 2015. Modified from the methodology to maintain the adult colony in laboratory conditions (see

"Colony Maintenance"), the bottom container was adapted to hold ABB larval instars until their metamorphosis to adults (**Figure 2A**; Supplementary Figure S3). First, 3 mm diameter holes were drilled in the bottom of the container to allow water drainage. Then, a layer of about 3 cm of autoclaved gravel (2 cm < particle size < 3 cm) was covered with 15 cm of 8:2 (w/w) autoclaved soil (Yarramundi Loam, from the site of collection of the beetle) and water. At this stage, the top container (as described in section, Colony Maintenance), containing ABB adults in potting mix, was inserted above the bottom part of the microcosm and beetles were allow to lay eggs in the layer of soil.

Every 2 weeks, the top container with beetles was placed on top of a new bottom container prepared as described above. About 5 cm of autoclaved potting mix was placed in the substituted container holding newly laid eggs (**Figure 2B**; Supplementary Figure S3). In order to provide second and third instar larvae with suitable food (King, 1977; King et al., 1981a), 1 g m^{-2} of long-rotation ryegrass *Lolium multiflorum* Lam. (Poales: Poaceae; cultivar Barberia, Heritage Seeds Pty Ltd, Australia) was sown in the potting mix. This cultivar is free of fungal endophytes harmful to the insect (e.g., Popay and Baltus, 2001). The central part of the container lid was removed and replaced with insect mesh net (Cyclone Insect Screen, Bunnings Warehouse, NSW, Australia) to prevent escape of emerging beetles. The containers were stored at 22°C and regularly watered to ensure there was enough moisture for the larval development (King et al., 1981c) and plant growth; stones at the bottom ensured the drainage of excess water. The presence of emerging ABB beetles was confirmed three times a week and degree-days to emergence were recorded (details provided in section, Egg Hatching Protocol).

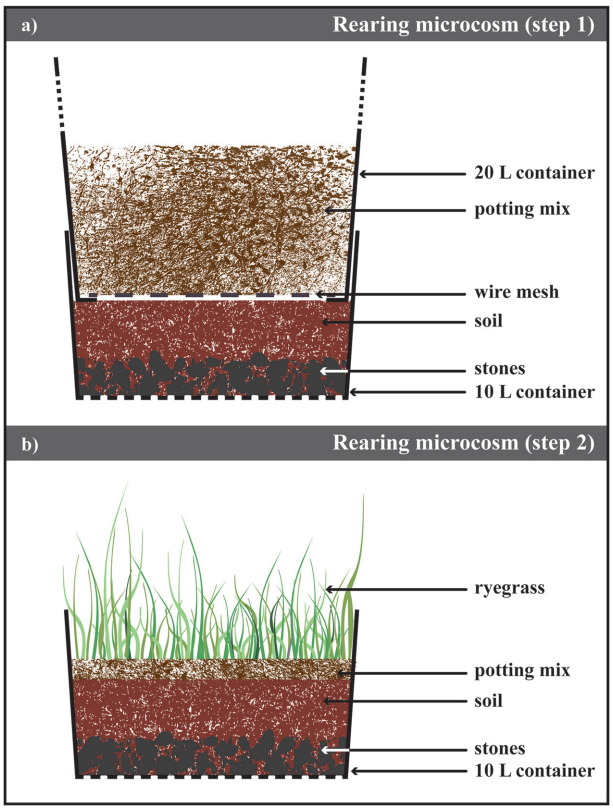


FIGURE 2 | Laboratory rearing of *Heteronychus arator*. (a) Schematic drawing of the microsm used to rear *H. arator* from eggs to beetles. It consisted of two containers inserted one into the other. The bottom of the top container was replace with a metal mesh allowing the beetles move to the bottom one containing soil from the field site of the insect collection and stones to ensure the drainage of the excess water. **(b)** After 2 weeks, the top container was removed and ryegrass sawn on a layer of potting mix. This microcosms were stored until adult emergence.

Statistical Analyses

All statistical tests described below were performed in R (R Development Core Team, 2015). Plots were computed using the function *visreg* ({visreg} package).

Beetle Oviposition and Egg Collection

The relationship between the abundance of beetles in each container and the number of laid eggs was tested by fitting an asymptotic regression to the data (*drm* function, {drc} package). The fitting of the model was tested with a lack-of-fit test (*modelFIT* function, {drc} package).

Egg Hatching Protocol

The effect of storage on egg hatching success was evaluated using generalized linear models (*glm* function, {*stats*} package) with a binomial distribution. The influence of storage on degree-days required for eggs to hatch was tested with the *lm* function ({*stats*} package).

Rearing Protocol

Differences in % beetle emergence between containers were analyzed with a Chi-Square test (*chisq.test* function, {*stats*} package). The impact of timing of egg laying (which varied across containers) on the degree-days required for the beetles to hatch was tested with an ANOVA (*lm* function with "containertID" as factorial descriptor, {*stats*} package) to evaluate any change in egg quality over the ovipostion period.

RESULTS AND DISCUSSION

In 2014, 370 beetles were captured in the field (172 males, 198 females). In the laboratory, a total of 170 beetles were observed in the soil in the bottom containers (where eggs were laid) in the egg-laying experiment. Assuming all of these were female (i.e., the most conservative estimate of fecundity), the minimum beetle fecundity was 19.98 eggs per female (3,398 recovered eggs). This is higher than fecundity levels observed in the field (ca. 12 eggs per female, Matthiessen and Ridsdill-Smith, 1991), which suggests the colony maintenance protocol used was successful. Indeed our assumption that all insects were female suggests that fecundity could well be higher than 19.98 per insect. The number of eggs found in the soil layer was positively correlated with the number of beetles present in that same layer (**Figure 3**,

$F_{17-3}1 = 0.9391$, $p = 0.5416$). Interestingly, the number of recovered eggs seemed to reach an asymptote around 165 eggs indicating that conspecific density might be used as a cue by ABB females to limit their oviposition, possibly to guarantee enough resources to their progeny (**Figure 3**). Such density-dependent fecundity has been observed in other beetle rearing set-ups (Peters and Barbosa, 1977) and should be accounted for while establishing an ABB laboratory colony. Preliminary attempts to collect eggs in the absence of the field soil layer failed, suggesting that ABB females require particular substrate conditions to lay eggs. This could be observed in other beetle species laying eggs in soil [e.g., Flower beetles (Coleoptera: Cetoniinae), McMonigle, 2012] and might be a required step as long as detailed knowledge on the biology and chemical ecology of the insect is not available.

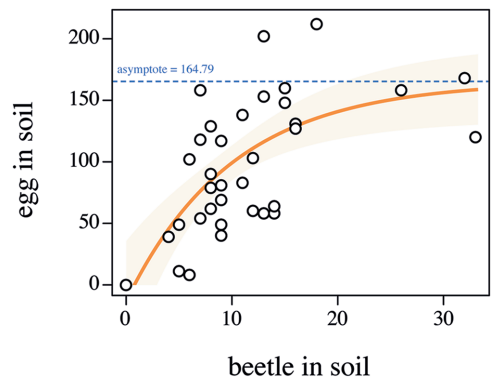


FIGURE 3 | Relationship between the number of *Heteronychus arator* beetles in the soil in the lower section of the microcosm and the number of laid eggs. Open circles indicate the observed data. The light orange zone represents the 95% confidence interval of the fitted model (orange solid line). The dashed blue line represents the model asymptote.

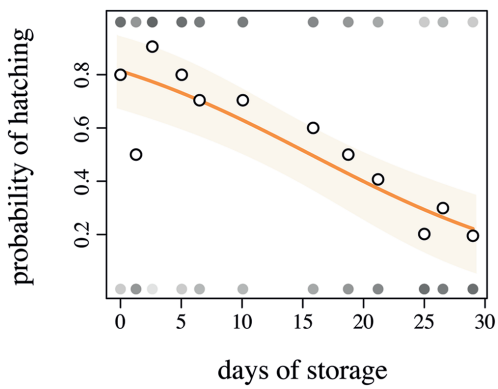


FIGURE 4 | Logistic regression between the days of storage at 6°C and the probability of *Heteronychus arator* eggs to hatch. Open circles indicate the observed data, closed circles indicate the number of hatched or non-hatched egg (in a shade of gray where white = 0% and black = 100% hatching). The light orange zone represents the 95% confidence interval of the fitted model (orange solid line).

In the 96-well plates, 46% of the eggs hatched. Storage time of the eggs, even in a cold environment, significantly impacted the hatching probability (**Figure 4**, $Z_{1-129} = -4626$, $p < 0.001$, $R^2 = 0.23$). Given the lower hatching probability, we would advise that the eggs should not be stored longer than 15 days at 6°C in order to guarantee higher hatching rates. The cumulative degree-days to hatch was slightly negatively correlated with the number of days in storage, yet this correlation was not significant (**Figure 5**, $F_{1-66} = 0.3349$, $p = 0.5648$). The negative slope of the model (–0.1759) suggests that ABB egg still very slowly developed at 10°C, yet this temperature seems to be an appropriate estimate of the developmental threshold of ABB. Storing ABB eggs at 10°C could extend the period of viability of eggs, as compared to storage at 6°C (**Figure 4**), and prolong shelf-life of the eggs. Storage in the fridge was originally tested in the hope of being able to delay hatching to ensure the availability of insects over an extended period of time, as it is done with some other insect species, especially with biological control agents [e.g., Trichogrammatidae species (Hymenoptera), Spínola-Filho et al., 2014]. As this approach was unsuccessful, it should be considered whether varying the temperature at which the beetle colonies are maintained in the laboratory can potentially delay the oviposition, but this has yet to be tested.

Despite a relatively good egg-hatching rate, the survival of the neonate ABB larvae in the specimen jars vas very low (ca. 5%, 4 larvae out of 68 hatched eggs). King et al. (1981d) demonstrated that younger ABB larval instars mainly feed on ubiquitous decaying organic matter. We hypothesize that the organic matter provided to the hatched ABB was too coarse or too fresh to be suitable for consumption by the larvae, resulting in a very low survival. An alternative explanation could be injuries caused by handling. Entomological forceps were used to manipulate ABB larvae and, despite their softness, the tweezers might have injured some larvae during transfer and handling, adding to the natural mortality.

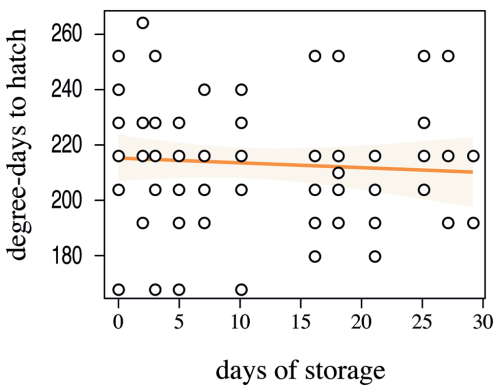


FIGURE 5 | Linear regression between the days of storage at 6°C and the degree-days required for *Heteronychus arator* eggs to hatch after removal from storage. Open circles indicate the observed data. The light orange zone represents the 95% confidence interval of the fitted model (orange solid line).

In 2015, a total of 90 beetles (52 males, 38 females) were captured from the field site. Based on the fecundity calculated in the laboratory set-up in 2014, an estimated total of 759 eggs were laid. In total, 148 adults emerged and were collected from the four containers. No differences in the number of emerged ABB adults were observed between containers ($\chi^2 = 3.521$, $df = 3$, $p = 0.318$). The observed survival in this laboratory set-up (19.5%) was similar to what has been measured in the field (Matthiessen and Ridsdill-Smith, 1991; Matthiessen and Learmonth, 1998; Matthiessen, 1999). This rate is ca. twofold higher than the estimated survival required to maintain a population to the next generation (9.6%, Matthiessen, 1999). No differences in the degree-days for ABB to emerge were recorded between the containers ($F_{1-3} = 0.586$, $p = 0.625$). The average cumulative degree-days for the adults to emerge was 1076.51 ± 8.04 SEM. This second approach offers a laboratory source of each ABB larval instars and adults. Varying the storage temperature would likely allow researchers to either accelerate or slow down the development of ABB, according to the experimental requirements (Bhuiyan and Nishigaki, 1995).

CONCLUSION

Because of their biology, it is difficult to collect soil-dwelling insect pests from the field in sufficient quantities for experimentation, and until now this has limited the use of a model insect root herbivore in studies of below-ground plant–insect interactions. For many years, we and others have spent many laborious hours digging larvae or trapping adults to conduct limited experiments to unravel root-feeding insect biology. The procedures we have described here allow ABB to be reared from eggs to adults in a laboratory setting, setting this species up to be used as a model insect root herbivore. While our procedures do not improve the survival of the insect above that observed in the field, these procedures save considerable time in the field and ensure the development of larvae under uniform conditions. Controlling temperature might allow some degree of manipulation of the insect development and therefore the availability of particular desired developmental stages (i.e., larval instars) over a longer period of time. Attempts to culture ABB under semi-artificial conditions were not successful and the current method still relays on natural rearing components instead of artificial diets or medium. Despite this current weakness, this rearing approach is easy to set up, cost effective and probably applicable to other root-feeding scarabs or coleopteran insects with soil-dwelling larvae (McMonigle, 2012). Adapting existing artificial diets used in the rearing of other scarab beetle larvae, such as *Popillia japonica* (Coleptera: Scarabaeidae; Klein and Allsopp, 1994), could help in the establishment of artificial conditions. Yet the development of insect artificial diet is a laborious task, which intrinsically requires a reliable source of insect.

By making available a model root herbivore, with a generalist diet, we hope to facilitate comparative studies of root defense and herbivory across diverse plant species and environments. This in turn will enable researchers to address a neglected but vitally important aspect of plant ecology, particularly in the context of changing agricultural and land management practices, and climate change.

AUTHOR CONTRIBUTIONS

IH, SJ, and BM conceptualized the study. IH designed the experimental set-ups, collected beetles in the field, and conducted the laboratory experiments. IH analyzed and interpreted the data and drafted the manuscript. All authors were involved in reviewing, revision, and final approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01316

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REVIEW
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A Review of Perennial Ryegrass Endophytes and Their Potential Use in the Management of African Black Beetle in Perennial Grazing Systems in Australia

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The major insect pest of Australian cool temperate pastures is the root-feeding insect *Heteronychus arator* (African black beetle, ABB). Significant pasture damage can occur even at low ABB densities (11 individuals per square meter), and often re-sowing of the whole paddock is required. Mitigation of the effects of pasture pests, and in particular subterranean species such as the larval form of ABB, can be challenging. Early detection is limited by the ability to visualize above-ground symptoms, and chemical control of insects in soil is often ineffective. This review takes a look at the historical events that molded the pastoral landscape in Australia. The importation route, changes in land management and pasture composition by European settlers may have aided the establishment of ABB in Australia. Perennial ryegrass *Lolium perenne* is discussed as it is one of the most important perennial agricultural grasses and is widely-sown in moderate-to-high-rainfall temperate zones of the world. Endophytic fungi from the genus *Epichloë* form symbiotic relationships with cool season grasses such as *Lolium perenne* (perennial ryegrass). They have been studied extensively and are well documented for enhancing persistence in pasture via a suite of bioactive secondary metabolites produced by the fungal symbionts. Several well-characterized secondary metabolites are discussed. Some can have negative effects on cattle (e.g., ergovaline and lolitrems) while others have been shown to benefit the host plant through deterrence of insect pests from feeding and by insecticidal activity (e.g., peramine, lolines, ergopeptines). Various control methods for ABB are also discussed, with a focus on the potential role of asexual *Epichloë* endophytes.

Keywords: endophyte, *Heteronychus arator*, pasture, pest management, control methods

INTRODUCTION

Food production is a basic requirement for a sustainable society and the reason why a significant area of land has been dedicated to agricultural practices worldwide. Within these practices, part is devoted to animal production systems as grassland for grazing animals and hay production (Conant et al., 2001). Although annual grasses and food crops have been selected for their productivity since the beginning of agriculture, perennial grasses have only been studied in the last century (Wilkins, 1991). The domestication and expansion of grasses have been associated with the early stages of primitive agriculture in the Fertile Crescent of the Middle East about 10,000 years ago (Balfourier et al., 2000). This scenario suggests that ryegrasses were probably spread as weeds of cultivated crops by farmers during migratory events.

It has been predicted that the world population will increase by 50% between 2000 and 2050 to nine billion people (Kingston-Smith et al., 2013). Pastures play an important role in agriculture as production of meat and milk products increases to supply the growing human population (Lasley et al., 2009). This represents a challenge for agronomists, as they will have to achieve the right balance for sustainable production, one that does not compromise food quality or the environment (Tilman et al., 2002). Ruminants are vital for mankind as they provide high protein food products from plant material that is otherwise unsuitable for humans (Kingston-Smith et al., 2013). Finneran et al. (2012) highlighted the importance of determining the annual cost of feed in order to achieve a self-sustainable grassland system, therefore decreasing the purchase of concentrated feed and increasing profitability. Additionally, a better understanding of the nutritional requirements of ruminants has allowed for an improvement in the quality of forage crops grown on pasture land, which has translated into improved animal performance (Kingston-Smith et al., 2013).

However, growers face biotic (invertebrate pest, plant pathogens, and weeds) and abiotic (temperature, water, soil type, and nutrients, etc.) stressors that can severely reduce crop production; some of these biotic factors can be managed by physical (cultivation, mechanical weeding, etc.), biological (cultivar choice, crop rotation, predators, etc.) and chemical measures (pesticides, herbicides, Oerke, 2006). This review focuses on the control methods for the economically important insect pest, the African black beetle (*Heteronychus arator*) primarily focusing on the use of perennial ryegrass endophytes. Physical, biological, and chemical measures described by Oerke (2006) are explained here in terms of cultural control methods, natural predators and parasites, and chemical control. This review also includes a background on the beginnings of agriculture in Australia and a sequence of events that has led to the current pasture-based grazing systems for animal production.

EARLY HISTORY OF GRAZING IN AUSTRALIA

The pastoral industry began in Australia at the time of European settlement. The first livestock to be introduced were bought from South Africa in 1787 and later transported to Australia (Clark, 1962). Due to the poor soil conditions and harsh grasses at the harbor side in Sydney the establishment of an agriculture enterprise was difficult (Younger, 1993). Initially, animals grazed on the cove of Port Jackson but were then moved to the head of the next cove after exhausting the limited feed in the area (Taylor, 1982). Even though early reports mentioned that most of the livestock imported survived after 6 months in Australia, all six cows and two bulls went missing after being left unattended (Younger, 1993). Food shortages and the lack of animals catalyzed the search for fertile soil leading to the settlement of the Rosehill area, 25 km west of Port Jackson, later that year (Hill, 2008).

In 1795, wild cattle were sighted in increasing numbers at the west bank of the Nepean River which were thought to be related to the cattle that escaped in the earlier years of the colony (Younger, 1993). It was not until 1803, however, that the government declared that the cattle could be maintained and numbers increased sustainably without relying on importation (Alexander and Williams, 1973).

Advances in food production were repeatedly affected by a series of droughts and insect plagues in 1810 forcing the colony to rely on imports from India, and in 1816 from Van Diemen's land (now Tasmania) (Stone and Garden, 1978). Food demand became a major concern as the number of convicts sent to Australia rapidly increased (Stone and Garden, 1978). Around 1820, meat production was the main focus for stockmen and other products, such as wool, were not as important at the time (Alexander and Williams, 1973). During the following years the agricultural industry experienced an expansion enabled by the convict system that provided cheap labor and inexpensive tracks of land (Stone and Garden, 1978). The earliest records of the dairy industry date back to the 1820s when dairy herds began to appear in the Illawarra district, New South Wales (Drane and Edwards, 1961).

The growth of the agricultural industry and the increase in investment allowed the establishment of the Australian Agricultural Company in 1824, which had large capital and land grants for expansion (Stone and Garden, 1978). The rapid expansion of the industry led to exploitation and grazing on unoccupied lands beyond the settlement boundaries despite the Governor's efforts (Billis et al., 1930; Alexander and Williams, 1973).

SEQUENCE OF AGRICULTURAL DEVELOPMENTS

Between 1830 and 1860, the Sydney-based colony expanded in all directions including southern Queensland, parts of the Riverina and across the Murray, Port Phillip, Adelaide, Swan River, and Albany in Western Australia (Pearson and Lennon, 2010). Agriculture in Australia suffered its ups and downs during this period. In the early 1840's an Australia-wide depression took place after a drop in prices of the main commodities at the time (wool and meat) due to a surplus of livestock (Alexander and Williams, 1973). During the gold rush in 1851, the pastoral

industry was initially detrimentally affected but then bounced back due to the high demand for meat caused by the increase of migration into the country (Pearson and Lennon, 2010). Fencing and management of native and introduced pastures also came into practice during the acute labor shortage caused by the goldrush (Schofield, 1990).

With the rapid expansion of the cities, dairy herds were established to supply the locality with milk (Alexander and Williams, 1973). The location of dairy farms was determined by a range of factors including climate, topography but more importantly a nearby market (Drane and Edwards, 1961). To be able to provide milk twice a day to the nearby city, dairy farms required a high rainfall and a long growing season to favor introduced pastures such as perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*, *T. pretense*) and prairie grass (*Ceratochloa unioloides*) (Alexander and Williams, 1973).

Until the 1880s, dairying remained a local industry as markets had to be near enough to enable the product to be transported before it spoiled (Drane and Edwards, 1961). In areas of high rainfall located away from the cities, cattle were kept for cheese and butter production (Alexander and Williams, 1973). Between 1880 and 1900, major technological advancements—including refrigeration for shipping, the Babcock system of estimating fat content of milk, and factory methods for manufacturing and preserving dairy products—allowed the rapid expansion of the dairy industry (Drane and Edwards, 1961).

From 1900 to the present time, the pastoral industry has gone through some challenging times: drought (1900), World War I (1910–1914), the Great Depression (1930–1940s), World War II (1939–1945), and recession (1990s) (Pearson and Lennon, 2010). During this period, however, scientific research focused on the selection and improvement of exotic and native grass cultivars to increase productivity and expand agriculture to areas which had not been exploited before (Parbery, 1967). In recent decades, the Australian industry has faced significant structural adjustment which has transformed the industry, driving productivity, and growth (Stott and Gourley, 2016). Milk production has intensified, with fewer farms and increased stocking rates, and there have been substantial increases in the use of bought-in feed and nitrogen (N) fertilizer to support increased milk production per cow and per hectare (CIE, 2011).

Currently agriculture makes a significant contribution to the Australian economy both nationally and in regional areas. The value of farm production was almost $54 billion in 2013–14. Agriculture contributed around 2% of Australia's Gross Domestic Product with milk being the third highest commodity (See **Figure 1**) (ABARES, 2014).

Dairy farming relies on high quality permanent pastures for year-round grazing (Stott and Gourley, 2016). Pastures are typically dominated by perennial ryegrass (*Lolium* spp.) and varying proportions of legumes (e.g., clover, *Trifolium* spp., Chapman et al., 2008; Jacobs, 2014).

AUSTRALIAN PASTURES

Prior to European settlement, extensive areas of vegetation were present either as grassland or as the understorey to *Eucalyptus* woodland in the south-eastern part of the continent (Groves et al., 2003). The typical plant composition of temperate grassland areas included perennial tussock, inter-tussock herbaceous flowering plants, with *Themeda, Poa,* and *Austrodanthonia* as the dominant grass genera (Lunt and Morgan, 2002). Vast areas of the temperate zone of south-eastern Australia had a long history of Aboriginal management before the first European settlers arrived on the continent (Gott, 2005).

Examples of land management by aboriginal people have been found in written reports of the earliest explorers:

> *"I found a considerable store of grass-seed, gum from the Mimosa, and other stores, carefully packed up in bags made from the skin of the kangaroo, and covered over with pieces of bark, so as to keep them properly dry. The weight of the bags containing the grass seed and gum was about 100 lbs; the seeds had been carefully dried after being collected from small grasses of the plains" (Coxen, 1866)*
>
> *"Dry heaps of this grass, that had been pulled expressly for the purpose of gathering the seed, lay along our course for many miles" (Mitchell, 1848)*

Additionally, Aboriginal people used fire to clear tracks and open hunting grounds (Rolls, 1999). It is no surprise that the temperate zone overlapped with the areas in which colonizers established and expanded their settlements (Pearson and Lennon, 2010). The extent of the temperate zone in the southern part of the continent incorporates Tasmania, most of Victoria, eastern NSW, areas of southern South Australia and the south-west of Western Australia (Dorrough et al., 2004). The European settlers exploited the fertile plains first, altering the native ecosystem to pasture and crops through agricultural practices (Lunt, 1991). Permanent changes in the vegetation composition such as pastures have occurred in response to the prolonged and intensive grazing (Groves et al., 2003). Large-scale conversion of grassland and grassy woodland to exotic pastures and crops took place on the fertile soils of south-eastern Australia as part of the colonization process (Fensham, 1998).

From the historical data we can appreciate how the improvement of pastures became an imperative in order to aid agricultural industries. The introduction of a number of plant species were promoted by the Acclimatisation societies, in conjunction with government botanists, during the late 1800s (Cook and Dias, 2006). Perennial pastures were first introduced into field trials in Victoria in 1860, as an initiative of the state government, with the purpose of evaluating the introduction and naturalization of pasture species (Cunningham et al., 1994). Perennial ryegrass was later reported as naturalized in Victoria before 1878 (Laffan and Ashton, 1964). Some studies date back to 1928 in Burnley, Victoria, when some pasture species were introduced and naturalized for agricultural purposes (Beilharz and Halloran, 1987). However, as pasture improvement moved further inland into drier regions the standard cultivars began to fail (Reed and Cocks, 1982).

In the 1950s, there was a large effort to develop perennial grass cultivars for the hotter, drier areas of south-eastern Australia. Grasses that could persist in a Mediterranean climate (average annual rainfall < 400 mm) were collected from Southern Europe

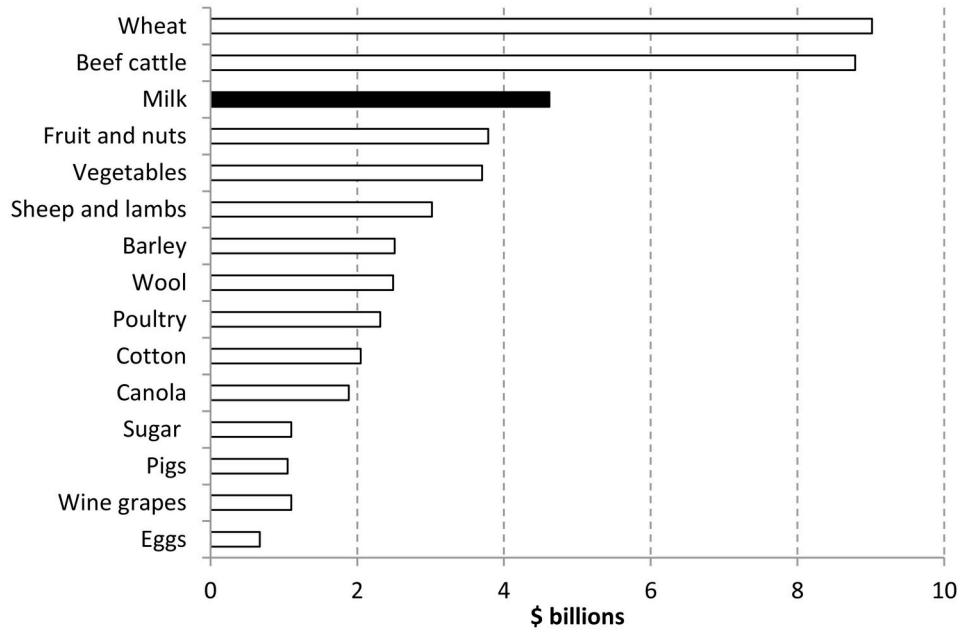


FIGURE 1 | Contribution of agriculture sectors in Australia 2013–2014 (ABARES, 2014).

and North Africa in order to provide new germplasm for breeding programs (Clark et al., 2016).

Cunningham et al. (1994) describes improvement programs of perennial ryegrass in Australia (1936–1995) from which different grass cultivars were developed and certified regionally (Victoria, New South Wales, Tasmania and South Australia) with the objective of improving their persistence, resistance to biotic and abiotic factors and quality.

More recently, in 1994, a joint effort by the Victorian Department of Agriculture, the U.S. Department of Agriculture and agencies in Morocco, Tunisia, and Italy collected widely in those three countries (Cunningham et al., 1997). Among the species collected were tall fescue, perennial ryegrass, phalaris and cocksfoot (Reed et al., 2016).

PERENNIAL RYEGRASS

Perennial ryegrass (*Lolium perenne*) is one of the most important perennial agricultural grasses world-wide. It is native to Europe, temperate Asia, and North Africa (Jensen et al., 2001) but it has been introduced in many countries including New Zealand, United States, and Australia for agricultural uses (Cunningham et al., 1994; Easton et al., 2001; Young et al., 2013), where it is widely-sown in moderate-to-high-rainfall temperate zones. Perennial ryegrass is an ideal forage grass due to its high digestibility, tolerance to grazing, and adequate seed production (Frame, 1989; Wilkins, 1991). Additionally, it is highly adaptive to different habitats and there is significant variation of traits in the wild populations providing room for genetic improvement (Wilkins, 1991).

There are three key factors that agronomists look to improve when breeding perennial ryegrass: dry matter yield, forage quality and persistence (temperature, drought, pests, disease, etc.) (Humphreys et al., 2006). As in most cool-season grasses, perennial ryegrass is an obligate outbreeder or self-incompatible plant that suffers from inbreeding depression (Cunningham et al., 1994). Self-incompatibility (SI) has been defined by Denettancourt (1977) as "the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination." Husband and Schemske (1996) defined inbreeding depression as "the reduction in fitness of progeny derived from inbreeding relative to those derived from outcrossing." The SI mechanism in grasses hinders the production of inbred lines and hybrids in plant breeding, but also preserves heterozygosity in wild populations (Yang et al., 2008).

The obligate outbreeding nature of perennial ryegrass is why the initial advancements in breeding programs were performed by gene selection through sexual recombination (Humphreys et al., 2006). Continuous selection of full or half-sibling families allows for the improvement of pastures as desirable traits are assessed after each generation but progress is rather slow (Wilkins, 1991).

Another factor taken into account in perennial ryegrass breeding programs is the presence of asexual *Epichloë* endophytes because of the benefits of this symbiosis to the host plant (Funk and White, 1997). Elite cultivars obtained from breeding programs are combined with selected asexual *Epichloë* endophyte strains which can be incorporated into grass plants by inoculation (Funk and White, 1997).

FUNGAL ENDOPHYTES

Epichloë (syn. *Neotyphodium*) spp. have been described in early publications as endophytic fungi present in grasses such

as perennial ryegrass and tall fescue (Sampson, 1933). They are an important group of filamentous fungi that infect cool season grasses, and consist of sexual (*Epichloë)* and asexual (*Neotyphodium)* species, previously classified as *Acremonium* sect. Albo-lanosa (Glenn et al., 1996). However, recent changes in fungal nomenclature rules have led to the renaming of the asexual (anamorphic) and sexual (teleomorphic) taxa into a single genus, designated *Epichloë* (Leuchtmann et al., 2014). The asexual growth of *Epichloë* endophytes in host grasses is characterized by seldom-branching hyphae in leaf sheaths which, in most cases, is aligned parallel to the leaf axis (Christensen et al., 2002). The endophyte is vertically transmitted through seeds by colonizing the developing flowers, ensuring their continuity from mother to daughter plant (Schardl, 1996). However, if the sexual stage of *Epichloë* species occurs, fungal stromata are formed on immature inflorescences "choking" affected flowering tillers and rendering them sterile (Moon et al., 2000).

The presence of asexual *Epichloë* endophytes in cool season grasses is not apparent and plants remain symptomless (Sampson, 1933; Wilson, 1993; Clay and Schardl, 2002; Iannone et al., 2011). However, infected plants can experience increased plant growth, reproduction and resistance to various biotic and abiotic stress factors (Clay and Schardl, 2002). For example, in symbiosis, *Epichloë* endophytes produce an array of secondary metabolites that benefit host plants through improved resistance against herbivores, pathogens, and drought (Siegel et al., 1987; Wilson, 1993; Zain, 2011). Christensen et al. (1993) demonstrated that alkaloid profiles are strain-specific producing the same chemical profile when the fungal symbionts are compared in the same plant. However, the chemical profile produced by an endophyte in symbiosis with a plant varies depending on the endophyte-plant combination (Panka et al., 2013). Evidence obtained from pot and field experiments have shown that endophyte-infected pastures perform better than endophyte-free swards under the above mentioned selective pressures (Prestidge and Gallagher, 1988b; Bacon, 1993; Crawford et al., 2010; Saikkonen et al., 2013).

Even though alkaloids produced by the endophytes can be beneficial for their host plant, they are also known to cause harm to vertebrate herbivores including livestock (Fletcher and Harvey, 1981; Crawford et al., 2010). Some groups of alkaloids have been identified as harmful, for example ergopeptine (ergovaline) and isoprenoid lolitrem (lolitrem B) which cause fescue toxicosis and ryegrass staggers, respectively (Smith et al., 1997). The effects of the different beneficial and harmful groups will be discussed in more detail in the section "Endophyte as a control method." These bioactive properties have driven research on the alkaloids produced by endophytic fungi because of the services they provide to their host plant and agricultural systems (**Table 1**).

PASTURE PESTS

Invertebrate activity can severely affect pastures by decreasing growth and establishment rate, impacting pasture composition favoring less palatable species and weeds, and enhancing damage caused by vertebrate grazers and predators by exposing areas to soil erosion (Bailey, 2007). However, some invertebrates which play a role in promoting pasture health (e.g., earthworms, termites, and ants) are regarded as soil engineers (Jouquet et al., 2006). Invertebrates that participate in biological, chemical and physical processes providing soil ecosystem services (e.g., recycling of nutrients, control of local microclimate, regulation of local hydrological processes, regulation of the abundance of undesirable organisms, and detoxification of noxious chemicals) as well interacting with other organisms in the substrate are recognized as beneficials (Altieri, 1999; Lavelle et al., 2006). It has been suggested that loss of biodiversity can prove costly for agroecosystems, as this directly affects basic regulation processes including soil fertility and pest control (Altieri, 1999).

In Australia, changes in land use from native pasture to intensive agriculture with exotic temperate pasture grass and legume species has led to addition of fertilizer and superphosphates to the soil to sustain such practices (King and Hutchinson, 1983). These landscape modifications have been associated with improved livestock production; however they also affect soil structure, water and nutrient cycling, as well as pasture productivity and palatability (Dorrough et al., 2004). Introduced pasture species influence their landscape by decreasing biodiversity of vegetation and invertebrate communities (King et al., 1985). In this large-scale intensive agriculture model, there is an increasing dependency on chemicals to manage pests which differs from the concept of sustainable agriculture (Tilman et al., 2002; Tscharntke et al., 2005).

European studies have found that improvement and management of pasture affects abundance and species richness of predators such as carabid beetles and spiders. Frequent use of the organo-phosphate pesticide chlorpyrifos was singled out as an important factor affecting predator richness (Rushton et al., 1989). In addition to the application of pesticides, grazing pressure has shown to have an impact on arthropod diversity of predator species (e.g., spiders) as well as affecting the abundance and diversity of pollinators such as bumblebee species (Tallowin et al., 2005).

In Australia there has been less research on the effect of agriculture on insect pollinators. Broad scale agriculture is thought to be associated with a low density of native bees, probably due to the absence of diverse nectar producing flowers, whereas the impact of pesticides on native bees is thought to play a more minor role as it is not well understood (Batley and Hogendoorn, 2009). Application of pesticides in perennial crops systems can be disruptive for beneficial insects, which is why refugia outside treated areas are essential (Landis et al., 2000). European studies suggest that mitigation of the negative effects of land management can be achieved by providing refugia adjacent to farmland to encourage the survival and reproduction of invertebrate predators (Macleod et al., 2004); changing grazing regimes to support beneficial species (Tallowin et al., 2005) and; reducing chemical sprays that impact on invertebrate predators (Rushton et al., 1989).

Although the relevance of European research to an Australian context is uncertain, Nash et al. (2008) found evidence to support transferability of some of this knowledge to Australian

TABLE 1 | Chronology of some of the papers published on ryegrass endophytes.

Subject	Research description	Reference
Animal health	Association of *Lolium* endophyte with ryegrass staggers.	Fletcher and Harvey, 1981
	Isolation of stagger-producing neurotoxins lolitrem A and B.	Gallagher et al., 1981
	Review on the effects and bioactivity of lolitrems, peramine, and paxilline. Lolitrem and paxilline have shown to be tremorgenic on vertebrates. In contrast peramine produces insect deterrence without affecting vertebrates.	Rowan, 1993
	Evaluation of the effects of penitrem, paxilline, and lolitrem B on sheep smooth muscle, show they cause low, mild, and persistence tremors, respectively.	Smith et al., 1997
	Review of mycotoxins important in ruminant feeding such aflatoxins, lolitrems, ergopeptine alkaloids, and others produced by fungi that are found in cattle feed.	D'Mello and MacDonald, 1997
	Evaluation of novel (AR37 and AR1) ryegrass endophytes showed improved persistence against insect pests without affecting cattle health.	Thom et al., 2013
Insect	Endophytes producing alkaloids responsible for ryegrass staggers in lambs (i.e., lolitrem B) were found to affect the growth rate of Argentine stem weevil (*Listronotus bonariensis*) larvae.	Prestidge and Gallagher, 1988a
	Endophyte infected plants exhibit increased insect resistance compared to uninfected conspecifics. Recommend that survey and selection of endophyte strains that do not affect cattle and benefit the host plant is necessary.	Clay, 1989
	Pot trials show that endophyte positive plants were significantly less damaged than endophyte free controls regardless of their alkaloid spectra.	Ball et al., 1994
	Bioassay based on mycotoxins found that only certain ergopeptine alkaloids deter adult African black beetle *in vitro*.	Ball et al., 1997b
	Absence of synergism between endophyte-infected perennial ryegrass and *Paenibacillus popilliae* against Japanese beetle (*Popillia japonica*).	Walston et al., 2001
	Pot trials found no effect of endophyte-infected ryegrass on redheaded (*Adoryphorus coulonii*) and blackheaded (*Acrossidius tasmaniae*) pasture cockchafers.	Watson, 2006
	Field trials examining the effects of selected endophyte strains (AR1 and AR37) and control against insect pests.	Popay and Thom, 2009
	Evidence of peramine and lolitrem B cascading up the food chain from aphids to ladybird increasing the duration of the pupal stage.	Fuchs et al., 2013
	Impact of selected endophytes (Wild-type, AR1 and AR37) and control against root aphids, African black beetle, Argentine stem weevil on field trials showed a decrease on insect pressure: Control > AR1 > Wild-type = AR37.	Thom et al., 2014
Plant performance	There is no effect of endophyte on photosynthesis and associated processes but there is evidence endophyte-infected plants are more tolerant of environmental abiotic stresses than uninfected grasses.	Bacon, 1993
	Leaf sheaths and leaf blades maintain similar peramine concentration, but decrease with leaf age. The seed from reproductive clones and younger sheaths and blades of leaves from vegetative tillers contained the highest concentrations, while the root, crown, and dead leaf tissue contained the lowest.	Ball et al., 1997a
	Grass-endophyte associations are based primarily on protection of the host from biotic and abiotic stresses.	Clay and Schardl, 2002
	Endophyte-infected plants promoted competitiveness, hindering weed invasion.	Saikkonen et al., 2013

agricultural systems in regards to conservation of predatory invertebrates. An Australian research team, Tsitsilas et al. (2006), highlighted that grassy shelterbelts adjacent to pasture may influence the number of pest organisms. More importantly, these shelterbelts carried low numbers of pest species but higher numbers of predatory mites and spiders. Collins et al. (2002) found that although refugia within a crop field (in this study refered to as beetle banks) supported polyphagous predators, they failed to prevent aphid outbreaks; the presence of refugia did appear to have a significant impact on reducing the aphid population up to a distance of 83 m from the refuge. Collins et al. (2002) concluded that to prevent economic losses, optimal density of predators and spacing of refugia in fields must be determined.

Australia's major pest groups of grass pastures and turf have been well described by Bailey (2007), who provides detailed information about the pest's food source and some of the different control methods available (**Table 2**).

Historically, pasture pests have taken a toll on Australian agriculture from as early as 1810 when caterpillar plagues and drought severely affected pastures (Stone and Garden, 1978). Hoffmann et al. (2008) reviewed pest outbreak bulletins from the 1980–1984, 1985–1989, 1990–1994, and 2006–2007 from south-eastern Australia and reported that the relative incidence of

TABLE 2 | Major pest groups of grass pastures and turf in Australia, adapted from Bailey (2007).

Pest	Common name	Scientific name
Mites (Acari)	Cereal rust mite	*Abacarus hystrix*
	Blue oat mites	*Penthaleus* spp.
	Red legged earth mite	*Halotydeus destructor*
	Bryobia pasture mite	*Bryobia praetiosa*
	Balaustium mite	*Balaustium medicagoense*
Springtails (Collembola)	Lucerne flea	*Sminthurus viridis*
Snails and slugs (Mollusca)	Common garden snail	*Cantareus aspersa*
	Slugs	Eupulmonata
Caterpillars (Lepidoptera)	Black cutworm	*Agrotis ipsilon*
	Corbie	*Oncopera intricate*
	Winter corbies	*O. rufobrunnea*
	Underground grassgrubs	*O. fasciculate*
	Ghost moths	*Fraus simulans*
	Oxycanus grassgrub	*Oxycanus antipoda*
	Armyworms	*Leucania* spp.
	Pasture webworms	*Hednota* spp.
	Cotton webspinner	*Achyra affinitalis*
	Pasture tunnel moths	*Philobota* spp.
Crickets and Grasshoppers (Orthoptera)	Black field cricket	*Teleogryllus commodus*
	Mole crickets	*Gryllotalpa* spp.
	Wingless grasshoppers	Orthoptera: Acrididae
Beetles (Coleoptera)	African black beetle	*Heteronychus arator*
	Blackheaded pasture cockchafer	*Acrossidius tasmaniae*
	Redheaded pasture cockchafer	*Adoryphorus coulonii*
	Argentine stem weevil	*Listronotus bonariensis*
	White fringed weevil	*Naupactus leucoloma*

lucerne flea, *Balaustium* mites, blue oat mites, redlegged earth mites, snails, and pasture cockchafers had increased during that period.

Scarabaeidae is one of the largest families of Coleoptera in Australia, comprising seven subfamilies and 3000 species (Allsopp, 1995). A number of these species are pasture beetles that share a similar lifestyle and behavior. For quite some time, all scarab larvae were commonly referred as "white grubs" because of their white/creamy color and curled shape during the larval stage (Cumpston, 1940). Members of the subfamilies Dynastinae, Rutelinae, and Melolonthinae are generally soil-dwelling, phytophagous, or phytosaprophagous, and in some cases the adults do not feed (Allsopp, 1995). However, there are still a number of soil-inhabiting pasture beetles whose larval forms have not yet been described (Berg et al., 2014). Pasture beetle larvae are predominantly a problem in grassland areas where they feed on humus and plant roots, decreasing plant persistence dramatically under stress conditions (e.g., grazing livestock, use of machinery, Blank and Olson, 1988; Berg et al., 2014). Some of the most cited crop and pasture pests in Australia from this family are summarized in **Table 3**. Most of these pasture beetles are endemic to Australia but that is not the case for the African black beetle, which, as its common name suggests, originates from Africa (Matthiessen and Ridsdill-Smith, 1991).

AFRICAN BLACK BEETLE

African black beetle (*Heteronychus arator*) is a univoltine (1 year life cycle) soil-dwelling scarab beetle predominately found in grassland (Matthiessen, 1999; Bell et al., 2011). Temperature seems to affect, directly or indirectly, the presence and distribution of African black beetle, as its incidence has been associated with areas with mean annual surface temperatures greater than 12.8°C (Watson, 1979). African black beetle is recognized as an agricultural pest in Australia, New Zealand, and South Africa (Matthiessen and Learmonth, 1998). The earliest record of its introduction in Australia is a specimen collected in Newcastle, NSW, in 1920, but it is presumed to have become established prior to 1920 (Wright, 1958). The earliest record in the Australian Pest Plant Database dates back to 1930 (Plant Health Australia, 2001), similar to the first reports in New Zealand in 1937 (Todd, 1959). In Australia, African black beetle has been reported throughout the coastal region of New South Wales, widespread in pastures of south-western Western Australia, in coastal South Australia, parts of Queensland, and Victoria (Plant Health Australia, 2001). Most of its lifecycle occurs underground, but during the adult stage, they emerge to mate and on some occasions swarm (Ormerod and Janson, 1889; Matthiessen and Learmonth, 1998; Bulinski et al., 2006). It has been suggested that flights not only occur to vary habitat between life stages, but also to have a dispersive role; as seen in most dynastids (members of the subfamily Dynastinae), flights are an adaptation to the fluctuation between seasons (i.e., wet and dry, as well as, cold and hot, Watson, 1979).

African black beetle is a polyphagous species, reported to affect a number of different plants such as, blue gum (Loch and Floyd, 2001), potatoes (Matthiessen and Ridsdill-Smith, 1991), tomatoes, grapevines (Bulinski et al., 2006), maize (Drinkwater, 2003), sugarcane, clover (*Trifolium* spp.), and grass species including, kikuyu, phalaris, *Paspalum* spp., and *Lolium* spp. (Bailey, 2007; Bell et al., 2011). Larvae feed on the roots (Bell et al., 2011) while adults have been reported to cause severe damage to subterranean stems of seedlings, including young stems of potatoes and summer-sown crops (Matthiessen and Ridsdill-Smith, 1991; Erasmus and Berg, 2014).

One of the reasons African black beetle is a difficult pest to control is because of its potential to cause a high level of damage per individual (Bulinski and Matthiessen, 2002). It has been suggested African black beetle can cause significant damage to crops at densities exceeding 10 individuals per square meter (Bailey, 2007). Densities of over 100 larvae per square meter can cause direct damage to turf grasses, however secondary damage caused by foraging birds preying on the grubs can be observed even at lower densities (Ford et al., 2001).

TABLE 3 | Scarabaeidae pests of crops and pasture in Australia.

Common name	Scientific name	Host plant	Reference
African black beetle	*Heteronychus arator*	Blue gum, potatoes, tomatoes, grapevines, sugarcane, maize, kikuyu, phalaris, clover (*Trifolium* spp.) and *Paspalum* spp. *Lolium* spp.	Matthiessen and Ridsdill-Smith, 1991; Loch and Floyd, 2001; Bulinski et al., 2006; Bailey, 2007; Bell et al., 2011
Redheaded cockchafer	*Adoryphorus coulonii*	Subterranean clover, annual and perennial grasses	Bailey, 2007; Berg et al., 2014
Blackheaded cockchafer	*Acrossidius tasmaniae*	Annual grasses, legumes and cereals	Mcquillan, 1985; Bailey, 2007
Yellowheaded cockchafer	*Sericesthis harti*	Pasture and cereals	Bailey, 2007
Wheat root scarab	*S. consanguinea*	Pasture and cereals	Bailey, 2007
Black beetle	*Metanastes vulgivagus*	Pasture and cereals	Bailey, 2007
Black soil scarab	*Othnonius batesii*	Pasture and cereals	Bailey, 2007
Cockchafer	*Heteronyx obesus*	Pasture and cereals	Bailey, 2007

The African black beetle life cycle (**Figure 2**) starts in spring when the eggs are deposited, then the larvae go through three instars of development from around September-November to late summer when pupation takes place (Matthiessen and Ridsdill-Smith, 1991). Adults appear in numbers from March to September when mating and then oviposition occurs; adults die soon after reproduction (Matthiessen and Ridsdill-Smith, 1991). Flight dispersal mainly occurs during the autumn months by immature adults, while mature adults generally crawl during spring (Matthiessen and Learmonth, 1998). Autumn flights seem to be associated with the first significant rainfall after pupation, dusk surface temperatures ($> 17°C$) and favorable wind conditions (Watson, 1979). It has been suggested that flights during autumn and spring may play a crucial role in infestation of new pastures during outbreak years (Bell et al., 2011).

African black beetle was reported to be a serious crop pest in South Africa as early as 1889 (Ormerod and Janson, 1889). However, only two epidemic outbreaks (1946 and 1977) have been reported in South Africa, both in maize (Taylor, 1951; Drinkwater, 1979). African black beetle has been defined by King et al. (1981) as "a sporadic but serious pest of pastures and crops in northern areas of New Zealand's North Island." The incidence of some outbreaks has been associated with warm conditions cause by a La Niña weather pattern (Eden et al., 2011). Warm spring temperatures greatly benefit African black beetle populations by allowing early oviposition followed by rapid egg and larval development, therefore increasing survival over summer (East et al., 1981). In Australia, African black beetle had been reported to reach plague levels in New South Wales as early as 1923 and subsequently in 1929–1933, 1936, 1940, 1944–1946, 1952–1954, 1957 (Wright, 1958). Additionally, a more recent study of pest outbreak reports from 1980 to 2006–2007 revealed that the relative incidence of pasture cockchafers, including African black beetle, increased during that period (Hoffmann et al., 2008).

CONTROL METHODS

Cultural Control Methods

Cultural control methods refer to activities carried out to control one or more pests by changing the habitat conditions to promote biological control and/or decrease habitat quality for the pest (Horne and Page, 2008). As described in Bailey (2007), some cultural methods that could be used against African black beetle include: delaying sowing until November-December following the end of the beetles' life cycle; reducing potential habitat by removing grass and weeds from headlands; avoiding sowing in pasture areas that may contain adults; and establishing a physical barrier by cutting a deep furrow with a vertical side toward the crop. Some of the earliest remedies used to control African black beetle in South Africa included manure traps, sprinkling with salt, and application of lime into the soil, the latter being the only one reported as successful (Ormerod and Janson, 1889).

In Australia, some of the most productive agricultural land is naturally acidic (Scott et al., 2000). It has been suggested that addition of lime into soils with a naturally low pH may lead to local extinction of endemic acidophilic species, therefore its application must be treated with caution (Oliver et al., 2005). Furthermore, it is still unclear what effects lime applications might have on pasture cockchafers incidence and the host plant's ability to overcome feeding damage (Berg et al., 2014).

Several scarab species are considered significant pests of eucalypts (Frew et al., 2013). These include stem-feeders (Abbott, 1993) such as African black beetle (Paine et al., 2011) and common defoliators such as the Christmas beetles (*Anoplognathus* spp.) (Johns et al., 2004). It has been suggested that the use of fertilizer with nitrogen (N) in eucalypt plantations could be used as a management option as it has been found to either moderate or negate the effect of severe insect defoliation on growth (Pinkard et al., 2006a). This is because the leaf structure and texture of eucalypts may play a role on levels of herbivory (Sanson et al., 2001; Steinbauer, 2001). For example, Pinkard et al. (2006b) showed that leaf density or thickness of *Eucalyptus globulus* increased following N application in response to artificial defoliation. Therefore, it has been argued that nitrogen (N) application will not increase future herbivory problems (Pinkard et al., 2006a).

Nonetheless, irrigation and fertilization practices applied on eucalypt plantations have been positively correlated with an increase in scarab populations as these practices (mostly fertilization) also affect the understory (Frew et al., 2013). Therefore, even though fertilization with N prior to defoliation

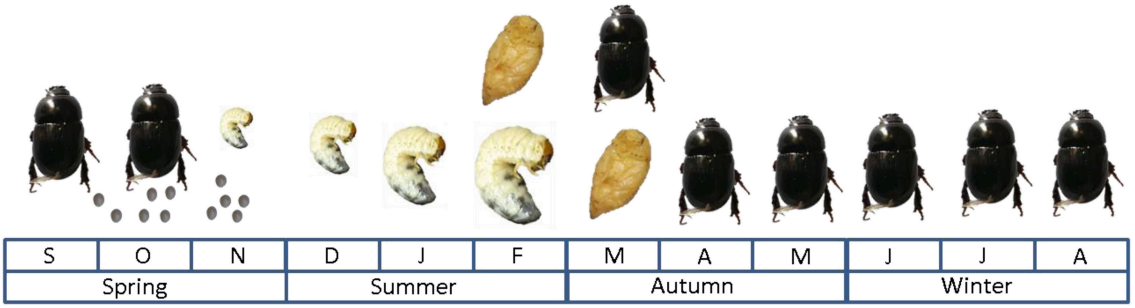


FIGURE 2 | African black beetle life cycle under Australian conditions as described by Matthiessen and Ridsdill-Smith (1991).

maintains stem growth and diameter at a similar rate to undefoliated unfertilized trees (Pinkard et al., 2007), it is concerning that the plantation understory of eucalypts and similar systems (e.g., orchards and oak woodlands) may serve as a potential niche for pasture beetles that will not only affect the plantation but also neighboring pastures and crops (Frew et al., 2013). Abbott (1993) highlighted that mat-forming grasses near plantations seem to favor African black beetle.

MONOCULTURE EFFECTS

Simplification of the environment on large expanses of land will cause an increase in the density of host plants, uniformity of crop population age structure and physical quality, and a decrease in biodiversity (Altieri et al., 1984). It has been suggested that pest problems in eucalypt plantations in south-western Australia may have been intensified by eucalypt monocultures (Loch and Floyd, 2001). Similarly, sown pastures are characterized by having lower diversity in vegetation and invertebrate communities than in naturally occurring pastures (King et al., 1985). In the case of pastures, it may be possible to break the lifecycle of the African black beetle through crop rotation; sowing non-host crops (e.g., brassicas, legumes, or chicory) in spring, thus causing a disruption of larval feeding as well as controlling grass weeds (Bell et al., 2011).

Even though evidence of monoculture effects has been found in agriculture (Altieri et al., 1984; Andow, 1991) this concept is not exempt from criticism (Emden and Williams, 1974; Goodman, 1975). Andow (1991) concludes that monocultures may influence pest abundance in different ways depending on the species (e.g., more in some, less in others) and that one hypothesis may not explain all insect-plant relationships. However, Root (1973) proposed two explanations for the monoculture effect and hypothesized that: (1) the level of complexity of the system is relative to the effectiveness of natural enemies to control herbivore populations (natural enemy hypothesis) and (2) specialized herbivores that can exploit the resources available in simple systems will reproduce in greater numbers than complex systems (resource concentration hypothesis). The facts that African black beetle is a polyphagous species (Bailey, 2007) and has the ability to disperse by flight (Matthiesse and Learmonth, 1998) should be taken into account when developing a management plan, as surrounding areas could represent a potential habitat for harboring this insect pest (Frew et al., 2013).

GRAZING

Grazing is a naturally occurring event that has an effect on the botanic diversity and structure of an area; for this reason, grazing is considered as a potential management tool in grassland conservation (Tallowin et al., 2005). However, it is essential to be able to foresee how grazing can affect vegetation in order to increase spatial heterogeneity rather than decrease it (Adler et al., 2001). Continuous selective grazing by livestock gradually deteriorates the quality and composition of grassland as it can cause the loss of the most palatable species of sward (Dorrough et al., 2004). Ultimately, grazing can alter landscapes by inhibiting regeneration of woody trees and native vegetation (Bennett et al., 1994).

Overbeck (2014) found that the influence of grazing on vegetation richness is relative to the productivity of the site, whereby low-productivity sites experienced a decrease in vegetation richness while high-productivity sites experienced an increase in vegetation richness. On the other hand, the level of grazing pressure has been found to affect beneficial invertebrates (e.g., bumblebees and spiders), as their abundance and species richness decreases under severe grazing regimes (Luff and Rushton, 1989; Tallowin et al., 2005).

It has been argued that plant and structural diversity in agricultural landscapes positively affects the abundance and diversity of natural predators of invertebrates thus offering improved biological control (Fiedler et al., 2008; Woltz et al., 2012). However, lack of knowledge on the biology and ecology of such predators represents a limiting factor when incorporating them into a management program (Horne and Page, 2008).

Some recommendations on grazing management for pasture beetle prevention include reducing cattle numbers in the affected paddocks early in the year when damage is at its peak (Blank and Olson, 1988; Berg et al., 2014) and reducing ground cover for egg-laying in early spring by heavy grazing and/or keeping pasture short (e.g., cut for silage, Douglas, 1972).

NATURAL PREDATORS AND PARASITES

The presence of natural predators affects pest-host plant relationships by hindering pests, causing them to utilize unsuitable areas that are less productive, or even cease feeding/reproduction completely; as a result, the outbreak phase can be delayed by controlling population numbers when they are below plague levels (Riechert, 1999). It has been suggested that pest regulation by natural predators plays a key role in the prevention of pest outbreaks in sustainable agricultural systems (Kromp, 1999). East et al., 1981 argues that predators of scarab pests are frequently insignificant in improved grassland. However, removal of overgrown grass has been shown to increase larval predation by birds (e.g., starlings, East and Pottinger, 1975). In order to prevent economic losses, optimal density of predators and distribution of refugia in fields must be determined (Collins et al., 2002).

Arthropod predators known to prey upon African black beetle in its native range and also in other regions include scoliid and tiphiid (Hymenoptera), tachinid flies and a number of beetles belonging to the families Carabidae, Staphylinidae, and Elateridae (Cameron et al., 1979). However, only carabids of the genus *Scarites* have been found to be significant as their populations are more abundant and stable than some of the other predators (Valentine, 1979). Even though carabids are considered potential pest-control agents because of their wide range of prey (Kromp, 1999), we currently have limited knowledge on their ecology in Australia and how efficient they are at controlling particular pest species, including African black beetle (Horne and Page, 2008).

Some of the vertebrate predators that have been reported to consume African black beetle include the Amur falcon (*Falco amurensis*), lesser kestrel (*Falco naumanni*) (Pietersen and Symes, 2010), starling (*Sturnus vulgaris*) (East and Pottinger, 1975), Hadeda ibis (*Bostrychia hagedash*), the cattle egret (*Bubulcus ibis*), Guinea fowl (Numididae), moles and rodents (Valentine, 1979). There is a lack of information on the vertebrate predators of African black beetle in Australia but from some publications the straw-necked ibis (*Thresbiornis spinicollis*), white ibis (*Threskiornis moluccus*) (Carrick, 1959), the Australian magpie (*Cracticus tibicen)* and the Australian raven (*Corvus coronoides*) (Ford et al., 2001) have been cited to consume them in highly infested areas. Consequently these bird species are often used as a cue for selecting beetle sampling sites. In addition, foxes (*Vulpes vulpes*) at times heavily rely on insect consumption, but in these particular cases it is usually related to availability of the insect species or population levels; remains of pasture beetles *Aphodius howitti* and *Rhopaea heterodactyla* have been found in stomachs of several foxes (Coman, 1973). Similarly, from field observations, fecal pellets (which are believed to be from fox) containing the exoskeleton of the beetles have been found on paddocks where African black beetles were abundant. However, the effectiveness of biological control by these predators is limited to their abundance and to the areas in which they coexist with their prey (East and Pottinger, 1975). Moreover, vertebrate predators may cause damage to pastures (i.e., scratch the top soil) in order to locate and subsequently feed on scarab larvae (Georgis et al., 2006).

ENTOMOPATHOGENS

Nematodes

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are often used as biological control agents of economically important insect pests due to the fact they are obligate parasites (Shapiro-Ilan et al., 2012). Unlike some parasitic nematodes, EPNs have a mutualistic relationship with pathogenic bacteria of the genera *Photorhabdus* for Heterorhabditidae, and *Xenorhabdus* for Steinernematidae (Lacey et al., 2001; Lewis et al., 2006). Both genera of symbiotic bacteria are motile and gram-negative Enterobacteria (Burnell and Stock, 2000). A number of pasture pests can be managed using nematodes including white grubs (Coleoptera: Scarabaeidae), mole crickets (*Scapteriscus* spp.), billbugs (*Sphenophorus* spp.), and the black cutworm (*Agrotis ipsilon*) (Georgis et al., 2006).

The process of infection is that juvenile nematodes seek out a suitable host to attach and penetrate (Lewis et al., 1993). Host penetration can occur through thin parts of the cuticle, spiracles (tracheae), mouth, and anus (midgut) (Koppenhöfer et al., 2000). The nematode-bacterial complex becomes lethal once it reaches the haemocoel, where the bacteria are released and multiply, killing the host within 48 h (Lewis et al., 1993; Lacey et al., 2001). When seeking potential pathogenic agents, Longworth and Archibald (1975) found that a nematode, *Neoaplectana* sp. (Steinernematidae), was present in African black beetle larvae.

However, there are a number of limitations for the use of EPNs as pest control agents. Factors that affect EPNs include accumulation of thatch in soil, soil temperatures below 20°C, soil texture (fine is better), moisture retention, and irrigation (Georgis and Gaugler, 1991). At present the costs of using EPNs are much higher than those associated with use of commercially available chemical insecticides (Georgis et al., 2006), making them economically nonviable. It has been suggested that EPNs may play an important role in integrated pest management (IPM) in the future as insects become more resistant to pesticides (Lacey et al., 2001). There is evidence of synergism between imidacloprid and EPNs against third instar scarab larvae (Koppenhöfer et al., 2000). However, imidacloprid efficacy decreases against scarabs in the latter stages of larval development (third instar), which are known to cause the most damage (Lacey et al., 2001).

Bacteria

There are a number of potential bacterial control agents for insect pests of pastures.

Bacillus thuringiensis

Bacillus thuringiensis (*Bt*) is a gram positive spore-forming bacterium that has been widely suggested as a biological control agent against agricultural pests (Kati et al., 2007). The insecticidal properties of *Bt* are associated with Cry proteins (δ-endotoxins) which are synthesized as parasporal crystals during sporulation of the bacteria (Deml et al., 1999, **Figure 3**). Different varieties

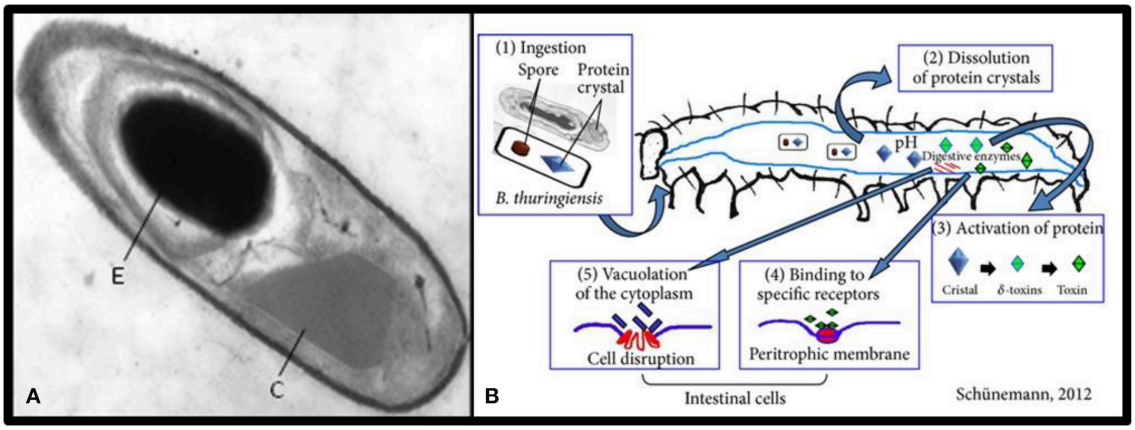


FIGURE 3 | (A) Transmission electron micrograph of a longitudinal section of *Bacillus thuringiensis* toward the end of sporulation; the spore (E) and the crystal inclusion (C) (Sanchis, 2010). **(B)** The mode of action of *Bacillus thuringiensis* (Schünemann et al., 2014).

of *Bt* produce different toxins that are specific to targets from Lepidoptera to Diptera and Coleoptera (Cidaria et al., 1991). Four major classes of insecticide crystal protein (ICP) genes have been identified: Lepidoptera-specific (CryI or Cry1), Lepidoptera- and Diptera-specific (Cryll or Cry2), Coleoptera-specific (CryIII or Cry3), and Diptera-specific (CryIV or Cry4) proteins (Chambers et al., 1991).

A study evaluating the effects of *Bt*-maize expressing Cry1Ab on non-target species found that there was no effect on mortality, mass, fertility, or fecundity of *Heteronychus arator* and *Somaticus angulatus* (Coleoptera) (Erasmus and Berg, 2014). However, there is evidence of cross-order toxicities occurring on target species, for example, Cry1Ab affecting mosquitoes *Aedes aegypti* (Diptera) (Haider et al., 1986), and CryIIIA having a comparable toxicity to CryIA in a number of caterpillars (Lepidoptera) (Deml et al., 1999). Van Frankenhuyzen (2009) highlighted that cross-order toxicities have been reported for 15 of the 87 insecticidal crystal protein families and that these numbers are likely to increase as testing across orders is expanded. Furthermore, *Bt* toxins have been found to affect mortality, development and longevity of parasitoid species that use target Lepidoptera as a host (Romeis et al., 2006).

Currently two methods are used to deliver *Bt* insecticidal proteins: formulated products prepared from naturally occurring or conjugated strains and development of transgenic plants which possess the genes responsible for the production of the toxin (Lacey et al., 2001). Some of the *Bt*-transgenic crops used include potatoes (Arpaia et al., 2000), eggplant (Arpaia et al., 2007), cotton (Sivasupramaniam et al., 2008), rice (Han et al., 2015), tobacco (Gore et al., 2005), and maize (Erasmus and Berg, 2014).

Paenibacillus sp.

Milky disease in scarab beetles receives its name from the milky aspect of the larva caused by a build-up of bacterial spores and parasporal bodies in the blood (Klein and Kaya, 1995). Milky disease comprises a number of species and strains of spore-forming rod bacteria which differ in morphology and virulence to specific hosts (Steinkraus and Tashiro, 1967). Milky disease bacteria can be found in scarab populations on all continents (Jackson and Klein, 2006). *Paenibacillus popilliae* and *Paenibacillus letinmurbos* (formerly *Bacillus*) are responsible for causing milky disease in Japanese beetle (*Popillia japónica* N.) and several other members of the scarab family (Dutky, 1940; Beard, 1956; Pettersson et al., 1999; Stahly et al., 2006). Bacterial spores are consumed by larvae while feeding on plant roots. Once the spores reach the gut, germination takes place followed by penetration of the haemocoel by vegetative cells (Harrison et al., 2000). Vegetative growing bacteria then sporulate in an asynchronous fashion leading to the death of the larvae (Rippere et al., 1998). Infectivity of *P. popilliae* varieties among scarabs tends to be higher in the species from which they were isolated (Klein and Kaya, 1995).

Dutky (1940) described the differences between the milky disease caused by *Paenibacillus popilliae* and *Paenibacillus letinmurbos*, describing them as type A and type B, respectively. Macroscopically they cannot be distinguished, however, the general appearance is quite different. In type A larvae tend to have a milk white coloration while in type B larvae turn muddy brown color. This coloration is due to the formation of haemolymph clots which block the insects' circulation resulting in gangrenous condition of the affected parts (Stahly et al., 2006).

Similar to *Bt*, *P. popilliae* produces parasporal crystals upon sporulation (Klein and Kaya, 1995; Deml et al., 1999). It has been suggested that parasporal crystal proteins may play a part in the mortality caused by milky disease because of the strong similarities and conservation of the hydrophobicity distribution of Cry proteins from *Bt* and *P. popilliae* (Zhang et al., 1997). While most of the insecticidal activity of *Bt* has been linked with the proteinaceous toxins located in parasporal inclusion bodies (parasporal crystals) (Lacey et al., 2001), *P. lentimorbus* causes a disease that is almost identical to that caused by *P. popilliae*, and has no parasporal inclusion (Stahly et al., 2006).

Despite the limitations of infectivity due to specificity from different varieties of *P. popilliae* (Klein and Kaya, 1995) this bacterium relies on the presence of viable spores to infect its

host as vegetative cells experience a decrease of viability in soil as well as deficient virulence (Stahly and Klein, 1992). Similarly with *Bt*, Head et al. (2002) demonstrated that Cry proteins accumulated in soil due to the continuous use of transgenic *Bt* cotton are subsequently incorporated into the soil resulting in no detectable immunological and biological activity. Furthermore, other factors such as application of insecticides and fungicides have been shown to affect spore viability in soil (Dingman, 1994).

Serratia sp.

Bacteria which cause Amber disease have been mentioned in the literature as a potential biological control option for scarabs (Jackson and Klein, 2006). *Serratia entomophila* (Enterobacteriaceae) is a gram-negative nonspore-forming, non-encapsulated, straight rod bacterium with peritrichous flagellae (Grimont et al., 1988). Pathogenic strains of *S. entomophila* infect their host by colonizing the larval gut and adhering to the crop; as a result starvation is induced causing the depletion of the fat bodies (Klein and Kaya, 1995). Consequently, this series of events ultimately causes the appearance of an amber color (Jackson et al., 1993), which gives its name to the disease (Klein and Kaya, 1995). It has been found that pathogenic strains of *S. entomophila* and *S. proteamaculans* causing amber disease contain a specific plasmid (Hurst et al., 2000). However, despite intensive testing, no other scarab species apart from *Costelytra zealandica* has been found susceptible to the plasmid-bearing strains (Jackson and Klein, 2006).

Rickettsiella sp.

Rickettsiella sp. is another bacterium that has been isolated from African black beetle and thus highlighted as a potential biological control agent for scarabs. Along with a protozoan, possibly *Adelina* sp., *Rickettsia* sp. was the most abundant pathogen isolated by Longworth and Archibald (1975). The genus *Rickettsiella* is made-up of intracellular bacterial pathogens of a wide range of arthropods (Leclerque et al., 2011). They are characterized by causing intracoelomic infections, multiplying in vacuolar structures within fat body cells and are often associated with protein crystals (Kleespies et al., 2011). However, infected larvae may live for several months (Longworth and Archibald, 1975).

PROTOZOA

Protozoan control agents offer persistence in host populations while decreasing overall fitness and reproduction of the target species, however, they produce low levels of immediate mortality (e.g., chronic infections) and *in vivo* production is required to prepare and release overwhelming amounts of the control agent (inundative application) (Lacey et al., 2001).

VIRUS

Isolations from diseased larvae and adults of African black beetle have revealed a number of pathogens, including a small isometric virus (30 nm in diameter) that develops in the cytoplasm of gut and fat-body cells (Longworth and Archibald, 1975). Longworth and Carey (1976) described this RNA virus as being icosahedral in shape without any obvious surface features. Moreover, it has a sedimentation coefficient of 137S, a buoyant density in CsC1 of 1.33 g/ml and RNA:protein ratio of 28.2:71.8. The virus was found to be infective for numerous species in the Lepidoptera and Coleoptera orders and also for *Drosophila melanogaster* cells in tissue culture (Crump and Moore, 1981). However, the low infection rate of the virus on African black beetle observed by Longworth and Archibald (1975) in the field was not enough to explain the mortality observed in the population.

FUNGI

Entomopathogenic fungi of the genera *Metarhizium* and *Beauveria* are omnipresent in soils; however, infectivity in scarabs is limited to certain strains mostly of the species *B. brongniarti* and the large-spored *M. anisopliae* var. *majus* (Jackson and Klein, 2006). *Beauveria* sp. has been isolated from African black beetle larvae (Longworth and Archibald, 1975) and has been considered as a potential biological control agent. Zimmermann (2007) summarized the infection pathway of *Beauveria* sp. and other entomopathogenic fungi in a sequence of events: attachment of the spore to the cuticle, germination, penetration of the cuticle, overcoming the immune response of the host, proliferation, saprophytic outgrow from the carcass and production of new conidia. However, when considering entomopathogenic fungi as biological control agents for soil-dwelling species, the ability of the entomopathogen to persist for an extended period of time as well as its infectivity to the host must be taken into account (Lingg and Donaldson, 1981). The survival and proliferation of these fungi can be affected by a number of abiotic factors such as temperature, humidity or moisture and solar radiation (Zimmermann, 2007).

CHEMICAL CONTROL

Management of subterranean pest species such as African black beetle is challenging because of the high damage potential per individual, therefore the success of any control method(s) depends on the reduction of the population to the minimum (Bulinski and Matthiessen, 2002). Many of the pesticides previously used for African black beetle control have either been withdrawn from the market or are no longer registered for that purpose. Traditionally, persistent broad-spectrum organochlorine products were deployed with cultivation and incorporated into the soil in order to protect crops from African black beetle (Bulinski and Matthiessen, 2002). In recent years, targeted insecticides such as insect growth regulators and neonicotinoid compounds have been developed (Jackson and Klein, 2006). Imidacloprid (Merit, Bayer, Kansas City, MO, USA) and halofenozide (Mach 2, RohMid, Parsippany, NJ, USA) have become widely used for preventive control of root-feeding scarabaeid grubs (Kunkel et al., 2001). However, Kunkel et al. (1999) found that imidacloprid and halofenozide may have disruptive effects on earthworms and some predatory invertebrates, but such effects are short-lived and unlikely to

cause pest outbreaks. In contrast, Prabhaker et al. (2011) found limited but detrimental effects of neonicotinoid compounds (imidacloprid and thiamethoxam) on some beneficial insects and maintained a more conservative approach, arguing that further investigation is required.

Imidacloprid and halofenozide are most effective against early larval instars (first and second instars), and must be applied before larval damage is visible (Jackson and Klein, 2006). However, third instar larvae are known to cause the most damage (Lacey et al., 2001). When evaluating spring and autumn applications of chlorpyrifos, alpha-cypermethrin, and diazinon for African black beetle control, Eden et al. (2011) concluded that the use of such pesticides is not recommended because of the difficulty in application timing, the inefficiency of treatments, and the likelihood that reinvasion will occur as these treatments do not prevent subsequent larval populations from causing damage.

SEED TREATMENT

It has been suggested that treated seeds (i.e., dressing, film coating, pelleting, or multilayer coating) present an environmentally safe method of protection for young plants against insect pests (Elbert et al., 2008). Seeds coated with insecticides (imidacloprid and furathiocarb) produce plants that are protected against stem borers (e.g., African black beetle) through systemic translocation of the insecticides (Drinkwater and Groenewald, 1994). However, Drinkwater (2003) found that in order to deter beetles, they have to feed on the plant first. Furthermore, a number of biological factors such as the age of the beetle influence the level of efficacy of the compound (Drinkwater, 2002). Drinkwater (2003) concluded all neonicotinoids evaluated significantly reduced insect damage to the host plant, but only imidacloprid reduced beetle abundance. Bell et al. (2011) suggested that treated seeds might play a crucial role in pasture establishment during outbreak years, as well as helping to control population numbers and avoid the risks of population build-up after pasture renewal.

SILICON SUPPLEMENTATION

Plant silicon is known to play a role in defense against pathogens and herbivores (Epstein, 2009). In grasses, silicon-based defenses provide a physical barrier that counters herbivores and pathogens (Massey et al., 2006; Massey and Hartley, 2009; Reynolds et al., 2009).

Massey and Hartley (2009) demonstrated that silica-rich diets increase mandible wear and decrease digestibility and absorption of nitrogen from food plants in African armyworm (*Spodoptera exempta*). In addition, silicon can also affect subterranean herbivores. Frew et al. (2016) found that silicon applications can play a significant role in defense against root-feeding pests such as greyback cane beetle larvae (*Dermolepida albohirtum*).

Silicon is the second most abundant element in soils (Epstein, 1994) but needs to be in the soluble form of monosilicic acid [$Si(OH)_4$] to be taken up by the plant roots (Guével et al., 2007). Once metabolized, silicon can provide a physical defense based on the mechanical properties of opaline silica (Garbuzov et al., 2011). Silicon concentrations within a grass species are not static but can increase when the plant is under herbivore attack (Massey et al., 2007), suggesting that there is a fitness cost associated with this defense (Garbuzov et al., 2011). It is thought that silicon defense fitness costs might place the plant at a disadvantage against its competitors in the absence of herbivores (Hanley and Sykes, 2009).

Silicon supplementation has shown promising results at deterring herbivores above and below ground (Massey and Hartley, 2009; Frew et al., 2016). It relies, however, on the availability of soluble silicon (Guével et al., 2007) and herbivore stimuli for plants to invest in this defense strategy (Massey et al., 2007). Silicon supplementation could complement other management strategies such as chemical defenses that can improve overall plant health and resistance (i.e., endophytes). However, it is important to take into account that silicon may reduce digestibility and grazing preference in vertebrates (e.g., sheep, Glenn et al., 1989).

ENDOPHYTE AS A CONTROL METHOD

Epichloë (syn. *Neotyphodium*) has been described in early publications as an endophytic fungus of grasses such as perennial ryegrass and tall fescue (Sampson, 1933). Although endophytes are inconspicuous *in planta* (Iannone et al., 2011), infected plants can experience increased growth, reproduction, and resistance to various biotic and abiotic stress factors (Clay and Schardl, 2002). Biotic resistance of endophyte-infected plants has been associated with an array of secondary metabolites (alkaloids) produced by the fungus that benefit the host plant as they provide resistance against herbivores and pathogens (Siegel et al., 1987; Wilson, 1993; Zain, 2011). Toxicosis in cattle and sheep has been associated with the ingestion of endophyte-infected pastures, decreasing animal performance, and in some cases causing death (Fletcher and Harvey, 1981; D'Mello and MacDonald, 1997). It has been determined that ergopeptine (ergovaline) alkaloids are responsible for causing tall fescue staggers or fescue toxicosis (Paterson et al., 1995), while isoprenoid lolitrem (lolitrem B) alkaloids are responsible for causing ryegrass staggers (Smith et al., 1997).

On the other hand, some alkaloids have proven to be beneficial, conferring insecticidal properties to the plant, such as the pyrrolopyrazine alkaloid peramine that acts as a feeding deterrent to the Argentine stem weevil (Rowan and Gaynor, 1986; Rowan, 1993) and epoxy-janthitrems (indole-diterpenes) which are produced by an endophyte variety called AR37 (Thom et al., 2014). Epoxy-janthitrems have been reported not to cause ryegrass staggers in cattle (Moate et al., 2012). In addition, loline has shown both feeding deterrence and insecticidal activity (Schardl et al., 2007), while only causing negative effects in mammals at extremely high concentrations (Strickland et al., 1994; Oliver et al., 1998).

When comparing livestock performance on endophyte-infected and endophyte-free swards, Prestidge et al. (1982) found that non-infected pasture was severely damaged by the Argentine

stem weevil, highlighting the importance of the endophyte (Prestidge et al., 1982). Endophytes have been reported to offer protection against a number of insect pests, including black cutworm (*Agrotis ipsilon*) (Baldauf et al., 2011), pasture mealybug (*Balanococcus poae*) (Pennell et al., 2005), Argentine stem weevil (Prestidge and Gallagher, 1988b), root aphids (Popay and Thom, 2009), and Japanese beetle and other white grubs (Scarabaeidae spp.) (Potter et al., 1992) including African black beetle (Bell et al., 2011).

In a field trial comparing different grass treatments, African black beetle populations in perennial ryegrass pastures harboring AR37, AR1, and wild-type endophyte remained low and their mean densities in these treatments were significantly less than those pastures without endophyte (Thom et al., 2014). Therefore, considerable research has been done on the alkaloids produced by fungi because of the services they provide to their host plant and agricultural systems (Clay and Schardl, 2002).

It has been found that the level of chemicals produced by an endophyte in symbiosis with a plant varies depending on the endophyte-plant combination (Panka et al., 2013). This is particularly important as endophyte-infected grasses containing ergot alkaloids (ergovaline) that are known to have detrimental effects on cattle (Smith et al., 1997; Bell et al., 2011) can also deter important pests such as African black beetle (Ball et al., 1997b). Therefore, screening endophyte-plant combinations to find a balanced chemical profile that protects the plants from pests without affecting cattle would be beneficial. In order to achieve this, it is necessary to understand the chemistry behind these processes (i.e., active compounds, intermediate compounds, and possible synergistic effects).

Ball et al. (1997b) tested alkaloid toxicity on adult African black beetle by incorporating them in an artificial diet, and found that ergopeptine alkaloids significantly reduced feeding at concentration of 5 μg/g, whereas ergopeptine epimer and its analogs were also active but to a lesser extent. In addition, he found that peramine, lolitrem B and a number of ergot alkaloids had no effect on deterring adult beetles, except for ergonovine which showed moderate activity.

As for insecticides (Jackson and Klein, 2006), the effects of endophyte infected grasses on African black beetle can vary depending on their different life stage. Previous studies have demonstrated that certain endophyte strains deter adult beetles from feeding (Ball et al., 1994), resulting in a decrease of survival and oviposition. However, commercially available endophyte strains do not seem to have negative effects on the larval form (Bell et al., 2011). Similarly, Watson (2006) found no evidence of alkaloids produced by endophyte-infected perennial ryegrass or tall fescue affecting redheaded cockchafer and black headed cockchafer larval stages. However, Bryant et al. (2010) found loline concentrations in excess of 1700 μg/g DM were particularly effective in reducing feeding and development of second instar redheaded cockchafer but not African black beetle larvae.

Endophytes have been screened to produce less toxic profiles to livestock, whilst maintaining other beneficial traits, such as the production of insect deterrent alkaloids (Johnson et al., 2013). In the case of vertebrates, it has been determined that some toxic alkaloids, such as ergot alkaloids, behave like neurotransmitters (i.e., dopamine, serotonin, and adrenaline) causing vasoconstriction, smooth muscle contraction, bewilderment, and hallucinations (Beaulieu et al., 2013). In addition, penitrem, paxilline, and lolitrem B are also known to be tremorgenic and have been associated with diseases of domestic animals and mice (Gallagher et al., 1981; Smith et al., 1997). However, the exact mechanisms of action of how endophytes affect soil-borne herbivores are still to be determined (Malinowski and Belesky, 2000). In a bioassay conducted on Argentine stem weevil, Rowan (1993) found definite but minimal structural requirements for insect deterrence activity caused by peramine and its analogs. All analogs tested were less active than peramine itself, suggesting some importance for the guanidinium group and the side-chain in obtaining the full biological response. A better understanding of the mode of action of alkaloids on soil-borne insects might provide valuable information for the development of novel endophytes to control the more resistant life-stage (i.e., larval form).

Clay (1989) suggested that an efficient biological control agent is characterized by its capacity to significantly decrease pest damage either by directly killing or damaging the pest, reducing its population growth, or by deterring the pest before it can do any damage. Endophytes have shown to offer insect deterrent activity to their host plant against certain pests as well as inducing resistance in their host plant to various other biotic factors (Clay and Schardl, 2002).

Even though there are a number of pathogens associated with scarabs, it appears that many occur at low levels and scarabs appear to show inherent resistance to many generalist pathogens (Jackson and Klein, 2006). In contrast, endophytes as a control method have a clear advantage, as they are present within host-plant grasses (Iannone et al., 2011) and they are transmitted vertically through seeds (Schardl, 1996). Therefore, in terms of presence, endophytes can be expressed in paddocks offering continued protection to their host plant. In addition, the chemical profile produced by an endophyte, in symbiosis with a plant, varies depending on the endophyte-plant combination (Panka et al., 2013) and as a result, endophyte-plant combinations could be selected according their chemical profile and the target pest affecting the plant.

Wild-type endophyte-infected grasses which contain lolitrem B and ergovaline offer insect control, but consumption by dairy cows may result in ryegrass staggers, reduction in feed intake, and losses in milk production (Thom et al., 2014). In recent years, development efforts have focused upon endophyte—grass host associations that produce little or no ergot alkaloids toxic to livestock yet still maintain pest resistance qualities of the more toxic profiles (Malinowski and Belesky, 2000).

Bell et al. (2011), however, states that endophytes could face limitations during African black beetle outbreaks, as insect-deterrence conferred by the best selected endophytes may not be sufficient to prevent larvae population build up or new infestations arising as a result of flight dispersal by adult beetles in late autumn or spring. Furthermore, Jackson and Klein (2006) concluded that while chemical control will be still used as a quick fix for scarab problems, integrated pest management (IPM) offers a better long-term solution. Integrated pest management

(IPM) refers to the synergistic use of multiple control strategies (e.g., cultural, chemical, and biological) based on surveillance information to assess and control pests in an ecologically and economically sound manner (All, 2005; Ehler, 2006). Kauppinen et al. (2016) proposed that *Epichloë* endophytes should be considered when developing sustainable management strategies for agriculture, as endophyte-infected grasses could be used as alternatives and/or in conjunction with synthetic plant protection products.

In view of the above, the use of endophytes may aid to control insect pest populations and therefore reduce the need for pesticide applications in the field. Prabhaker et al. (2011) argues that even though neonicotinoid compounds (imidacloprid and thiamethoxam) used for soil-borne insects are generally assumed to be safe they can have negative effects on beneficial insects (e.g., via food chain toxicity; following feeding on plant tissue or excretions) if they are exposed to the pesticide.

Consequently, the use of endophytes might allow the recruitment of natural predators which have been suggested to play a key role in the prevention of pest outbreaks in sustainable agricultural systems (Kromp, 1999). Riechert (1999) argues that the presence of natural predators can affect pest species by deterring pests, causing them to utilize unsuitable areas that are less productive, or even cease feeding completely; ultimately, delaying the outbreak phase by controlling population numbers when they are below plague levels.

Endophytes may play a crucial role in IPM in sustainable agricultural systems, as they not only enhance host plant resistance to biotic factors but also to abiotic factors (Clay and Schardl, 2002). Some examples of IPM approaches for scarab control include the combination of entomopathogenic nematodes *Heterorhabditis megidis* and *Steinernema glaseri* with *Metarhizium anisopliae* (Ansari et al., 2004) and also the combination of imidacloprid with entomopathogenic nematodes (Koppenhöfer et al., 2000). However, the combination of two different control strategies does not necessarily result in the desired outcome. Walston et al. (2001) found a lack of synergism between endophyte infected perennial ryegrass and *P. popilliae* on Japanese Beetle. Perhaps a combination of chemical agents, such as targeted pesticides (e.g., imidacloprid) applied as seed treatments plus the application of soluble silica could help establish endophyte-infected grass when renewing pastures, and supplement entomopathogenic nematodes and other natural predators in an IPM strategy.

To conclude this literature review it is apparent that African black beetle is not a pest that can be controlled with a single strategy and requires a more holistic approach. The design of an IPM program that works on-farm is necessary. To achieve this, different methods of control that are synergistic should be aligned with farmers needs and capabilities. Based on the advantages described in this review, selection of an ideal endophyte-grass combination could be a first step to develop such an IPM program.

AUTHOR CONTRIBUTIONS

MK: researched the relevant literature and wrote the body of the article. AY: contributed with information regarding insects and ecology, as well as, editing the article. SR: contributed mainly in the biochemistry part of the article, information about secondary metabolites, as well as, editing the article. KG: contributed with information about ryegrass endophyte, as well as, editing the article. KP: edited the final version of the article. JE: edited the final version of the article. GS: provided overall direction for the program of work.

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Temperature and Plant Genotype Alter Alkaloid Concentrations in Ryegrass Infected with an *Epichloë* Endophyte and This Affects an Insect Herbivore

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Asexual *Epichloë* endophytes colonize agricultural forage grasses in a relationship which is mutually beneficial and provides the host plant with protection against herbivorous insects. The endophyte strain AR37 (*Epichloë festucae* var. *lolii*) produces epoxy-janthitrem alkaloids and is the only endophyte known to provide ryegrass with resistance against porina larvae (*Wiseana cervinata* (Walker)), a major pasture pest in cooler areas of New Zealand. This study examined the effect of temperature on concentrations of epoxy-janthitrems in AR37-infected ryegrass and determined how the resulting variations in concentration affected consumption, growth and survival of porina larvae. Twenty replicate pairs of perennial (*Lolium perenne* L.) and Italian ryegrass (*L. multiflorum* Lam.) plants with and without endophyte were prepared by cloning, with one of each pair grown at either high (20°C) or low (7°C) temperature. After 10 weeks, herbage on each plant was harvested, divided into leaf and pseudostem, then freeze dried and ground. Leaf and pseudostem material was then incorporated separately into semi-synthetic diets which were fed to porina larvae in a bioassay over 3 weeks. Epoxy-janthitrem concentrations within the plant materials and the semi-synthetic diets were analyzed by high performance liquid chromatography. AR37-infected ryegrass grown at high temperature contained high *in planta* concentrations of epoxy-janthitrem (30.6 μg/g in leaves and 83.9 μg/g in pseudostems) that had a strong anti-feedant effect on porina larvae when incorporated into their diets, reducing their survival by 25–42% on pseudostems. In comparison, *in planta* epoxy-janthitrem concentrations in AR37-infected ryegrass grown at low temperature were very low (0.67 μg/g in leaves and 7.4 μg/g in pseudostems) resulting in a small anti-feedant effect in perennial but not in Italian ryegrass. Although alkaloid concentrations were greatly reduced by low temperature this reduction did not occur until after 4 weeks of exposure. Alkaloid concentrations were slightly lower in Italian than in perennial ryegrass and concentrations were higher in the pseudostems when compared with the leaves. In conclusion, epoxy-janthitrems expressed by the AR37 endophyte show strong activity

against porina larvae. However, when ryegrass plants are grown at a constant low temperature for an extended period of time *in planta* epoxy-janthitrem concentrations are greatly reduced and are less effective against this pasture pest.

Keywords: endophyte, *Epichloë festucae* var. *lolii*, AR37, epoxy-janthitrem, ryegrass, temperature, porina, bioactivity

INTRODUCTION

Cool season grasses of the family Poaceae harbour fungal endophytes of the genus *Epichloë*. Asexual *Epichloë* endophytes grow as unbranched hyphae within the above ground tissues of the host plant and are transmitted between reproductive generations within the seed of its host. There is an ongoing debate over the nature of the relationship between endophytes and their host (Saikkonen et al., 1998, 2010). The relationship between agricultural forage grasses and asexual *Epichloë* endophytes, however, is thought to be defensive mutualistic. Defensive mutualism was first proposed by Clay (1988) and involves both organisms benefiting from the relationship. The endophyte gains from its host shelter, nutrients and a means of transmission (Saikkonen et al., 2004). In return the plant gains increased protection from biotic stresses including insects (Prestidge et al., 1982; Ball and Prestidge, 1992; Pennell et al., 2005; Popay et al., 2012), mammalian herbivores (Edwards et al., 1993; Cosgrove et al., 2002), pathogens (Pańka et al., 2013) and nematodes (Eerens et al., 1997; Bacetty et al., 2009) as well as increased tolerance to abiotic stresses such as drought and nutrient stress (Ravel et al., 1997; Kane, 2011; Nagabhyru et al., 2013).

Plants infected with an asexual *Epichloë* endophyte can have increased resistance against herbivorous insects due to the production of alkaloids which can have anti-feedant and/or toxic effects (Rowan et al., 1990; Jensen et al., 2009; Popay et al., 2009). Understanding bioactive alkaloids, their distribution within the plant and their effects on insects enables endophytes to be used in pest management strategies in both farming systems and turf. Fungal endophytes have been recognized as an important part of New Zealand's pastoral sector since the early 1980s, as New Zealand contains a number of herbivorous pasture pests which can cause severe pasture damage.

The common toxic endophyte (*Epichloë festucae* var. *lolii*) strain found naturally infecting ryegrass (*Lolium perenne* and *L. boucheanum* syn. *L. hybridum*) in New Zealand produces alkaloids which provide the host with protection against a number of important pest insects (Prestidge et al., 1982; Popay and Baltus, 2001; Pennell et al., 2005). It also, however, produces lolitrem B an alkaloid which causes ryegrass staggers, a neurological impairment (Cunningham and Hartley, 1959; Fletcher and Harvey, 1981; di Menna et al., 2012) and the alkaloid ergovaline which causes vasoconstriction in grazing livestock (Dyer, 1993; Klotz et al., 2007). Due to these harmful effects on livestock endophyte research in New Zealand has focused on identifying different *E. festucae* var. *lolii* strains from European grasslands, where there is a greater chemical diversity, in an attempt to select those with a favorable chemical profile. Endophyte strains that are found to produce beneficial alkaloids, to deter insects, but not the detrimental alkaloids are then inoculated into New Zealand ryegrass cultivars (Johnson et al., 2013). These strains are known as 'selected endophytes.' One selected strain of *E. festucae* var. *lolii* is AR37. The only known alkaloids to be produced by AR37 are the epoxy-janthitrems (Tapper and Lane, 2004; Finch et al., 2007, 2012), a group of five compounds within the indole diterpene class of alkaloids. The epoxy-janthitrems are lipophilic compounds and are not easily translocated around the plant. Therefore, concentrations are thought to be highest in the pseudostem where endophyte mycelia are concentrated. AR37 provides ryegrass with protection against many of New Zealand's major ryegrass pests including; African black beetle adults [*Heteronychus arator* (F.) Coleoptera: Scarabaeidae] (Ball et al., 1994), Argentine stem weevil larvae [*Listronotus bonariensis* (Kuschel), Coleoptera: Curculionidae] (Popay and Wyatt, 1995), root aphid [*Aploneura lentisci* (Passerini), Aphididae: Fordinae] (Popay et al., 2004; Popay and Gerard, 2007) and porina larvae (*Wiseana* spp. Hepialidae: Lepidoptera) (Jensen and Popay, 2004).

Porina are a group of seven closely related moth species endemic to New Zealand. The larvae of many of these species are a pest of cultivated grasses (Dugdale, 1994), particularly in the lower half of the North Island and in many parts of the South Island of New Zealand. Temperature is one of the main environmental factors which influences the location of porina in New Zealand. A study by Allan et al. (2002) looked at survival of larvae to pupation and then adulthood at four temperatures. Larval survival was found to be significantly lower when larvae were grown at 20°C compared to those grown at both 10 and 15°C. But survival was higher at 20°C than 5°C. Porina larvae are nocturnal and emerge at night from vertical burrows created beneath the soil surface (Barlow et al., 1986). Larvae can be highly destructive as they feed by cutting ryegrass tillers off at the base of the plant or by grabbing low lying leaves before dragging the herbage back into their burrow (Harris, 1969). The 'selected' endophyte AR37 has been shown to provide ryegrass with resistance against porina larvae in pot trials (Jensen and Popay, 2004), choice bioassays (Jensen and Popay, 2004) and field trials (Popay et al., 2012). In addition, when pure and semi-pure epoxy-janthitrem I, produced by AR37, was incorporated into a semi-synthetic diet and fed to porina larvae, larval diet consumption and growth were significantly reduced (Finch et al., 2010; Hennessy, 2015).

Several abiotic and biotic factors including plant growth temperature (Ball et al., 1995; Eerens et al., 1998; Salminen et al., 2005) and plant genotype (Adcock et al., 1997; Easton et al., 2002; Faeth et al., 2002) are known to affect alkaloid

Abbreviations: HT, high temperature (20°C); LT, low temperature (7°C).

concentrations within endophyte-infected ryegrass. What effect these factors have on epoxy-janthitrem concentrations in ryegrass is not known. In this paper, the results of two experiments, a ryegrass pot trial and a porina larval bioassay, were designed to investigate the effect of high (20°C) and low (7°C) growth temperature on epoxy-janthitrem concentrations in AR37-infected perennial (*L. perenne* L.) and Italian (*L. multiflorum* Lam.) ryegrass and to examine how any resulting variations in concentration would affect consumption, growth and survival of porina larvae.

MATERIALS AND METHODS

Establishment of Ryegrass Plants

Diploid perennial (cv 'Grasslands Samson') and Italian [cv 'Grasslands Asset' (PG255)] ryegrass plants were germinated from AR37-infected and endophyte-free seed in a Petri dish lined with moist filter paper. Germinated seedlings were sown into trays filled with potting mix (a commercial potting mix composed of N.Z. pine bark fines and fiber, pumice, coco fiber, controlled release fertilizer and a wetting agent (Daltons commercial)) on the 23rd of September (spring) and left to establish in a glasshouse. After seven and a half weeks plants were tested for endophyte infection using a tissue print immunoassay technique (Simpson et al., 2012). Thirty plants of each plant/endophyte treatment (AR37-infected perennial ryegrass, endophyte-free perennial ryegrass, AR37-infected Italian ryegrass and endophyte-free Italian ryegrass) were cloned (split in two) and planted into individual pots (12.5 cm by 10 cm) filled with potting mix (Daltons commercial). Plants were left to establish in a screenhouse for 16 weeks and were maintained with regular watering, trimming and fertilizing (1.8 g/L Thrive® and 1.3 g/L urea).

Establishment and Maintenance of a Porina Larval Colony

Forty female porina moths were collected in November–December 2013 from Allanton, near Mosgiel, in the South Island of New Zealand using an incandescent light as an attractant. Moths were held in 60 mL specimen vials overnight, to allow female moths to lay their eggs. The bursa copulatrix of the female moth was examined to determine the species of porina (Dugdale, 1994). Larvae from eggs laid by *Wiseana cervinata* moths were selected for this study. Porina eggs were sent to AgResearch, Ruakura Research Centre, Hamilton, New Zealand where they were surface sterilized with a copper oxychloride solution (Carpenter, 1983). Sterilized eggs were placed in a Petri dish lined with moist filter paper and left to hatch in an 18°C controlled environment (CE) room. Hatched larvae were placed into plastic rectangular containers (1000 mL) quarter filled with fine sized bark chips (40 larvae per container). Larvae were fed a semi-synthetic diet (Popay, 2001) which was cut into small pieces and evenly spread over the surface of the bark. Larvae were initially maintained at 15°C, but the temperature was later decreased to 7°C to slow larval growth. Larvae were maintained for 8 months with weekly diet changes.

Effects of Temperature on Epoxy-Janthitrem Concentrations

The ryegrass pot trial contained eight treatments: Endophyte (AR37-infected or endophyte-free) × Temperature [high (20°C) or low (7°C)] × Plant species (Perennial or Italian ryegrass). Twenty healthy pairs of cloned plants from the original 30 cloned for each treatment were selected for the experiment. One of each cloned pair was randomly assigned to CE rooms, set at either 20 or 7°C with both set at a 12:12 h light:dark cycle. Plants were set up in identical randomized block designs in each room, with the same proximity to lights.

A herbage sample was taken from each plant at the beginning of the trial and after 4 weeks to compare changes in epoxy-janthitrem concentrations between treatments. At each of the two time points (Weeks 0 and 4) two tillers per plant were removed, the leaves and pseudostems (base of the plant to the first emerging leaf) were separated and material from five replicate plants combined to produce four replicate composite samples to be analyzed for epoxy-janthitrems. Immediately after samples were harvested they were put into sealed plastic bags and placed inside a chilly bin containing a cold pack. Samples were then frozen at −20°C approximately 20 min after harvest. After 10 weeks of growth in the CE rooms all plant material was harvested by replicate over a period of 2 weeks. Ryegrass was harvested by cutting all tillers off at the base of the plant; care was taken to ensure the meristem was included in the sample. Dead material was removed from the sample and live pseudostems and leaves were separated. All ryegrass samples taken were frozen soon after their harvest and later freeze dried and ground to a very fine powder. Total epoxy-janthitrem concentration (all five epoxy-janthitrem compounds) was determined by high performance liquid chromatography (HPLC).

To obtain a representative ryegrass sample of each treatment to be tested on porina larvae in the larval bioassay an approximately equal amount of ground plant material from the final harvest of each plant in a treatment (20 plants) was combined and mixed thoroughly. Three samples (3 g each, one for each week of the 3 weeks porina larval bioassay) of plant material from each treatment were weighed into separate glass vials and set aside for use in the porina larval bioassay.

Larval Bioassay

Plant material harvested from the eight treatments in the pot trial described above was fed to porina larvae in a bioassay. Tillers were separated into pseudostems and leaves and were tested separately to give a total of 16 treatments, with 12 replicate larvae per treatment. Porina larvae (32 weeks old), weighing between 226 and 692 mg, were selected from 27 parent moths. Larvae were removed from their containers and starved overnight before being weighed and assigned to a replicate so that larvae within a replicate were of similar weight. Within each replicate, larvae were randomly allocated to a treatment. Individual larvae were then placed into specimen containers (150 mL polystyrene) three quarters full with fine sized bark chips. Larvae were fed plugs (14–15 mm diameter cut with a cork borer, average weight of

788 mg) of a semi-synthetic diet containing ground plant material from each of the treatments. Fresh diets were made weekly and diets changed over in each larval container on days 4 and 7 of each week. Diets were kept at 4°C between diet changes. Consumption was estimated by change in diet weight between diet changes. Larvae were checked for survival at each diet change and weighed again after 3 weeks at the completion of the trial. Total epoxy-janthitrem concentration in fresh diets and remnant diets (diets larvae had fed on for 3–4 days) were determined by HPLC.

The insect bioassay was conducted in a CE room at 15°C. Specimen containers were placed into polystyrene trays that were covered with black polythene to exclude light. These conditions were necessary as epoxy-janthitrems degrade when exposed to light.

Semi-Synthetic Diet

Fresh carrot (500 g) was blended with Milli-Q water (1000 mL) and strained to obtain carrot juice (750 mL). Carrot juice was mixed with agar (18 g) and warmed in a microwave until boiling point. Diet was kept warm in a water bath, to prevent agar setting, while individual diets were made. Sixteen batches of diet (27 mg) were weighed out separately into warm glass beakers. One of the ground ryegrass samples (3 g) was added to each beaker, mixed thoroughly and then poured into a Petri dish and smoothed flat. Petri dishes were wrapped in tin foil to exclude light.

Alkaloid Analyses

Epoxy-janthitrem concentrations in both herbage and diet samples were quantified by HPLC. Epoxy-janthitrems were extracted from ground herbage (20 mg) or diet samples (50 mg) with water-acetone (1:4, 1 mL) using an over-over mixer at 30 rotations/min for 1 h. The extract was then centrifuged (1 min, 5600 *g*, Eppendorf, Hamburg, Germany) and analyzed by HPLC. Epoxy-janthitrems were quantified by comparison with a reference standard (N-benzyl-1, 8-naphthaleneimide, 5 μg/mL) which had previously been compared with a pure epoxy-janthitrem I standard (Finch et al., 2012, 2013). Due to the instability of epoxy-janthitrems the use of an epoxy-janthitrem standard is not practical for routine analysis. Samples were protected from light during extraction and analysis. For analysis of extracts a 4.6 mm × 250 mm ODS C18 column (Phenomenex, Torrance, CA, USA) fitted with a 4 mm × 3 mm Phenomenex Security GuardTM containing two C18 cartridges (Torrance, CA, USA) was used with an eluent of water-acetonitrile (1:19, 1 mL/min). Eluting compounds were detected with an Agilent Series 1100 fluorescence detector (excitation at 333 nm, emission detection at 385 nm).

Statistical Analyses

Data on epoxy-janthitrem concentration, larval diet consumption, mortality and growth collected during the bioassay were analyzed using GenStat 16th and/or 17th edition. Epoxy-janthitrem concentrations in ryegrass plants at the beginning of the trial, after 4 weeks and after 10 weeks of growth in the CE rooms were analyzed using 3-way analysis of variance (ANOVA) blocked by replicate, with treatment factors Temperature, Plant species, and Plant part. All variables were natural log transformed prior to analysis to stabilize the variance. Larval diet consumption data (average diet consumed per day) were analyzed using a REML linear mixed model, with replicate a random effect, with fixed effects of Endophyte by Temperature by Species by Plant part. To take into account the higher variance of data from the AR37 high temperature treatments compared with data from low temperature treatments, a separate residual variance was defined for the AR37 high temperature treatments. Larval growth data (not transformed) were analyzed using 4-way ANOVA blocked by replicate, with treatment factors Endophyte, Temperature, Species, and Plant part. In all analyses differences were compared using protected Fisher's least significant difference *post hoc* tests, conducted at the 5% significance level.

RESULTS

Effects of Temperature on Epoxy-Janthitrem Concentrations

Epoxy-janthitrem concentrations within the leaves and pseudostems of AR37-infected Italian and perennial ryegrass were determined at the beginning of the trial and then after 4 and 10 weeks to monitor changes in concentration over time at the different temperatures (**Figure 1**). When ryegrass was grown at high temperature (HT) epoxy-janthitrem concentrations were greatly increased. Concentrations were 2–3 times higher than the initial concentrations after 4 weeks and 3–7 times higher after 10 weeks. In contrast to this, concentrations declined in ryegrass pseudostems grown at low temperature (LT) although the decrease was small over the first 4 weeks.

After 10 weeks epoxy-janthitrem concentrations were highly variable among treatments and plants within a treatment especially in the two high temperature pseudostem treatments, which contained high epoxy-janthitrem concentrations.

On average, epoxy-janthitrem concentrations at the beginning of the trial were significantly higher ($P < 0.05$) in perennial ryegrass than in Italian ryegrass and this difference was maintained throughout the trial (**Table 1**). Concentrations were also significantly higher ($P < 0.05$) in the pseudostems when compared with the leaves of ryegrass plants at all three sample points. An interaction between Species and Plant part was significant at the beginning of the trial. In this interaction epoxy-janthitrem concentrations in perennial ryegrass leaves were significantly higher than those in Italian leaves. But there was no significant difference between perennial and Italian pseudostems. Temperature and the Temperature by Plant part interaction had a highly significant ($P < 0.001$) effect on epoxy-janthitrem concentration after 4 and 10 weeks, with concentrations significantly higher in pseudostems grown at high temperature.

Larval Bioassay

There were statistically significant effects of Endophyte, Temperature, Plant species, and Plant part on both larval diet consumption and larval growth (**Table 2**).

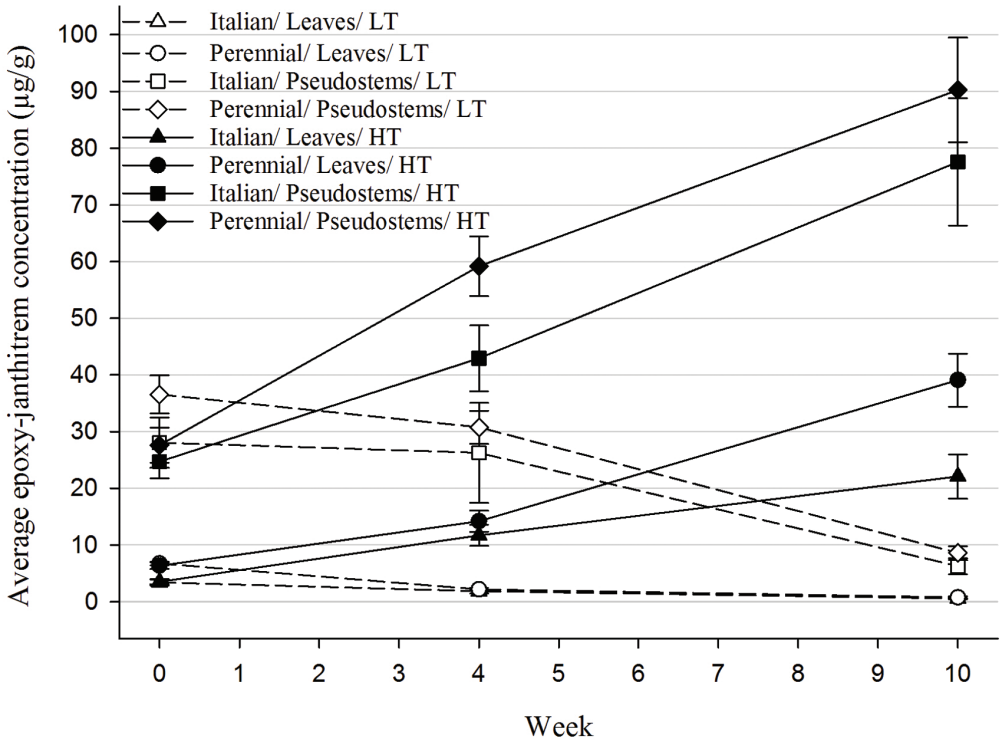


FIGURE 1 | *In planta* epoxy-janthitrem concentrations (µg/g) for each of the AR37-infected ryegrass treatments at week 0 (sample 1), week 4 (sample 2), and week 10 (final harvest) (error bars are ±SEM of raw data). HT, high temperature (20°C); LT, low temperature (7°C).

TABLE 1 | *P*-values for the effects of Temperature (high and low), Species (perennial and Italian), Plant part (pseudostems and leaves) and their interactions from the analysis of epoxy-janthitrem concentration in ryegrass at the beginning of the trial, after 4 weeks and after 10 weeks of growth in the controlled environment rooms.

Source of variation	*P*-value		
	Week 0 (Sample 1)	Week 4 (Sample 2)	Week 10 (Final harvest)
Species	**<0.001**	**0.029**	**<0.001**
Plant part	**<0.001**	**<0.001**	**<0.001**
Temperature	0.181	**<0.001**	**<0.001**
Species × Plant part	**0.005**	0.523	0.429
Temperature × Plant part	0.205	**<0.001**	**<0.001**
Species × Temperature	0.315	0.884	0.701
Species × Temperature × Plant part	0.849	0.877	0.089

Bold values are the statistically significant ($P < 0.05$) values.

TABLE 2 | Interactions between Endophyte (AR37 or endophyte-free), Temperature (high and low), Species (perennial and Italian), and Plant part (pseudostems and leaves) for larval diet consumption and larval growth data within the larval bioassay.

Source of variation	*P*-value	
	Diet consumption	Larval growth
Endophyte	**<0.001**	**<0.001**
Temperature	**<0.001**	**<0.001**
Plant part	**<0.001**	**<0.001**
Species	0.866	0.994
[1.693pt] Endophyte × Species	**0.005**	**0.006**
Endophyte × Temperature	**<0.001**	**<0.001**
[1.693pt] Species × Temperature	0.996	0.224
Endophyte × Plant part	0.056	**0.002**
Species × Plant part	0.461	0.597
Temperature × Plant part	**0.006**	**<0.001**
Endophyte × Species × Temperature	**0.033**	**0.002**
Endophyte × Species × Plant part	**0.006**	**0.022**
Endophyte × Temperature × Plant part	0.316	**0.022**
Species × Temperature × Plant part	0.989	0.656
Endophyte × Species × Temperature × Plant part	0.170	0.112

Bold values are the statistically significant ($P < 0.05$) values.

Larvae fed AR37-infected (E+) ryegrass grown at HT consumed significantly ($P < 0.05$) less diet and gained significantly less weight than larvae fed E+ ryegrass grown at LT and endophyte-free (E−) ryegrass at both temperatures (**Figure 2**). In the LT treatment, however, only larvae fed E+ perennial ryegrass consumed less diet ($P < 0.05$) and gained less weight ($P < 0.05$) than larvae in the equivalent E− treatment with no differences for the Italian ryegrass. In E− perennial ryegrass treatments significantly more diet was consumed and larval growth was higher in the LT treatment than the HT treatment. No such difference was found in the

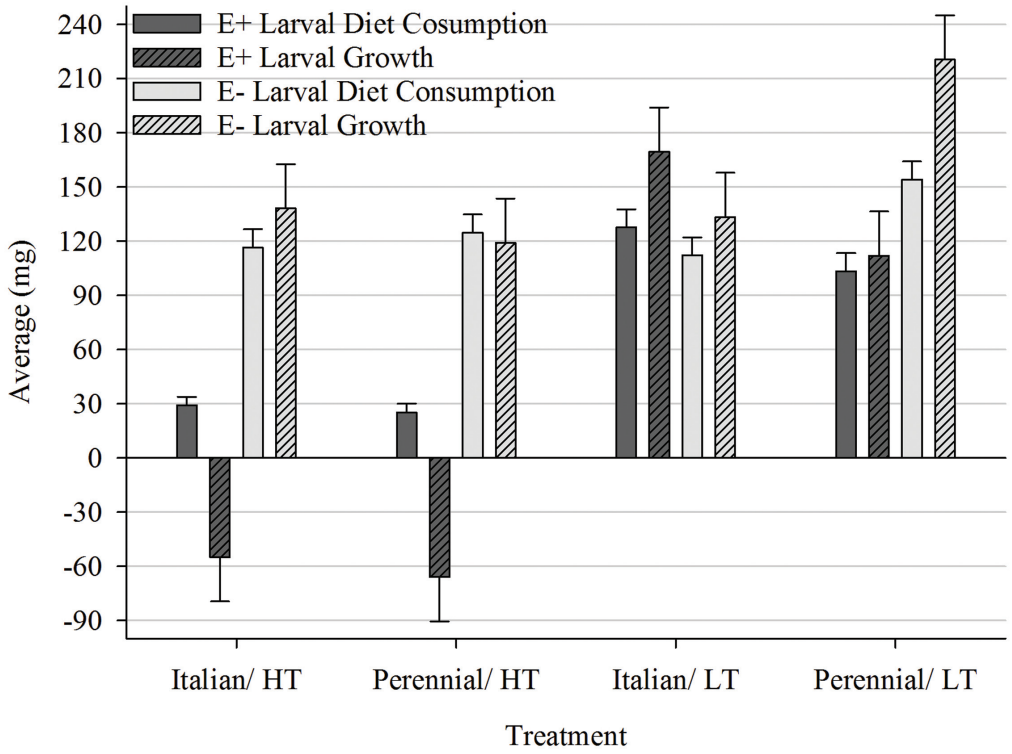


FIGURE 2 | Comparison of average diet consumption (mg; ±SE) and average larval growth (mg; ±SED) within the Endophyte (E+ = AR37-infected or E− = endophyte-free) × Temperature [HT = high (20°C) or LT = low (7°C)] × Species (perennial or Italian) interaction.

corresponding Italian ryegrass treatments. When comparing perennial with Italian treatments grown at LT larvae fed E− ryegrass consumed more and gained more weight on perennial. In contrast, when fed E+ ryegrass there was no difference ($P < 0.05$) in consumption but larvae gained significantly more weight on Italian.

Both pseudostems and leaf blades from E+ plants grown at HT caused larvae to lose weight, with pseudostems having a significantly greater ($P < 0.05$) effect than leaf blades (**Figure 3**). In comparison, all larvae fed E+ ryegrass grown at LT gained weight but those fed pseudostems gained less weight ($P < 0.05$) than those fed leaf blades. There was no significant difference ($P > 0.05$) in growth between larvae fed E+ ryegrass grown at LT and the equivalent E− treatment, for both pseudostems and leaves. Larvae gained more weight ($P < 0.05$) when fed E− ryegrass pseudostems than leaves from plants grown at HT whereas the opposite occurred for the LT E− plants.

The greatest larval mortality occurred in the HT pseudostem treatments where larval mortality was 41.7% in the perennial ryegrass treatment and 25% in the Italian. Mortality in all remaining treatments was less than 8.3%.

Epoxy-Janthitrem Concentrations within Insect Diets

Epoxy-janthitrem concentrations were analyzed by HPLC in freshly prepared diet (day 0), diet added to containers on day 4 (stored at 4°C from days 0 to 4) and in remnant diets (recovered from insect containers on days 4 and 7) to ensure the fresh diet concentrations were similar at each diet change and to check that the concentrations in the diet were not substantially degraded when diet plugs were exposed to porina larvae. Epoxy-janthitrem concentrations in diet added to containers on day 4 were not substantially different (average 10.7%) from fresh diet concentrations (**Table 3**). Furthermore, epoxy-janthitrem concentrations were not substantially degraded (average 9.1%) during the time diets were in the insect trial. At the end of the trial, samples of the endophyte-free diets (week 3) were analyzed for epoxy-janthitrem to confirm that there was no contamination. No epoxy-janthitrems were found.

DISCUSSION

This experiment has shown that when AR37-infected ryegrass was grown at 20°C epoxy-janthitrem concentrations were greatly increased, resulting in a strong anti-feedant effect on porina larvae that led to high weight loss and in the case of pseudostems, increased mortality. In contrast, epoxy-janthitrem concentrations declined markedly in plants grown at 7°C causing low level deterrence and a small reduction in weight gain of larvae fed perennial ryegrass. Although epoxy-janthitrem concentrations were greatly reduced by low temperature this reduction did not occur until after 4 weeks of exposure.

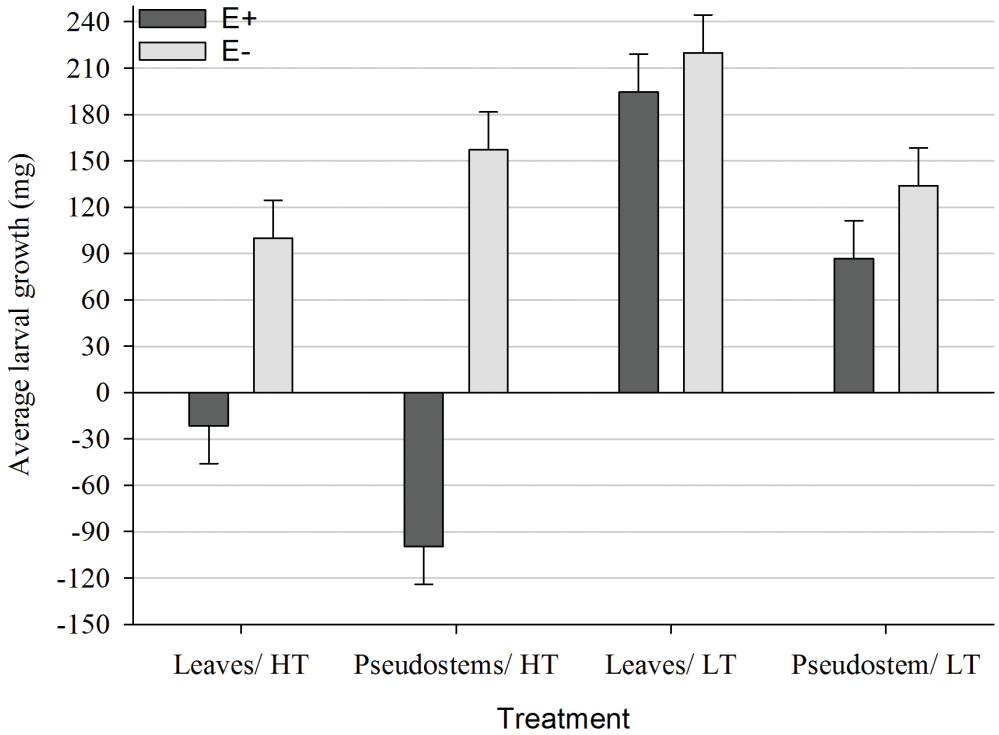


FIGURE 3 | Comparison of average growth (mg; ±SED) within the Endophyte (E+ = AR37-infected or E− = endophyte-free) × Temperature [HT = high (20°C) or LT = low (7°C)] × Plant part (pseudostems or leaves) interaction.

TABLE 3 | Average epoxy-janthitrem (EJ) concentration (μg/g) in fresh diets, the range and estimated dry weight concentrations of epoxy-janthitrem (μg/g).

Ryegrass species	Temperature	Plant part	Average EJ concentration (μg/g)	Range	Estimated dry weight conc. (μg/g)
Italian	Low	Leaves	0.08	0.07–0.10	0.66
Italian	Low	Pseudostems	0.85	0.82–0.88	7.02
Perennial	Low	Leaves	0.10	0.09–0.10	0.83
Perennial	Low	Pseudostems	1.62	1.59–1.65	13.38
Italian	High	Leaves	2.33	2.27–2.40	19.24
Italian	High	Pseudostems	11.14	11.02–11.31	91.99
Perennial	High	Leaves	3.78	3.60–3.93	31.21
Perennial	High	Pseudostems	13.68	12.89–14.18	112.96

Wet weight-dry weight conversion = 8.258.

When fed to larvae E+ perennial ryegrass grown at LT reduced larval consumption and growth but Italian ryegrass did not. This is likely explained by the higher epoxy-janthitrem concentrations in perennial ryegrass insect diets, although this effect was exaggerated by the large increase in consumption and growth of larvae fed E− perennial ryegrass (cv 'Grasslands Samson') that did not occur in larvae fed E− Italian ryegrass (cv 'Grasslands Asset'). It is possible that differences in the ratios of the five epoxy-janthitrem compounds between perennial and Italian ryegrass may have contributed to the differences in bioactivity, particularly if certain compounds, or combinations of compounds are more bioactive than others. It is also possible that there was an unknown alkaloid produced in higher concentrations in perennial than Italian ryegrass.

Results from this study have shown an anti-feedant effect of the endophyte AR37 on porina larvae when ground herbage was incorporated into an insect diet. Epoxy-janthitrems within AR37 are likely to be responsible for this bioactivity as pure and semi-pure epoxy-janthitrem I have previously been shown to have an anti-feedant effect on porina when incorporated into semi-synthetic diets (Finch et al., 2010; Hennessy, 2015).

Although the results from this experiment clearly show an anti-feedant effect of AR37 it could not be determined whether this endophyte also has a toxic effect on larvae. Here toxicity is defined as a reduction in growth and survival of an insect

above that which can be attributed to starvation. Pseudostems of AR37-infected ryegrass grown at HT, which contained the highest epoxy-janthitrem concentrations, reduced larval survival. A reduction in survival could indicate toxicity but it is also possible that larvae may have died due to starvation caused by the strong anti-feedant effect of AR37. Further research is required to resolve this.

Plant growth temperature is known to affect the concentrations of other important endophyte alkaloids. Seasonal concentrations of lolitrem B, which like the epoxy-janthitrems is in the indole diterpene class of alkaloids, and peramine were monitored by Ball et al. (1991). Lolitrem B concentrations were found to be highest during the summer months and lowest during the winter when rainfall is higher and temperatures are cooler. Peramine concentrations were comparatively stable, but were also significantly lower during winter when compared to summer and autumn. Although caution must be applied when relating results of pot trials to field conditions the results of this study suggest that epoxy-janthitrems could respond to temperature in a similar way. However, for epoxy-janthitrem concentrations to decrease to the low levels observed in this experiment plants would have to be exposed to constant low temperatures for an extended period of time (at least 4 weeks). Under field conditions temperatures will constantly fluctuate which may mean that epoxy-janthitrem concentrations are not decreased to the extent as that observed in this study.

The reduction in epoxy-janthitrem concentrations in plant material grown at low temperatures suggests that AR37 may not provide the highest level of protection against porina larvae during the winter months in parts of New Zealand. Porina are major pasture pests particularly in the southern areas of both the North and South Island of New Zealand where they are capable of causing severe pasture damage. Several species of porina are known pasture pests, the moths of which have different peak flight periods. Moths of *W. cervinata*, the species tested in this experiment, fly between October and December in the South Island (Barratt et al., 1990). Young larvae of this species will begin feeding on ryegrass during the late spring and summer months, when temperatures are warm. Results from this study suggest that during this period AR37-infected ryegrass is likely to contain relatively high epoxy-janthitrem concentrations which should provide good control over larvae. Larvae of the later flying species, *W. copularis*, which can fly as late as February (Barratt et al., 1990) begin feeding on AR37-infected ryegrass when temperature and alkaloid concentrations are likely to be lower and less effective at controlling larval populations.

The mechanisms by which temperature and plant genotype affected alkaloid concentrations in perennial and Italian ryegrass plants in this study are not known. These factors may have indirectly affected alkaloid concentrations by influencing the ratio of endophyte mycelial biomass to plant biomass, resulting in changes in alkaloid concentration (di Menna and Waller, 1986; Breen, 1992; di Menna et al., 1992; Ju et al., 2006). Alternatively, alkaloid biosynthesis, metabolism, or degradation rates may have been directly affected by temperature or plant genotype (Spiering et al., 2005).

No published information is available comparing epoxy-janthitrem concentrations in the leaves and pseudostems of AR37-infected ryegrass plants. In this study, concentrations were found to be markedly higher in the pseudostems than the leaves at both temperatures and for both cultivars. This distribution is not uncommon and has also been found for lolitrem B (di Menna et al., 1992; Davies et al., 1993; Keogh et al., 1996; Ball et al., 1997). Alkaloids such as lolitrem B and the epoxy-janthitrems are lipophilic compounds and are not easily translocated around the plant (Ball et al., 1993; Munday-Finch and Garthwaite, 1999; Spiering et al., 2005) thus distribution tends to be similar to that of the endophyte, which is generally higher in the pseudostem and lower in the leaves (Musgrave, 1984; Musgrave and Fletcher, 1984; Keogh and Tapper, 1993). Maintaining high alkaloid concentrations in the pseudostem is advantageous for both the host plant and the endophyte as the meristem, the tissue containing undifferentiated cells and where growth occurs is located at the base of the ryegrass plant (Popay, 2009). Tiller death will occur if an insect severely damages the meristem. Insect damage to the leaves of ryegrass plants is not as harmful to the plant itself, as ryegrass is adapted to animal grazing (Popay, 2009). However, the more leaf material the insect is able to consume the less that is available for both plant photosynthesis and consumption by grazing livestock, resulting in reduced plant growth and animal productivity.

The endophyte AR37 is very important for the control of porina in New Zealand as although other endophytes such as AR1 and the common toxic strain provide protection against some pest insects (Prestidge et al., 1982; Popay et al., 1999; Pennell et al., 2005; Popay and Gerard, 2007; Popay and Thom, 2009) it is only AR37 which provides ryegrass with protection against porina (Jensen and Popay, 2004; Popay et al., 2012). Control against porina, which are a major pasture pest in parts of New Zealand, currently involves an integrated pest management strategy involving planting ryegrass infected with the AR37 endophyte and the application of insecticides at particular times of the year (Barratt et al., 1990). The results of this paper support the continued use of integrated pest management strategies to control porina populations in the field.

Leading on from this study field trials should be conducted to determine how temperature affects epoxy-janthitrem concentrations in AR37-infected ryegrass in the field and how these concentrations then impact on porina populations. If concentrations are found to be reduced under certain environmental conditions the next step could be to identify existing ryegrass cultivars and/or plant genotypes, from which a new breeding line could be produced, that produces higher alkaloid concentrations when grown at low temperature.

AUTHOR CONTRIBUTIONS

LH carried out this research as a part of her Masters of Science (Research). AP was a co-supervisor and the main supervisor of all experimental work. SF was a co-supervisor and oversaw all of the chemical analyses. MC was the University supervisor and VC provided statistical expertise.

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Aploneura lentisci (Homoptera: Aphididae) and Its Interactions with Fungal Endophytes in Perennial Ryegrass (*Lolium perenne*)

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Aploneura lentisci Pass. is endemic to the Mediterranean region where it is holocyclic, forming galls on its primary host, *Pistacia lentiscus* and alternating over a 2-year period between *Pistacia* and secondary hosts, principally species of Gramineae. This aphid is widely distributed in Australia and New Zealand on the roots of the common forage grasses, ryegrass (*Lolium* spp.) and tall fescue (*Schedonorus phoenix*) where it exists as permanent, anholocyclic, parthenogenetic populations. Previous studies have indicated that infestations of *A. lentisci* significantly reduce plant growth and may account for differences in field performance of *Lolium perenne* infected with different strains of the fungal endophyte *Epichloë festucae* var. *lolii*. These obligate biotrophs protect their host grasses from herbivory via the production of alkaloids. To confirm the hypothesis that growth of *L. perenne* is associated with the effect of different endophyte strains on aphid populations, herbage and root growth were measured over time in two pot trials that compared three fungal endophyte strains with an endophyte-free control. In both pot trials, aphid numbers were lowest on plants infected with endophyte strain AR37 at all sampling times. In plants infected with a common toxic strain naturalized in New Zealand, aphid numbers overall were lower than on uninfected plants or those infected with strain AR1, but numbers did not always differ significantly from these treatments. Populations on AR1-infected plants were occasionally significantly higher than those on endophyte-free. Cumulative foliar growth was reduced in AR1 and Nil treatments relative to AR37 in association with population differences of *A. lentisci* in both trials and root dry weight was reduced in one trial. In four Petri dish experiments survival of *A. lentisci* on plants infected with AR37 declined to low levels after an initial phase of up to 19 days during which time aphids fed and populations were similar to those on plants without endophyte. Aphids on AR37-infected plants became uncoordinated in their movement and developed tremors before dying suggesting a neurotoxin was responsible for their mortality. Results support the hypothesis that differences in *A. lentisci* populations due to endophyte infection status and strain affects plant growth.

Keywords: *Epichloë festucae* var. *lolii*, root aphid, populations, plant growth, endophyte strain

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INTRODUCTION

There is an abundance of literature on the interactions between above-ground herbivores and their host plants, but comparatively little on root herbivores (Brown and Gange, 1990; Hunter, 2001), despite the profound effects that the latter can have on plant growth and physiology, and on the determination and regulation of soil communities (Anderson, 1987; Brown and Gange, 1990; Hunter, 2001; Wardle, 2002). The consequences that root herbivory have for individual plants, depending on the type of feeding and its severity, include reductions in above and below-ground plant growth, changes in biomass allocation, and effects on nutrient acquisition, water relations and physiological and morphological parameters of the plant (Brown and Gange, 1990; Hunter, 2001; Wardle, 2002). At the community level, root herbivory may alter plant competitiveness and diversity and the rate and direction of plant succession (Brown and Gange, 1990; Hunter, 2001; Wardle, 2002). As a major component of the soil foodweb, root herbivory also has major repercussions for soil microbial and invertebrate populations (Bardgett et al., 1999; Denton et al., 1999; Wardle, 2002).

Species of *Lolium* and *Festuca* are often infected with asexual clavicipitaceous endophytic fungi belonging to the genus *Epichloë* [previously *Neotyphodium* (Leuchtmann et al., 2014)]. These endophytes are obligate biotrophs and form, in most cases, a mutualistic relationship with their hosts in which they produce secondary metabolites that are deterrent or toxic to herbivorous insects (Popay and Bonos, 2005). There is no external stage and they are transmitted via seed. Much of the research into the effects of the *Epichloë* infection on insect herbivores has focused on those that feed above-ground. In part this relates to the location of endophyte infection in the meristematic and basal leaf sheath tissue along with the alkaloids that are also concentrated in above-ground tissues (Ball et al., 1997a,b; Lane et al., 1997) but also reflects the difficulties inherent in monitoring below-ground herbivory.

The particular alkaloids produced by *Epichloë* fungi are a characteristic of each different strain (Lane et al., 2000), although several factors moderate the quantities that are produced. These factors include plant genotype (Ball et al., 1995a,b; Easton et al., 2002), nutrient status (Rottinghaus et al., 1991; Azevedo et al., 1993) and environmental and seasonal factors (Ball et al., 1995a,b; Hennessy et al., 2016). Location of alkaloids within plants, however, appears to be mainly an attribute of the compounds themselves (Ball et al., 1995a, 1997a,b; Keogh et al., 1996; Lane et al., 1997) although this may also be modified to a degree by plant genotype (Popay et al., 2003).

In New Zealand pastures, there is a high incidence of endophyte infection of ryegrass by naturalized strains of the fungus (*Epichloë* festucae var. *lolii*; referred to here as Common Toxic (CT) but also known as wild-type or standard endophyte) that share a common chemical profile (Easton, 1999). Of the alkaloids they produce, ergovaline and lolitrem B are toxic to grazing mammals (Fletcher and Easton, 1997) as well as having effects on insect herbivores (Popay and Bonos, 2005); a third alkaloid, peramine, is a powerful deterrent to a major pest *Listronotus bonariensis* (Rowan et al., 1990) with no known effect on mammals (Fletcher, 1999; Tapper and Latch, 1999). In order to resolve the animal health problems associated with infection of ryegrass by the CT strains while retaining the anti-insect properties that infection provides, endophytes with different metabolic profiles have been investigated (Tapper and Latch, 1999). One of these, AR1, which produces peramine but not the mammalian toxins lolitrem B and ergovaline, was made commercially available to New Zealand farmers in 2001. In 2007, a second endophyte strain, AR37, which lacks the ability to produce peramine, ergovaline or lolitrem B was also commercially released to farmers. This strain produces indole-diterpenoid compounds, related to lolitrem B, known as epoxy-janthitrems (Finch et al., 2012). The role these particular compounds have for animal health and on insect pests is still being defined.

In New Zealand, fungal endophyte infection is necessary for plant survival in some areas and can considerably improve *Lolium perenne* growth, effects which have been attributed to protection against insect herbivory that the endophyte confers (Popay et al., 1999; Hume et al., 2007, 2009; Popay and Hume, 2011; Thom et al., 2014). In Australia, infection of *L. perenne* has delivered similar benefits for plant performance although the role of insect pests has been less well studied (Lowe et al., 2008; Hume and Sewell, 2014). The CT strain reduces predation of ryegrass by three pests, Argentine stem (*Listronotus bonariensis*), African black beetle (*Heteronychus arator*) and pasture mealybug (*Balanococcus poae*), of which Argentine stem weevil is the most significant. AR1 provides a similar spectrum of effects except it has a much weaker effect on African black beetle whereas AR37, in addition to these pests, also reduces populations of porina caterpillars (*Wiseana* spp.) and a root aphid, *Aploneura lentisci*. In trials comparing the agronomic benefits of these different strains in the same cultivar, AR37 has consistently out-performed both the CT and AR1 strain with those advantages attributed to the strong effects AR37 has in suppressing populations of *A. lentisci* (Hume et al., 2007; Thom et al., 2014). Perennial ryegrass infected with the endophyte strain AR37 also significantly reduced infestations of a root aphid *A. lentisci* in a pot trial with associated increases in plant growth (Popay and Gerard, 2007). This aphid is also adversely affected by *Epichloë* infection of tall fescue (*Schedonorus phoenix*) and meadow fescue (*L. pratense*) (Schmidt and Guy, 1997; Jensen and Popay, 2007).

Aploneura lentisci is endemic to the Mediterranean and Middle East region where it is holocyclic, forming galls on its primary host, *Pistacia lentiscus* (Anacardiaceae), alternating over a 2-year period between *Pistacia* and secondary hosts, principally species of Gramineae (Cottier, 1953; Wool, 2005). This aphid has a wide geographical range on its secondary hosts, on which it exists as permanent, anholocyclic, parthenogenetic populations (Wool and Manheim, 1986). Winged morphs of *A. lentisci* have been trapped in Australia and New Zealand (O'Loughlin, 1962; Lowe, 1968) but have not been observed in the field (A.J. Popay unpublished). Mobile young nymphs can be found on the herbage (Rasmussen et al., 2008a) while mature aphids are largely sedentary living amongst copious amounts of flocculent white wax which likely protects them from soil moisture extremes, microbes and predators. This species

is reported to be abundant in grassland in Britain (Purvis and Curry, 1981), occurs throughout New Zealand (A. J. Popay, C. Pennell, D. E. Hume, unpublished observations) and is common in Australian pastures (Salmon et al., 2008; Moate et al., 2012). It has been reported to cause severe damage to young wheat plants (Mustafa and Akkawi, 1987) although Cottier (1953) considered it to be of no economic importance on Gramineae in New Zealand.

We have found no published information on the population dynamics of *A. lentisci* on the roots of its secondary hosts and no direct evidence of its effects on grass growth over an extended period of time. Here we report on populations of *A. lentisci* sampled on individual plants on several occasions in two pot trials. Plant growth has also been measured to test the hypothesis that *A. lentisci* has detrimental effects on host plant growth related to different effects of fungal endophyte strain on population size. One pot trial also investigated the effect of container size on populations after observations indicated aphid colonies were common on the roots growing at the interface between the potting media and the container. From this it was postulated that a greater accumulation of roots at this interface for plants in smaller containers may influence population size and the effects of endophyte strain. Aphid response to ryegrass with and without endophyte to determine if endophytes had a deterrent and/or a toxic effect was also investigated in a series of Petri dish experiments.

MATERIALS AND METHODS

The hypothesis that plant growth would be differentially affected by fungal endophyte strain according to the effects of each strain on populations of *A. lentisci* was tested in two pot trials, namely a plant growth trial (PG) and a root mass trial (RM). In both trials root and herbage growth was quantified along with the root aphid populations on individual plants in successive samplings over 2 years in the PG trial and 10 months in the RM trial. In addition, to more closely examine effects of endophyte infection on aphid behavior and population development, aphids were closely monitored on plants for short periods of time in four Petri dish experiments.

Plant Preparation and Maintenance

In all trials, *L. perenne* cv. Grasslands Samson without endophyte (Nil) or infected with endophyte strains AR1, AR37 or CT were used. All plants were grown from seed obtained from the Margot Forde Germplasm Centre, AgResearch, Palmerston North, New Zealand. Seed was germinated in the dark on damp filter paper in Petri dishes held at 20°C for 5–7 days. Germinated seed was planted into a 2:1 soil:sand mixture into individual pots (120 mm diameter × 100 mm deep) in the PG trial and into a commercial potting mix in polystyrene trays (300 × 500 × 90 mm) for the RM trial and Petri dish experiments. Plants were maintained in ambient light and temperature conditions under automatic overhead watering in a screenhouse. Nutrients were supplied to plants in two forms. At planting Osmocote® slow release fertilizer (19% nitrogen, 2.6% phosphorous, 10% potassium) was incorporated into the top 50 mm of the planting medium at a rate of approximately 2.0 g per plant. Once established, plants received a nutrient solution comprised of a commercially available nutrient mix, Thrive™, prepared at the recommended rate (approximately 8 g per 4.5 L of tap water) with additional nitrogen (approximately 5 g per 4.5 L) in the form of urea (46% nitrogen). Both pot trials were conducted outdoors under ambient conditions. Irrigation was deliberately kept to the minimum needed to prevent the plants from wilting and dying during prolonged dry weather during the summer-autumn period between December and April. During this period, in 2000/01 and 2001/02, respectively, average monthly rainfall was 84.5 and 93.3 mm; minimum monthly rainfall was 28 and 21 mm; average number of rain days was 10.2 and 13.2; average maximum/minimum air temperature was 22.8/12.4°C and 22.3/12.2°C. As required, plants were watered by hand with a hose held for 4 s over each plant, or using a sprinkler.

The endophyte status of all plants used in the trials was determined by taking a single tiller from each 6- to 9-week-old plant to test for the presence of endophyte using a tissue print immunoblot method (Simpson et al., 2012). A tiller was cut near the base, and the freshly cut surface was blotted onto the surface of nitrocellulose paper. A development process was then used that exposed protein produced by the endophyte to polyclonal antibodies resulting in a color change if the tiller was infected with endophyte.

Plant Growth Trial

When plants were 6 months-old, ramets comprising six tillers were planted individually into a soil/sand growing medium (2:1) in polythene planter bags (90 mm × 90 mm × 200 mm). To enable root growth to be measured periodically without disturbing the plant, additional pairs of holes (5 mm diameter, 25 mm apart) were made in each planter bag at 30 mm, 70 mm, and 110 mm from the top of the bag and aligned with existing holes. Twenty replicate plants were arranged randomly on a sand base within a large tub. Initially sand was placed around the planter bags until it was level with the planting medium in the bags. After the first root sampling in August 2000, each plant was isolated from others by placing the small planter bag inside a larger one (160 mm × 160 mm × 370 mm), with the space between each bag filled with sand (**Figure 1**). Root 'outgrowth' was determined by severing roots where they exited the holes in the smaller bag into the larger planter bags. Sampling of herbage above 50 mm and root outgrowth was carried out on five occasions; in late winter and early summer 2000, autumn and spring 2001 and in mid-summer and autumn 2002. The plant roots in the small planter bag in which plants were originally planted were also harvested at the final sampling in autumn 2002. Root aphid populations were measured on each occasion.

After a further check of the endophyte status of all plants in late spring 2001, 20 months after the first test, both AR37 plants in one replicate and one in another were found to have lost their endophyte and data for both replicates of this treatment were then excluded from all analyses.

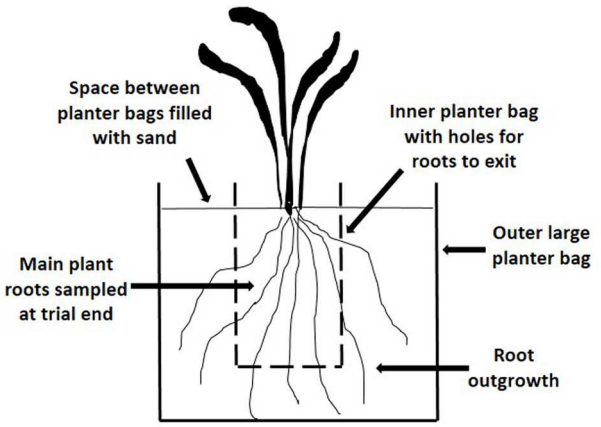


FIGURE 1 | Diagram of the arrangement for plants in the Plant Growth Trial (PG) to enable root growth and aphid populations to be monitored.

Root Mass Trial

Although root aphid colonies occur throughout the root system of infested plants, they appeared from casual observations to be more concentrated on roots at the interface between the potting medium and the container. Thus this second trial was designed to investigate the effect of container size and possible interactions with endophyte strain on infestations of root aphid and associated plant growth effects.

To take account of plant genotype/endophyte interactions known to affect root aphids (Popay and Easton, 2006) 6-month-old plants were cloned by taking two ramets of six tillers from each of 15 replicate plants. One of each cloned pair of treatments was planted into a small planter bag (120 mm × 120 mm × 230 mm containing 2484 cm^3 of 2:1 sand/soil medium) and the other into a large bag (140 mm × 140 mm × 280 mm containing 4900 cm^3 of 2:1 sand/soil medium). Plants were arranged in two rows with cloned pairs of plants adjacent to each other in separate rows and treatments randomly arranged within each replicate. A square of weed mat was placed underneath each planter bag so that any roots which grew through the base of the bag could also be sampled. Herbage growth was determined by harvesting tillers above 5 mm on seven occasions through the course of the trial. Roots were sampled on three occasions (spring 2002, mid-summer and early winter 2003) by destructive sampling of five replicates of each treatment. Root dry weight and root aphid populations were measured on each sampling occasion.

Herbage and Root Sampling

For both trials herbage was harvested at 4 cm and root material was captured in a three-stage washing process that was also designed to remove invertebrates from the samples. Roots together with the planting medium were first placed in a bucket and agitated while filling the bucket with water. After a short standing period, the suspension containing the invertebrates was then decanted off while the remaining sand was washed through a 2.5 mm^2 mesh from which roots were retrieved. Before drying, roots were washed more thoroughly under running water over a 1 mm^2 mesh to remove any further sand and debris. Root and herbage samples were either frozen and then freeze dried before weighing if they required chemical analysis or oven dried. Herbage samples were oven dried at 60°C for 36–48 h and roots at 80°C for 48–60 h. All samples were weighed immediately after drying.

Aphid Inoculation and Sampling

Soon after plants were set up in each trial, they were inoculated with root aphids by inserting a small piece of infested root down the side of the planter bag, although it was noted that many plants had become naturally infested prior to this. The number of aphids inoculated was not determined because of the difficulty in doing so and the risk of damaging the aphids. The root aphids were sampled at each plant growth assessment in 2001 and 2002 in the PG trial and at each of the three destructive harvest times in the RM trial. Aphids were extracted by flotation in water and wet sieving. After roots were initially washed in a bucket as described above, the suspension was decanted through three sieves (2.00 mm, 710 μm, and 210 μm). The two larger sieves were rinsed thoroughly but gently before all material that had collected on the 210 μm sieve was washed into a 70 mL specimen container. Samples were stored at 4°C until counting.

For counting, samples were transferred to a beaker and diluted if necessary to give an amount between 30 and 60 mL. The total amount depended on the size of the original sample and the number of aphids present. The sample was stirred thoroughly to distribute the aphids in the sample before a 10 mL subsample was removed to a Petri dish base (90 mm diameter) in five 2 mL aliquots, using a pipette. The base of the Petri dish was marked with a grid (approximately 1 mm^2) to facilitate counting of the aphids in the dish. Counting was carried out under a stereo microscope at 16× magnification.

Petri Dish Experiments

Four experiments (A–D) were conducted on plants in Petri dishes to enable regular observations of root aphid behavior and population dynamics on perennial ryegrass with and without endophyte. The effect of plant genotype was also investigated by using cloned plants in Experiments A and B which were tested for their effects on root aphid at different times, and then using cloned plants again in Trial C which were tested concurrently.

For each trial, the base of a 90 mm diameter Petri dish was firmly packed with a 60 mL volume of perlite mixed with 25 mL of tap water and approximately 2.0 g of Osmocote® slow release fertilizer. Plants or tillers from plants were placed in the Petri dishes so that the base of the tiller was level with aligned holes (approx. 10 mm wide) cut in the side of the base and lid of each dish. Roots were splayed out on the surface of the perlite before the lid was put in place and sealed with a 20 mm wide piece of parafilm. Replicate groups of Petri dishes were placed upright in random order and fastened together with a rubber band. A piece of black polythene with a slit in the center where the tillers emerged was placed over each group of Petri dishes to

exclude light from the roots and fastened in place with another rubber band. Each replicate was then partially buried in potting mix in a polystyrene planter box and kept outside under ambient light and temperature conditions.

After a period to allow plants to establish, mature and immature root aphids taken from potted plants were transferred with a fine paint brush on or close to roots of the plants in the Petri dishes. Maturity was arbitrarily based on size (immature < 1 mm > mature). Aphids were later checked and replaced if damaged in any way before lids and parafilm were replaced.

To count and observe root aphid in each trial, lids were removed from the Petri dishes and the surface of the perlite and roots were inspected under a stereo microscope (16× magnification). The number of live and dead aphids was recorded and dead aphids were removed. These observations probably underestimated the aphid populations as counting was done with minimal disturbance of the roots and perlite. Location of the aphids on or off roots (on perlite and not in contact with roots) was noted at each inspection in all trials, and their preference for new (i.e., roots grown since planting) or old roots was determined at all assessments in Trial B. In Trial A, 5–10 mL of water was added to each Petri dish at every second inspection, which kept the perlite damp. In subsequent trials water was added only as necessary to maintain the perlite in a moist condition. At the completion of each trial root aphid numbers in each Petri dish were counted. The endophyte status of at least one tiller from each plant was confirmed by staining and microscopic examination.

Trial A: A single healthy tiller was removed from each of five 1-year-old plants of each treatment and planted into separate Petri dishes to give five replicates of each endophyte treatment. One-week after planting, 10 mature and five immature aphids were released onto each plant. The trial was terminated after 25 days.

Trial B: This was planted at the same time as Trial A using clones of the same plants with five replicates of each endophyte treatment. Plants were inoculated with 10 mature and five immature aphids 4 weeks after planting. Petri dishes were inspected regularly for 25 days.

Trial C: For each endophyte treatment, five cloned pairs of plants were tested by taking two ramets of two tillers, matched for root size, from five individual 1-year-old plants and planting them separately into Petri dishes. Four weeks after planting five mature and five immature root aphids were released into each Petri dish. The experiment was assessed for 21 days.

Trial D: This trial tested the effects of the different endophyte treatments in 10-week-old ryegrass plants. They were tested for endophyte before 20 plants of each endophyte treatment were planted into Petri dishes. Four weeks after planting, the 10 healthiest plants of each treatment were inoculated with 12 root aphids, of which at least five were mature and five immature. The Petri dishes were checked regularly for 21 days and then left without checking for a further month during which time they were watered individually as necessary.

Statistical Analysis

Root aphid numbers/plant and number/g of root (aphid loading) for each of the pot trials were log transformed to stabilise the variance. All log transformations used a constant that was based on the minimum number of aphids possible for each data set based on the dilution of samples prior to counting. For example if the original sample of 20 mL was diluted to 40 mL for counting, one aphid counted in the diluted sample was equivalent to two in the original sample; hence the log transformation was L(n + 2). Data were analyzed using a general analysis of variance in Genstat Releases 6.1–17, testing for main effects of endophyte in the PG trial, and plant container size and harvest date in the RM trial. Block strata for the analysis of the PG trial was based on the randomized block design for each replicate of endophyte treatments. Similarly, in the RM Trial, the analyses were structured to take into account the randomized block design of the trial and the cloned plants within each replicate. Cumulative herbage and root dry weight data were analyzed in a similar way but did not require log transformation. In the RM trial, the cumulative herbage growth prior to each root sampling was analyzed separately; i.e., for the first root sampling, three herbage cuts had been taken on all 15 replicates; in the second, five herbage cuts on 10 replicates; at the third root sampling, there were seven herbage cuts on five replicates. Means were separated using Fisher's protected least significant difference test.

In the Petri dish experiments, an analysis of variance was also carried out on log transformed aphid numbers structured to also investigate the effect of time, and blocked by replicate. Pearson's correlation analysis in Excel investigated the effect of plant genotype.

RESULTS

Plant Growth Trial

The most consistent and statistically significant result in this trial was the strong suppression of *A. lentisci* population growth on ryegrass plants infected with AR37. This is shown for both aphids per plant and aphid loadings (number/g of root; **Figure 2**). AR37 had significantly lower populations than the other three treatments ($P < 0.001$) for both aphid numbers per plant and aphid loadings on root outgrowth in April 01 and May 02, and for the main plant roots also sampled in May 02. In September 01, AR37 and CT had fewer aphids per plant ($P = 0.007$) and lower aphid loadings ($P < 0.001$) than AR1 and Nil; in January 02, aphid populations on AR1 were similar to AR37 and both were lower than on Nil, with AR37 populations also lower than CT. The AR1 strain had consistently high infestations of root aphid, except in January 02, with significantly higher loadings than on Nil on three of five sampling occasions. By comparison with Nil treatments, root aphids tended to be less numerous on CT-infected plants but for most samplings this difference was not significant.

Aphid plant populations were highest in September (**Figure 2A**) when actual mean numbers/plant were 515, 55, 269 and 254 for AR1, AR37, CT and Nil, respectively. The average aphid numbers across all samples in the trial including

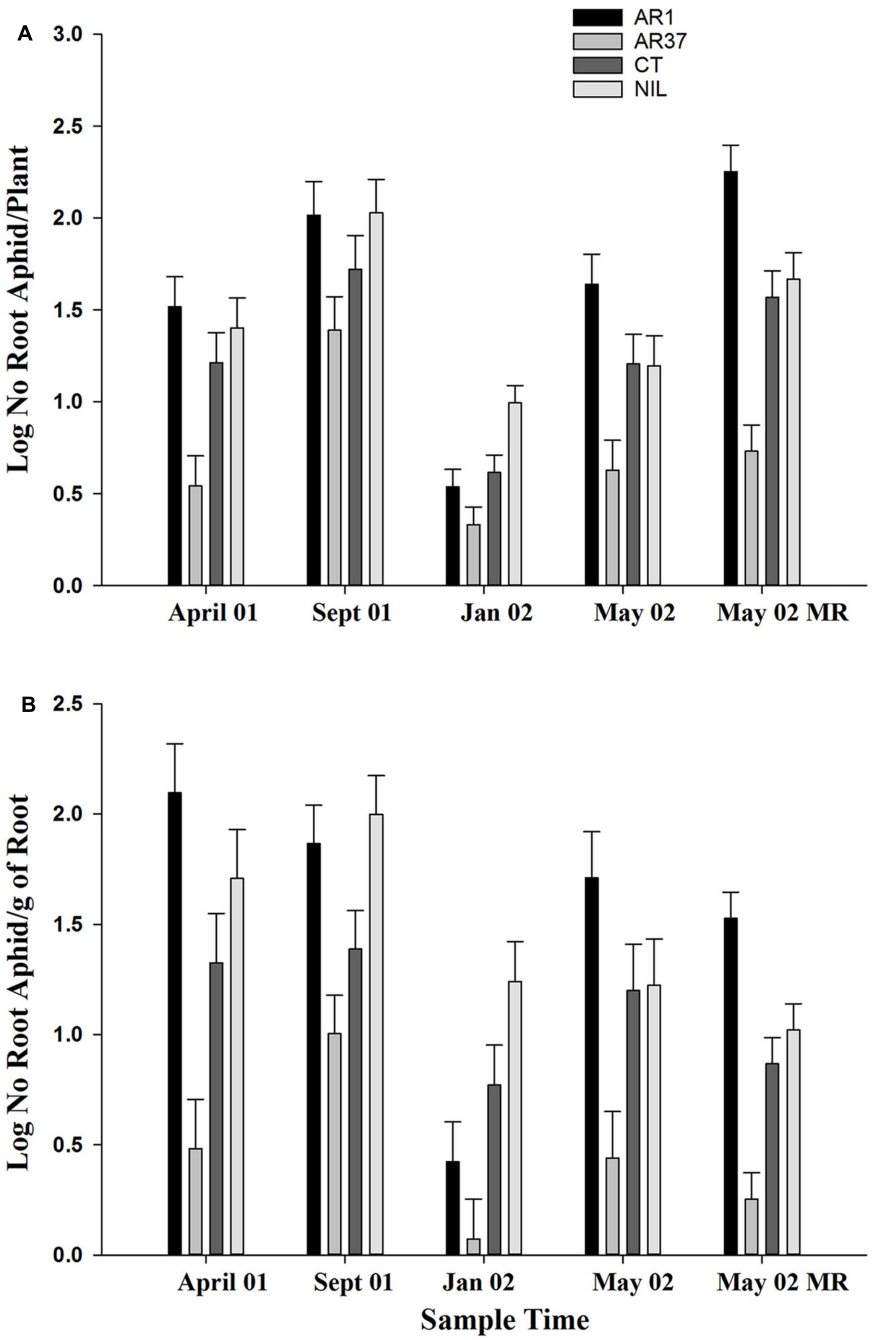


FIGURE 2 | Effect of different strains of fungal endophytes in perennial ryegrass on *Aploneura lentisci* in the PG trial (A) Number of root aphids per plant (B) Aphid loading. Sample times April 01 to May 02 aphids were taken from root outgrowth; sample May 02 was from the main plant roots: Error bars = SED

the final plant assessment was 347, 15, 105, and 148/plant. The highest aphid loading occurred on root outgrowth of AR1 plants in April 2001 (**Figure 2B**).

Root aphid populations varied widely among individual plants infected with AR1 and Nil, varied less on CT but showed little variation on plants infected with AR37. On AR1, aphid numbers ranged from 0 to 1116 on the root outgrowth of different plants at the first autumn sampling, compared with 0–750 for Nil plants, 0–573 for CT and 0–12 for AR37. The same level of variability was also seen in aphid loadings (**Figure 3A**)

Cumulative herbage dry weight from six consecutive harvests during the trial for AR37-infected ryegrass exceeded that of all the other treatments ($P < 0.001$; **Table 1**). In contrast to this, cumulative root growth did not differ ($P > 0.05$) between endophyte strains, although AR37 recorded the highest root dry

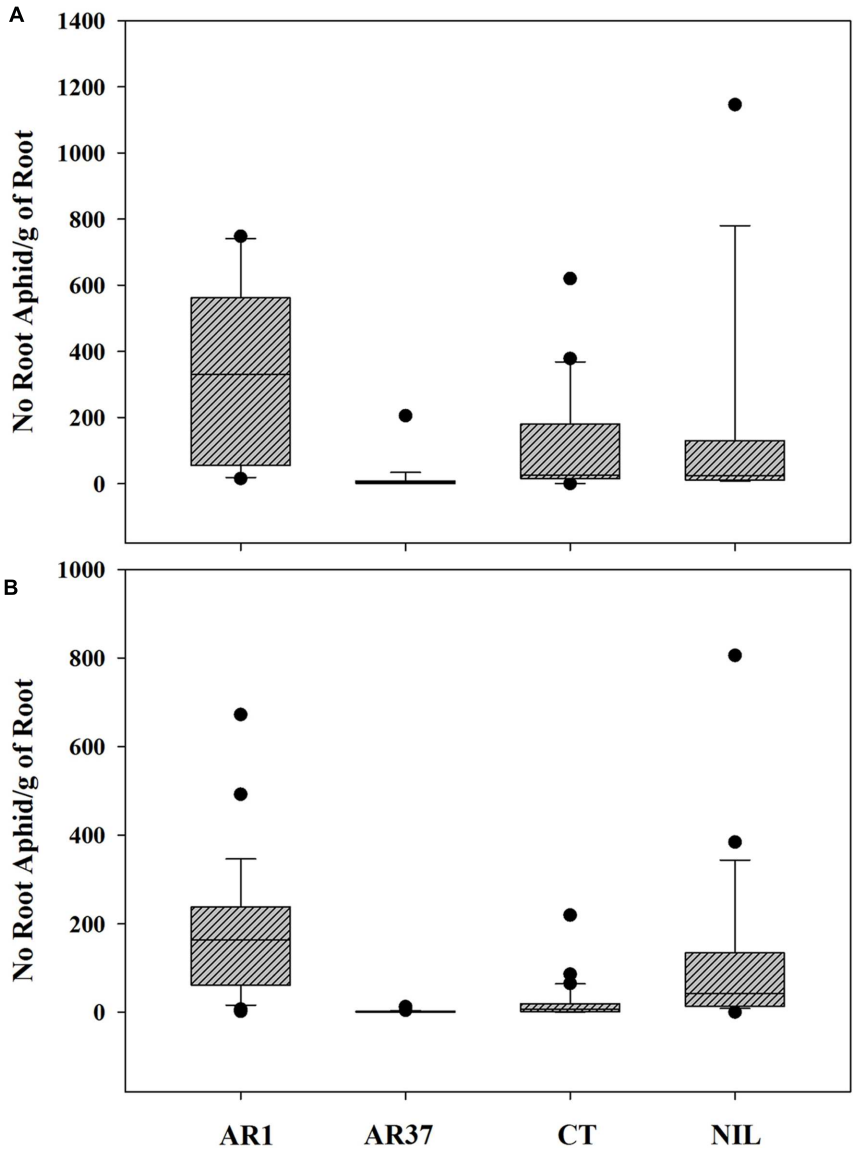


FIGURE 3 | Variability in root aphid loading per plant among individual ryegrass plants without endophyte (Nil) or infected with AR1, AR37 or CT in the (A) September 01 sampling of the PG and (B) the three samplings in the Root Mass Trial.

weight. Likewise, the dry weight of main plant roots in the small planter bag at the final harvest was not significantly affected by endophyte treatment ($P > 0.05$).

Root Mass Trial

Over all assessments, AR37 had fewer aphids/plant and lower aphid loadings than all other endophyte treatments ($P < 0.05$) while there were more aphids and greater aphid loadings on AR1 and Nil than on CT ($P < 0.05$; **Table 2**). Aphid populations per plant for AR1 and Nil did not differ significantly whereas aphid loading across all harvests was greater on AR1 than on Nil ($P < 0.05$).

Container size had no significant effect on root aphid populations either for individual endophyte treatments or overall ($P > 0.05$). In a significant interaction between endophyte and container size, however, aphid loadings for AR1 plants were greater in small containers than in the large ones [Log No.aphids/g of root: Small 2.220, Large 1.853; LSD (5%) 0.3060, df 46 $P < 0.05$]. For the other endophyte strains, aphid loadings were very similar (respectively, for small and large containers Log No./g of root: AR37 0.690, 0.740; CT 1.201, 1.016; Nil 1.649, 1.712).

There was a significant effect of harvest time on root aphid populations but no significant interaction between endophyte and harvest date. There were more aphids on AR1 and CT plants sampled in January 2003 than at other harvests, although aphid loadings showed less seasonal variation for these two treatments. In Nil plants the highest aphid populations and loadings occurred

TABLE 1 | Dry weights (g/plant) of herbage and roots from *Lolium perenne* without endophyte (Nil) or infected with three different endophyte strains from (A) Plant Growth Trial (PG) and (B) Root Biomass Trial.

	AR1	AR37	CT	Nil	SED	df	LSD (5%)
A. Plant growth trial							
Herbage	24.1	31.7	25.0	25.5	1.84	44	3.71
Root growth	5.2	7.6	6.8	6.3	0.90	44	NS
Main root	10.5	9.1	8.8	9.2	1.14	123	NS
B. Root mass trial							
Herb Sept 02[1]	6.6	9.0	9.9	7.4	0.481	36	0.98
Herb Jan 03[2]	13.4	16.4	18.1	13.1	0.966	24	2.00
Herb Jun 03[3]	20.0	23.7	26.0	19.2	2.289	12	3.43
Root Sept-02	1.6	3.8	4.3	2.5			
Root Jan-03	3.3	4.6	6.9	4.9	1.21	36	2.41
Root Jun-03	3.3	8.5	6.7	6.6			
Root Mean	2.6	5.5	5.8	4.4	0.68	36	1.39

A: Cumulative herbage and root growth from six samples and dry weight of main roots from plants at final harvest; B: Root biomass taken at three destructive harvests and cumulative herbage growth from successive samples taken up to the time of each harvest. Cumulative herbage dry weights from [1]three samples taken from 15 replicates, [2]five samples taken from 10 replicates; [3]seven samples taken from five replicates.

TABLE 2 | Root Mass Trial (RM) Effect of *L. perenne* without endophyte (Nil) or infected with AR1, AR37, and CT on overall mean aphid populations and aphid loading/plant (pooled for both planter bag sizes) and for each harvest date.

	Mean		September 02		January 03		June 03	
	Log[1]	Actual[2]	Log	Actual	Log	Actual	Log	Actual
No/plant								
AR1	2.338	406	2.261	315	2.7	605	2.054	296
AR37	0.872	6	1.075	11	0.788	3	0.753	3
CT	1.596	137	1.25	19	1.899	258	1.637	133
NIL	2.215	382	2.276	555	1.973	172	2.397	419
SED	0.1789		0.3271		0.3271		0.327	
df	36		46		46		46	
LSD (5%)	0.3628		0.2680		0.2680		0.2680	
No./g/of root								
AR1	2.037	173	2.125	234	2.258	198	1.727	88
AR37	0.715	2	0.820	4	0.680	1	0.646	1
CT	1.108	21	0.902	5	1.322	44	1.101	16
NIL	1.681	102	1.935	214	1.422	35	1.685	57
SED	0.1297		0.2377		0.2377		0.237	
df	36		46		46		46	
LSD (5%)	0.2630		0.4784		0.4784		0.4784	

[1]*Analysis carried out on log (n + 4) transformed data.*
[2]*Arithmetic means.*

in September 2002. Over all treatments aphid loadings were lowest in June.

The extreme variability in root aphid numbers per plant which characterized the PG trial was also evident in this trial for AR1 and Nil plants (**Figure 3B**). Individual plants within each treatment had been cloned between the large and small containers enabling the role of host plant genotype to be explored. An analysis of log transformed aphid loading for each clonal pair of plants within each treatment showed significant correlations between the cloned pairs of CT and AR1-infected plants (Pearson's correlation coefficient 0.77 and 0.76, respectively; $P \leq 0.002$) whereas neither parameter was correlated in Nil plants (Coefficient 0.47; $P > 0.05$).

For all three destructive harvests of this trial the cumulative total herbage dry matter removed was significantly higher from CT and AR37-infected plants than from Nil and AR1 ($P < 0.001$; **Table 1**). At all three time points, cumulative herbage dry weight from plants growing in the larger planter bags was significantly greater than that in the small bags with no significant interactions between this and endophyte treatment (data not presented). Endophyte effects on mean root dry weight was similar to the effects on herbage, with the overall mean root weight from the three harvests greatest in CT and AR37 treatments and

significantly ($P < 0.001$) more than for Nil and AR1 with the latter two also significantly different (**Table 1**). Root weights were significantly greater for plants grown in larger planter bags than the small ones in all endophyte treatments.

Petri Dish Experiments

There were overall effects of endophyte in each trial, with AR37 having significantly fewer aphids than at least one other treatment in each trial ($P < 0.02$) and, likewise, CT also having low numbers in Trials A and C. On plants infected with AR37, root aphid survival declined to very low levels in all four trials, after an initial phase in which numbers were similar in all treatments (**Figure 4**). In Trials A and C root aphid numbers on CT plants followed a similar pattern of decline to those on AR37 whereas in Trials B and D aphid performance on CT was similar to that on AR1 and Nil treatments. Differences between AR37 and other treatments did not become significant until Day 19 in Trial A, Day 15 in Trial B, Day 20 in Trial C, and Day 7 in Trial D. In Trial A, it was also Day 19 before CT had significantly reduced numbers compared with AR1 whereas in Trial C it was Day 16, slightly earlier than AR37.

The role of plant genotype was considered by comparing aphid performance on cloned plants in Trials A and B and again in Trial C. For AR1, final numbers/plant were highly correlated between individual cloned plants in Trials A and B and again between the cloned plants in Trial C (**Table 3**). For CT-infected plants the strongly contrasting differences in aphid performance between Trials A and B showed no evidence of a plant genotype effect while in Trial C plant genotype effects could not be tested for when aphid numbers fell to low levels on all five plants. Aphid numbers on Nil plants were not correlated between either Trials A and B (−0.44) or in Trial C (−0.22).

A large majority of root aphids were located on roots regardless of treatment or aphid maturity, providing no evidence of deterrent effects of AR37 or CT (**Table 4**). There was no effect of assessment time on this aspect (data not presented). In Trial B, both nymphs and mature aphids also displayed a marked preference for new roots in all treatments and at all assessments.

On Day 7 in Trial B, two aphids on separate AR37 plants were trembling quite violently and continued to do so over the ensuing 24 h period. Both had died within 36 h of the time they were first observed. Over that period both aphids remained stationary, one with its stylet inserted into the root throughout. Following this, aphids in other experiments were closely observed and others were also found to be trembling and their movements uncoordinated but only in AR37 treatments. No aphids were subsequently found with tremors as severe as those first observed. Trembling aphids were recorded at Day 5 in Trial D but not until Day 13 in Trial C.

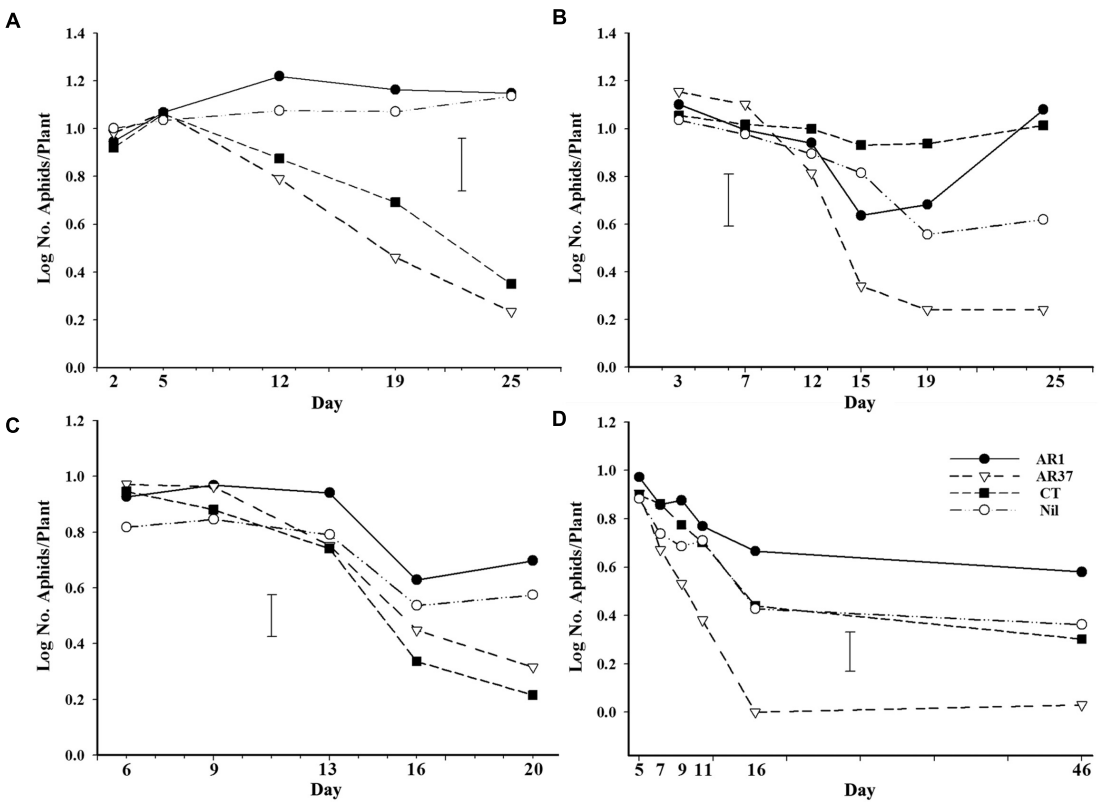


FIGURE 4 | Numbers of root aphid/plant on ryegrass plants without endophyte or infected with AR1, AR37, or CT at different times after inoculation with aphids in Petri dish experiments (A–D). Error bars = SED.

TABLE 3 | Petri Dish Experiments Effect of plant genotype on root aphid: final number of aphids/plant for cloned pairs of ryegrass plants infected with AR1 tested at two different times in Experiments A and B and at the same time in Experiment C.

Rep	Trials A and B – Cloned Pairs		Trial C – Cloned Pairs	
	A	B	A	B
1	2	0	0	1
2	59	35	38	23
3	46	15	7	6
4	27	10	12	0
5	1	2	0	6
Correlation[1]	0.96		0.87	

[1]*Pearson's correlation coefficient.*

TABLE 4 | Petri Dish Experiments Percentage of immature and mature root aphids located on roots (rather than away from roots) in Experiments B and C and on new roots (rather than old roots) in Experiment B.

	Trial	Endo	Immature	Mature	Mean	*N*[1]
% Aphids on roots	B	AR1	86.7	89.0	87.6	315
		AR37	77.6	76.0	77.1	153
		CT	85.7	82.5	84.6	331
		Nil	88.7	84.7	87.1	248
	C	AR1	93.6	91.3	92.8	470
		AR37	89.7	95.5	91.9	357
		CT	84.7	91.7	87.9	256
		Nil	92.7	90.9	91.9	385
% Aphids on new roots	B	AR1	98.0	85.7	93.2	161
		AR37	83.5	80.8	82.5	149
		CT	89.0	84.2	86.9	130
		Nil	89.3	68.3	86.5	144

N[1] *- Number of aphids observed*

DISCUSSION

Interactions between insect herbivores and their host plants at any one time depend on host quality, defined by Leather (1994) as "those plant attributes, chemical or physical, that contribute either negatively or positively to the fitness of the insect population or individual insect that feeds upon the plant's tissues." Insect performance is therefore governed by a balance between those chemical factors that positively influence its fitness and those that have a negative effect while other elements of host quality include resource availability. *Epichloë* endophyte infection of grasses changes the host quality in terms of its chemistry for those insects that utilize the infected plant as a food source. Differences in chemistry, however, may go beyond the presence or absence of certain alkaloids with more fundamental changes in the plant hosting the endophyte (Rasmussen et al., 2008a,b). The response of any one insect species can vary from negative, where the presence of alkaloids impair the performance of the insect, to neutral, where the insect is not affected to positive, where insect fitness appears to be better on infected plants than on uninfected (Saikkonen et al., 1999; Bultman and Bell, 2003). Effects may be endophyte-strain specific and be transitory rather than stable.

The effects of host quality on insect performance are exemplified in the results of the trials with *A. lentisci*. Populations of this aphid exhibited a marked response to host ryegrass plants ranging from negative to positive that were driven not only by the presence or absence of *Epichloë* infection but also by the strain of endophyte. At the negative end of the scale, ryegrass infected with AR37 was highly resistant to *A. lentisci*. This effect was stable, showing only minor seasonal variation with some increases in populations in spring but little variation in the level of resistance among individual plants. At the other end of the spectrum, ryegrass infected with AR1 was often more susceptible to root aphid than endophyte-free plants. Aphid populations were highly variable on AR1 both on individual plants and over time. In addition, ryegrass infected with other endophytes chemically similar to AR1 have shown similar levels of vulnerability to this aphid (Popay and Gerard, 2007). For Nil plants there was considerable inter-plant and temporal variation in the number of root aphids/plant, and overall aphid performance on this treatment could be considered to range from neutral to positive. Aphids tended to be less numerous on CT than on Nil plants but not always significantly so. Thus aphid performance on ryegrass with CT endophyte was mostly neutral with what appears to be transient negative effects. Inter-plant and temporal variations in number of aphids on CT were much less than on Nil and AR1.

The amount of roots may provide one explanation for differences between treatments but aphid loadings generally reflected the numbers/plant and did not change relative

differences between endophyte treatments suggesting that this was not a limiting factor. As a measure of resource availability, however, root weight may not be sufficient because it takes no account of differences in root morphology and age which may be equally, if not more important, for aphid performance. This was evident from the Petri dish experiments in which aphids exhibited a strong preference for new roots suggesting that the availability of new roots, rather than the total root weight *per se,* is more important for population development. In this regard, the design of the pot trials in allowing repeat sampling of new root growth was useful. The strong preference to inhabit young roots also suggests that actively growing plants are likely to stimulate population growth. Growth affects the quantity and quality of phloem, both of which are factors that contribute to aphid performance (Whitham, 1978). The preference root aphid showed for new roots may be explained by changes in chemistry as roots age but equally may be due to physical factors such as increasing lignification that may make it difficult for the aphid to probe older roots. Respiration rates are higher and uptake of nutrients and water more efficient in new than in old roots (Eissenstat and Yanai, 1997; Bouma et al., 2001) but there is little other information on physiological changes in maturing roots that may explain aphid preference.

If habitat was not limiting aphid populations then plant chemistry is the most likely basis for the differences observed among endophyte treatments. The effects of AR37 on *A. lentisci* were most likely attributable to the production of a metabolite by the fungus that was toxic to the aphid. The tremors induced when the aphid feeds on plants infected with AR37 indicated that the compound was a neurotoxin. In all the Petri dish trials there was an initial phase lasting up to 2 weeks after aphids were released onto the plants in which the aphid behavior, feeding and reproduction appeared normal. Such a delayed effect suggests that the toxin was either a slow-acting constitutive compound or one that is inducible. The proportion of aphids recorded on roots provided no evidence of a deterrent response to AR37.

The effect of the CT strains may also be due to the presence of a secondary metabolite. In Experiments A and C in Petri dishes, the rapid decline in aphid numbers on CT was symptomatic of the presence of a toxin but there was no indication of this in Experiments B and D. Plants in Experiment B were clones of those in A, ruling out plant genotype as a factor in the different population responses. Plants in B and C had been grown in the Petri dishes for a similar length of time prior to inoculation with aphids and were kept under similar ambient conditions. Experiment C was conducted a month after Experiment B in spring when temperatures were warmer (mean maximum/minimum temperatures were: B 15.2/5.3°C; C 17.0/7.0°C) but there was no indication in the pot trials that aphid performance on CT varied with seasons or temperature. The alkaloids produced by CT endophyte with known anti-insect activity are lolitrem B, ergovaline and peramine (Popay and Bonos, 2005). Peramine was ruled out as affecting aphids since it is the only one of the three compounds that is also produced by AR1. In an experiment by Popay and Gerard (2007) that compared endophytes with different alkaloid profiles, ergovaline was implicated in low root aphid populations although the reason for the transient effects observed is unknown. Ergovaline concentrations in plants vary seasonally and with environmental conditions (Ball et al., 1995a; Lane et al., 1997) and are also linked to plant genotype (Easton et al., 2002). Despite being a lipophilic compound this alkaloid does occur in roots in concentrations that can be as high as those in the pseudostem in 'Grasslands Samson' infected with CT (A. J. Popay unpublished).

A strong host plant genotype influence on aphid fitness on CT and AR1-infected plants was previously reported for the PG trial by Popay and Easton (2006). A similar analysis of aphid populations on the cloned plants in small and large planter bags in the RM trial also provided evidence of a plant genotype effect but again only for those plants infected with CT and AR1. The weakness of the link between plant genotype and aphid performance in Nil indicates that a host plant genotype/endophyte interaction may be moderating aphid performance more than plant genotype itself. A similar high degree of variability associated with inter-plant genotypic differences has been found in the amount of damage inflicted on AR1-infected plants by black beetle adults (Easton et al., 2000). Alkaloid production is linked to endophyte concentration in the plant and is markedly influenced by host plant/endophyte interactions (Ball et al., 1995a,b; Easton et al., 2002). Other aspects of plant growth and mineral uptake have also been shown to vary according to interactive effects of endophyte and host plant genotype (Malinowski and Belesky, 1999; Malinowski et al., 2000; Cheplick and Cho, 2003).

Composition and concentration of amino acids and concentration of sucrose in the phloem are important determinants of aphid performance (Douglas, 1993; Karley et al., 2002) and levels of soluble nitrogen are often causally linked to inter- and intra-plant differences in aphid fitness, site preferences, host alternating behavior and seasonality (Leather, 1994). Endophyte infection of *L. perenne* can modify the metabolic profiles of their hosts, interacting with nitrogen supply and host plant genotype, in ways that influence herbivore response (Rasmussen et al., 2008a,b). Differences in some of these factors may account for not only the apparent differences in aphid performance between AR1 and Nil, but also the extreme variability between individual plants.

Unlike many foliar-feeding aphid species, there was no discernible pattern in aphid numbers over time or season. In the PG trial, numbers were highest in autumn 2001 but then fell to very low levels in summer 2002. Seasonality was not the cause since aphid populations were generally high in the RM trial in summer 2003. Anderson (1987) noted that root herbivores are often chronic pests and this would appear to be true for *A. lentisci*. Observations also suggest that, like many root herbivores, *A. lentisci* are highly aggregated in their distribution, forming sometimes large colonies on roots where they cocoon themselves in wax secretions. They show no preference for a particular depth in the soil profile but exploit large pore spaces in the soil structure where there is often a proliferation of new roots; hence their apparent prevalence at the interface between the growing medium and container. At times in the field and in potted plants, aphids have been observed feeding at the soil surface, clustered around the base of tillers. A similar behavior

in another root aphid *Pemphigus bursarius* on lettuce plants has been associated with the production of sexupariae that develop into winged morphs in readiness for flight to their primary host (Dunn, 1959). Winged *A. lentisci* were not observed in the course of this study but have been trapped in both New Zealand and Australia (O'Loughlin, 1962; Lowe, 1968). Early instar nymphs are highly mobile in the soil and have also been sampled from the foliage of ryegrass (Rasmussen et al., 2008b). Thus dispersal mechanisms are likely to involve movement of these nymphs along the surface but, given their very small size, may also include dispersal by wind.

There is considerable information in the literature on the detrimental effects of foliar aphids on plant growth and fitness (e.g., Giménez et al., 1997; Riedell et al., 2007) but much less information on root aphids. Hutchison and Campbell (1994) demonstrated a severe effect of the sugar beet root aphid, *Pemphigus betae* on yield and sugar content including a 54% reduction in total recoverable sugar/ha. Similarly the lettuce root aphid, *P. bursarius*, and the cabbage root aphid *Pemphigus populitransversus* cause significant economic yield loss (Royer and Edelson, 1991; Liu et al., 2011). Here, using comparisons of *L. perenne* growth between the resistant AR37 and the other treatments in the PG trial, *A. lentisci* reduced overall foliar growth by between 20 and 23% but did not reduce cumulative root growth. In addition to this, however, survival of AR1 and Nil plants was reduced by 35% whereas there was no mortality of CT and AR37 plants during the trial (data not presented). In autumn and spring 2002, tillers of AR1 and Nil plants were also infested with a mealybug (data not presented) in the PG trial but given that cumulative foliar growth on CT was similar to that for Nil and AR1, root aphid can be considered to be the primary factor affecting this. In the RM trial, herbage growth of AR1 and Nil was 16–27% less than AR37 over the different harvests while average root mass of AR37 over the three harvests was over 50% greater than for AR1 and 22% greater than Nil. In contrast to the PG trial, growth of CT plants has matched or exceeded that of AR37. This difference between the two trials reflects a difference in root aphid loadings which were considerably lower on the CT treatment in the RM than in the PG trial (mean 21 cf. 142/g of root). Effects of infestations of *A. lentisci* have also been demonstrated in the field (Hume et al., 2007) where very high populations of aphids can occur causing a chronic loss of vigor and large yield loss of ryegrass. The presence of this aphid year round on plants will be a constant drain on the plant's resources, resulting not only in reduced plant performance but also poor survival. Thus the results reported here have supported the hypothesis that differences in plant growth of *L. perenne* without endophyte or infected with different strains of endophyte are associated with their effects on populations of *A. lentisci*.

AUTHOR CONTRIBUTIONS

AP designed and carried out all the experimental work reported here; NC provided statistical expertise.

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Does White Clover (*Trifolium repens*) Abundance in Temperate Pastures Determine *Sitona obsoletus* (Coleoptera: Curculionidae) Larval Populations?

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To determine if host plant abundance determined the size of clover root weevil (CRW) *Sitona obsoletus* larval populations, a study was conducted over 4 years in plots sown in ryegrass (*Lolium perenne*) (cv. Nui) sown at either 6 or 30 kg/ha and white clover (*Trifolium repens*) sown at a uniform rate of 8 kg/ha. This provided a range of % white clover content to investigate CRW population establishment and impacts on white clover survival. Larval sampling was carried out in spring (October) when larval densities are near their spring peak at Lincoln (Canterbury, New Zealand) with % clover measured in autumn (April) and spring (September) of each year. Overall, mean larval densities measured in spring 2012–2015 were 310, 38, 59, and 31 larvae m^{-2}, respectively. There was a significant decline in larval populations between 2012 and 2013, but spring populations were relatively uniform thereafter. The mean % white clover measured in autumns of 2012 to 2015 was 17, 10, 3, and 11%, respectively. In comparison, mean spring % white clover from 2012 to 2015, averaged c. 5% each year. Analysis relating spring (October) larval populations to % white clover measured in each plot in autumn (April) found the 2012 larval population to be statistically significantly larger in the ryegrass 6 kg/ha plots than 30 kg/ha plots. Thereafter, sowing rate had no significant effect on larval populations. From 2013 to 2015, spring larval populations had a negative relationship with the previous autumn % white clover with the relationship highly significant for the 2014 data. When CRW larval populations in spring 2013 to 2015 were predicted from the 2013 to 2015 autumn % white clover, respectively, based on their positive relationship in 2012, the predicted densities were substantially larger than those observed. Conversely, when 2015 spring larval data and % clover was regressed against 2012–2014 larval populations, observed densities tended to be higher than predicted, but the numbers came closer to predicted for the 2013 and 2014 populations. These differences are attributed to a CRW population decline that was not

accounted by % white clover changes, the CRW decline most likely due to biological control by the Braconid endoparasitoid *Microctonus aethiopoides*, which showed incremental increases in parasitism between 2012 and 2015, which in 2015 averaged 93%.

Keywords: insect pest management, biological control, dairy pasture, pasture persistence, *Microctonus aethiopoides*

INTRODUCTION

Weevils belonging to the genus *Sitona* include a number of species that are recognized pests including *Sitona lineatus* L. (Cantot, 1989; Lohaus and Vidal, 2010), *S. discoideus* Gyllenhal (Aeschlimann, 1978; Goldson et al., 1988), *S. hispidulus* F. (Quinn and Hower, 1986; Dintenfass and Brown, 1988) and *S. obsoletus* Gmelin (Murray and Clements, 1998; Gerard et al., 2007). *S. obsoletus* (formerly described as *S. lepidus* and *S. flavescens*), is a Palaearctic species first detected in the North Island of New Zealand in 1996 (Barratt et al., 1996). It was first discovered in the South Island of New Zealand in 2006, with discrete populations located near Richmond, Rai Valley and Christchurch (Phillips et al., 2007). The weevil shows a strong preference for white clover (*Trifolium repens* L.), although overseas it has also been considered a pest of red clover (*T. pratense* L.) (Brudea, 1982; Murray and Clements, 1994). Worldwide, white clover is recognized as a valuable component of pastures because of its ability to fix nitrogen and provide quality feed for improved animal production. In New Zealand, ryegrass (*Lolium* spp. L.) and white clover are the predominant plant species in improved grasslands (Woodward et al., 2003; Tozer et al., 2014), so the potential impact from *S. obsoletus* was considerable.

Sitona obsoletus is found almost all year round and depending on season, both adults and larvae can be present in pasture where white clover is growing. Under severe larval infestations, a decrease in percent foliar N levels in spring can occur, with subsequent reductions in herbage dry matter (DM) yield levels (Gerard, 2002). Under severe infestations, and in combination with livestock herbivory, plant stress or poor fertility, white clover plants can disappear from the sward. Although this is often temporary, it does have implications for pasture productivity and cost to the farmer through a reliance on artificial N to compensate for the loss of white clover. New generation adults commence emergence in spring (November), with peak emergence occurring in the following 4–6 weeks. Following emergence, adults feed extensively on the foliage, and can be damaging to both seedling clover and plants in mature stands, particularly when populations are high in early-mid-summer. During this period, flight muscles and the reproductive system are maturing. Following mating, females exhibit reproductive maturation with egg laying commencing in mid-summer, and continuing through winter into spring. The percentage of reproductively mature females is highest over winter–spring with eggs laid indiscriminately in the foliage. Eggs accumulate in the litter, with larval development occurring at temperatures above 9.8°C (Arbab and McNeill, 2011). First instar larvae emerge and burrow down into the soil to locate the root system. There are five larval instars, with the first instar dependent on root nodules for establishment and survival (Gerard, 2001) and inoculated nodules with viable rhizobia preferred to uninoculated nodules (Hackell and Gerard, 2004). Later instars feed on the general root system including the stolons. There are two larval generations per year in the Canterbury region of New Zealand, with first major peak occurring in October–November (spring), and a second smaller peak occurring in April–May (autumn) (M. R. McNeill, unpublished data).

The arrival of clover root weevil (CRW) and its natural and anthropogenic-assisted spread across New Zealand, coincided with farmer reports of white clover production and consequent animal production declines as population numbers built up in newly colonized regions (e.g., McNeill et al., 2012). This was also confirmed by small plot study that showed up to a 35% decline in DM production under a modest population of c. 300 larvae m^{-2} (Gerard et al., 2007). Management recommendations to minimize impacts included increased applications of artificial nitrogen, especially in spring, pasture management to encourage white clover production, and a prolonged fallow or crop to eliminate larval populations prior to resowing of new pastures. As part of the management program to control *S. obsoletus*, the Braconid endoparasitoid *Microctonus aethiopoides,* was introduced in 2006 (Gerard et al., 2006; Phillips et al., 2007). Originally from Ireland, the parasitoid attacks the adult weevil, with parasitism resulting in sterilization and the eventual death of the weevil upon emergence of the parasitoid larvae. For female CRW, parasitism prevents further egg laying, with a subsequent impact on the number of larvae that establish. The parasitoid is able to complete 3–4 generations per year, attacks multiple weevils and has a winter diapause, occurring as a first instar larva inside the adult weevil. As of June 2016, CRW is widely distributed across New Zealand pasture containing white clover, and depending on season, either adults or larvae or both stages occur concurrently.

The importance of root nodules to *Sitona* spp. larval development has been shown in several contributions (e.g., Danthanarayana, 1967; Quinn and Hower, 1986; Wolfson, 1987; Murray and Clements, 1998; Gerard, 2001; Lohaus and Vidal, 2010), although the relationship was less clear for *S. discoideus* first instar survival (Aeschlimann, 1986). The result of nodule feeding and destruction of the root system, is that the plant can become stressed causing overcompensation in nodule production (Quinn and Hall, 1992), reduction in leaf and root N (Murray et al., 2002), modification to C:N ratios (Murray et al., 1996) and overall reductions in DM production (Dintenfass and Brown, 1988; Goldson et al., 1988). As CRW egg deposition and

subsequent larval establishment is a function of time and place, female CRW have an important role in distributing the eggs in an environment that is conducive to larval survival (Johnson et al., 2006). For this reason, the number of adults and resultant egg laying effort could be expected to be higher in swards supporting a greater white clover content compared with pastures with a low white clover content. Gerard et al. (2007) reported that plots with good clover cover had more than twice the number of larvae m^{-2} compared to plots with low % clover. Therefore, it was hypothesized that the density of CRW larvae is a function of white clover content, with % white clover an indicator of root resources, especially root nodules. In other words, high CRW larval populations occur in swards with a high % white clover, conversely low populations will occur in swards with a low % white clover. In addition, it was considered that the % white clover measured in autumn, at the start of the ovipositional effort, would be a primary determinant of subsequent CRW larval populations in spring.

The establishment of a DairyNZ field trial in March 2010, to investigate ryegrass persistence under a range of sowing rates also provided an opportunity to measure CRW larval densities each spring in order to assess any impact of CRW on white clover production. In addition, it offered an opportunity to see if the presence of *M. aethiopoides* had a long term impact on CRW larval populations. CRW adults were first detected on the Lincoln University Research Dairy Farm in late 2009. The Irish *M. aethiopoides* was first recorded in CRW collected from a paddock on the farm on 14 March 2011 with a parasitism rate of 46%. This high level of parasitism indicated that the parasitoid was possibly already established at the beginning of 2011.

MATERIALS AND METHODS

Research Site

The research was undertaken on plots within the Longitudinal Persistence Experiment (Sub-project 3 of FD1004) run by DairyNZ (Lee et al., 2016). The experiment was on the Lincoln University Research Dairy Farm (S43.636721, E172.460865), with research investigating the effect of ryegrass seed rates on plant size, competitive ability and persistence, as well as establishment and subsequent presence of white clover in the sward. Ryegrass and clover were drilled into cultivated seedbed between 30 March – 4 April 2011. The DairyNZ experiment comprised four ryegrass cultivars [cv. Alto AR37 (diploid), cv. Commando AR37 (diploid), cv. Halo AR37 (tetraploid) and cv. Nui wild-type endophyte (diploid)]. There were five ryegrass seeding rates for each cultivar: 6, 12, 18, 24, and 30 kg/ha of Ultrastrike®– insecticide treated seed. All plots were drilled with 8 kg/ha of Superstrike® – insecticide treated white clover seed (cv. Tribute) (equivalent of 5 kg/ha of bare seed). Treatments were arranged in a randomized split block design with five block replicates. The site was irrigated and grazed by dairy cows (see grazing details below).

In the study reported here, CRW larval and % clover measurements were restricted to the ryegrass cv. Nui plots sown at 6 and 30 kg/ha.

Sampling CRW Larvae and Adults

Soil cores (10 cm diameter by 14 cm deep) were taken from each of the five replicates of both 6 and 30 kg/ha ryegrass-white clover plots. In the laboratory, cores were hand sorted and second through to fourth instar larvae counted to determine the number of CRW larvae m^{-2}. Because first instar larvae are difficult to locate in the soil or hidden in the clover root nodules these were not counted. Therefore, recorded numbers are an underestimate of actual larval densities. Sampling to monitor initial establishment and build-up of larvae first occurred on 13 December 2011 approximately 8 months after the trial was sown. with a subsequent sample on 2 May 2012. On each occasion, only five cores were taken per plot. Thereafter, sampling occurred 25 October 2012, 24 October 2013, 22 October 2014, and 20 October 2015 with 10 cores taken from each plot.

Sampling to detect the presence of adult CRW was first carried out on 13 December 2011. Sampling on this occasion was undertaken using a vacuum cleaner to remove weevils from 0.25 m^{-2} quadrats, with five quadrats per replicate, giving 50 samples in all. This method provided an estimate of ground density at the start of immigration by spring-emerged adults. Thereafter, adults were sampled in May or July each year, using a modified blower vac (Echo ES-2400, 24 cc, Kioritz Corporation, Tokyo) to collect the weevils from the foliage and litter. CRW adults were extracted from the sample, counted and dissected under a binocular microscope to determine reproductive condition and parasitism status. The period May to July provides an accurate fix on parasitism, as *M. aethiopoides* enters diapause in late autumn, overwintering as first instar larvae inside the weevil. The blower vac was specifically utilized for collecting sufficient numbers of weevils for dissection and not to ascertain adult densities.

White Clover Measurements

Details on the collection and assessment of botanical composition are detailed in Lee et al. (2016). Briefly, representative samples of herbage from each sub-plot were collected the day before grazing in autumn (April) and late spring (August/September). Cuts were taken using hand shears, returned to the laboratory and a sub-sample dissected into the following categories: perennial ryegrass leaf, perennial ryegrass reproductive stem (including seed-head and flowers), white clover, unsown species and dead material. The percentage white clover was measured on 16 April 2012, 22 April 2013, 14 April 2014, and 31 March 2015 (autumn) and then 27 August 2012 (late winter), 23 September 2013, 23 September 2014, and 27 October 2015 (spring).

Fertilizer and Grazing Management

Maintenance fertilizer was applied to the Canterbury site in June 2012 (61, 38, 74, and 135 kg ha^{-1} of P, K, S, and Ca; Lee et al., 2016). Nitrogen (N) fertilizer was applied as urea with total yearly N applications across the 4 years being 198 kg ha^{-1} (2011/12), 264 kg ha^{-1} (2012/13), 280 kg ha^{-1} (2013/14), and 225 kg ha^{-1} (2014/15), respectively, spread over three to six applications. Plots were rotationally stocked by dairy cows, grazed at the same time

to a desired residual and as required, mown post-grazing to maintain a uniform pasture (Lee et al., 2016).

Data Analysis

Larval Density by Year

This analysis compared the larval populations in October from 2012 to 2015. Analysis took a generalized estimating equations (GEE) approach, using a generalized linear model assuming group-specific negative binomial distributions through log link function. The groups were defined by a single factor Year (four levels: October 2012 to October 2015). The GEE analysis also took account of potential correlation among larval numbers in soil cores taken from each individual plot in each year.

Percentage White Clover by Year

The % white clover values were analyzed and compared between the 4 years; 2012 to 2015, using analysis of variance (ANOVA) with a single factor: Year (four levels: October 2012 to October 2015). The ANOVA was applied to % clover in autumn and spring, respectively.

Larval Density and % White Clover within a Year

As CRW larvae require white clover to survive, it was hypothesized that the percentage white clover measured in the pasture was an indicator of the size of the CRW larval populations (e.g., high clover content indicated a high larval population). This was based on the premise that CRW adult populations would colonize swards with high white clover content. Furthermore, % white clover measured in autumn would reflect both the availability of plant material for adult CRW and nodule resources for the putative larval population going into winter. Therefore, low % white clover in autumn would be expected to result in low CRW larval populations in spring. An assessment using spring measurements of % white clover was also carried out to examine the relationship between the larval populations and % white clover in spring. In order to test this hypothesis, larval populations measured each October 2012 to 2015, irrespective of ryegrass sowing rate, were analyzed using GEE, against % white clover measured in autumn (April) and again in late winter and spring (August–September). Each GEE analysis used a generalized linear model assuming negative binomial distribution through log link function, and the relationship of larval population [larval density (m^{-2})] in each year to % white clover was modeled as an exponential equation:

$$\text{Larval density } (m^{-2}) = 127.4 \times \exp(A + B \times \% \text{ white clover})$$

where 127.4 was a constant to convert the estimated larval density per soil core into the density m^{-2} and A and B were intercept and slope coefficient to be estimated, respectively. This GEE analysis also took account of potential correlation among larval numbers in soil cores taken from each individual plot in each year. All GEE analyses and ANOVAs were carried out using SAS (version 9.3).

Larval Density and % White Clover between Years

Within any year the range of % white clover was small, however, between years the range was much greater. Therefore, in order to further investigate whether there was any relationship between spring larval density (m^{-2}) with % white clover measured in autumn or spring, a repeated measures analysis of the per plot data across all 4 years was performed. The correlation between mean larval densities in an individual plot over time was modeled using an autoregressive model of order 1. Additional uniform correlation within plots was also allowed for. Larval density was $\log_{10}+10$ transformed prior to analysis to stabilize the variance. Ten was added prior to $\log_{10}$ transformation to accommodate the presence of zero densities observed in 2015. The model was fitted using restricted maximum likelihood (REML) in Genstat (version 17).

A simple linear regression model was also fitted to the 2012 $\log_{10}$ (spring larvae) data, with either the % clover in spring, summer or autumn as the explanatory variable. From the fitted model, the $\log_{10}$ (spring larvae) in 2013, 2014, and 2015 was predicted. Similarly, using 2015 spring larval data with % clover in either in spring, summer or autumn as the explanatory variable, the 2012, 2013, and 2014 larval populations were predicted. However, in contrast to the 2012 larval data, the 2015 spring larvae data didn't require transformation prior to analysis in order to linearize the relationship.

RESULTS

CRW Larval Populations

There were no CRW larvae found in the cores taken on 13 December 2011, which is consistent with our understanding that colonization of the newly established plots by spring emerged CRW adults would have only just begun and the lack of reproductively mature females would have meant an absence of larvae. Larval recruitment between December 2011 and May 2012 was rapid, and by 2 May 2012, mean (±SEM) larval populations in the 6 and 30 kg/ha plots were 234 ± 86.6 and 246 ± 124.9 larvae m^{-2}, respectively, and not significantly different ($P = 0.887$).

Mean larval densities measured in all plots in October 2012–2015 were 310 ± 43.3, 38 ± 7.6, and 59 ± 14.131 ± 8.1 larvae m^{-2}, respectively. There was a significant decline in larval populations between 2012 and 2013 ($P < 0.001$), thereafter larval densities between the October 2013–2015 samples were not significantly different ($P > 0.05$).

Ryegrass sowing rate had a significant influence on the larval populations in October 2012, with mean densities of 431 ± 28.7 and 188 ± 28.9 larvae m^{-2} in the 6 and 30 kg/ha plots, respectively ($P < 0.001$; **Figure 1**). Sampling in October 2013, found a significant decline in larval populations (**Figure 1**) at both sowing rates compared to 2012, but sowing rate had no significant effect on larval densities ($P = 0.064$), a result that was repeated in the following 2 years (**Figure 1**).

CRW Adults and Parasitism

Sampling on 13 December 2011, found a mean (±SEM) of only 1.0 ± 0.33 adults m^{-2} with all eight individuals immature and unparasitised. Subsequent dissection of weevils collected in May–July from 2012 to 2015, found parasitism by *M. aethiopoides* was high to very high (38–95%) and generally increased over the study

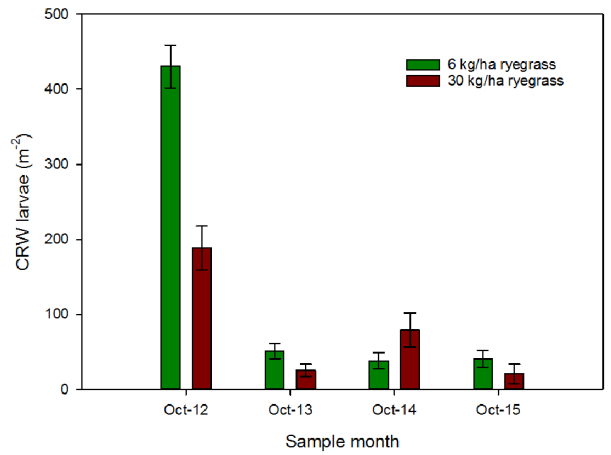


FIGURE 1 | CRW larval densities (±SEM) measured in ryegrass/white clover swards consisting of ryegrass sown at 6 or 30 kg/ha and white clover sown at 8 kg/ha.

period. Dissection of CRW adults collected on 14 May 2012 and on 16 May 2013, showed high rates of parasitism of 79% ($n = 39$) and 84% (31), respectively. Sampling on 01 July 2014, found an average parasitism rate of 58% (range 38–94%). However, in 2015, the mean parasitism rate across all plots was 93%, indicating both a significant increase in overall parasitism compared to 2014 and subsequent impact on CRW oviposition potential.

Percentage White Clover by Year

The mean % white clover measured in autumn of 2012 to 2015 was 17 ± 2.2, 10 ± 2.3, 3 ± 2.3 and 11 ± 2.3%, respectively. The declines between 2012 and 2013, and between 2013 and 2014 were both significant ($P = 0.043$ and 0.025, respectively). By comparison, there was a significant increase between 2014 and 2015 ($P = 0.016$).

The mean % white clover measured in spring of 2012, 2013, and 2014 was relatively constant at c. 5 ± 1.2 for each of the 3 years but in 2015 averaged 6.4 ± 1.2, the slight increase possibly related to the later sampling and the onset of spring clover growth.

Ryegrass sowing rate had a significant influence on the % white clover measured in autumn, with white clover content higher in plots sown with ryegrass at 6 kg/ha compared to the 30 kg/ha sowing rate (**Figure 2A**). This was most notable in the April 2012 assessment, where the mean % white clover measured in the 6 kg/ha and 30 kg/ha plots was 23.4 ± 2.7 and 10 ± 2.7%, respectively, and significantly different ($P = 0.002$). Thereafter, the % white clover was not significantly different at the two ryegrass sowing rates. Overall, at the 6 kg/ha ryegrass sowing rate, autumn % white clover showed a significant decline each year, while for the 30 kg/ha sowing rate the decline was only significant between April 2012 and April 2014 ($P = 0.043$).

By comparison, the mean % white clover measured in late winter and spring (August 2012, September 2013–2015), across both sowing rates showed no significant difference between years or ryegrass sowing rate (**Figure 2B**).

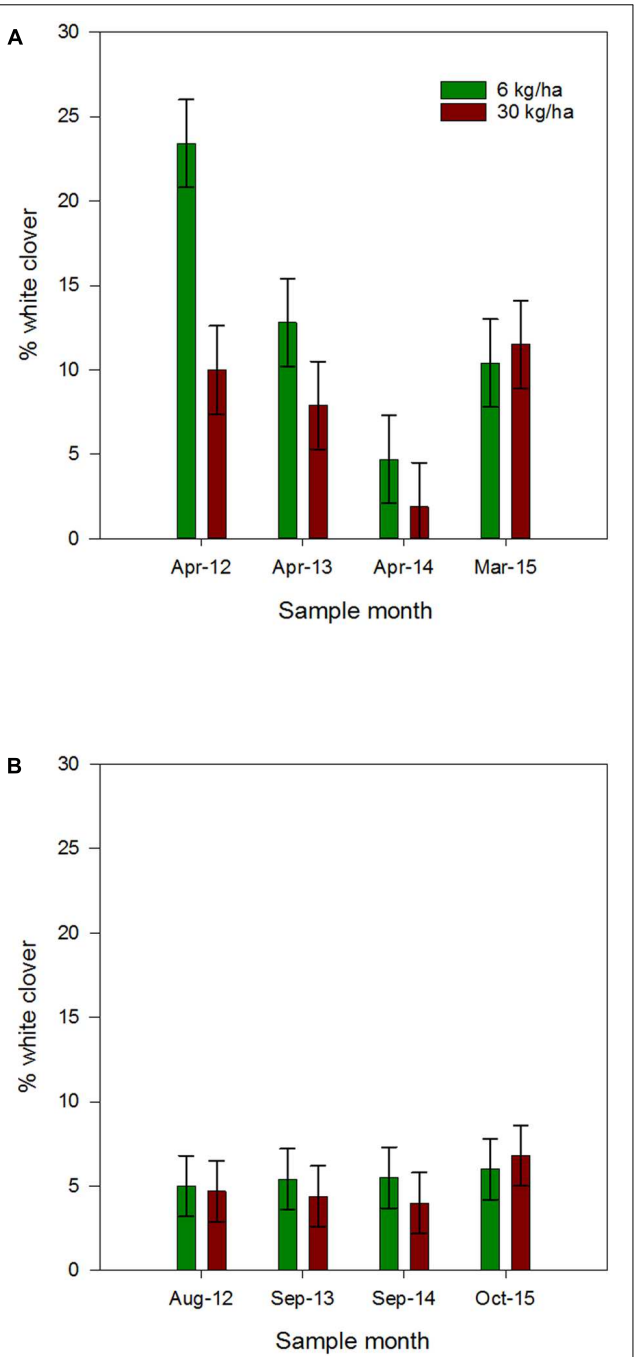


FIGURE 2 | Mean autumn (A) and spring (B) % white clover (±SEM) measured in plots containing ryegrass sown at 6 or 30 kg/ha and white clover sown at 8 kg/ha. Autumn values indicated in **(B)** for comparison.

CRW LARVAL POPULATIONS AND INTERACTION WITH % WHITE CLOVER

From 2012 to 2015, CRW larval populations showed a relationship with % white clover that varied across years and by season (**Figures 3–6**; **Table 1**).

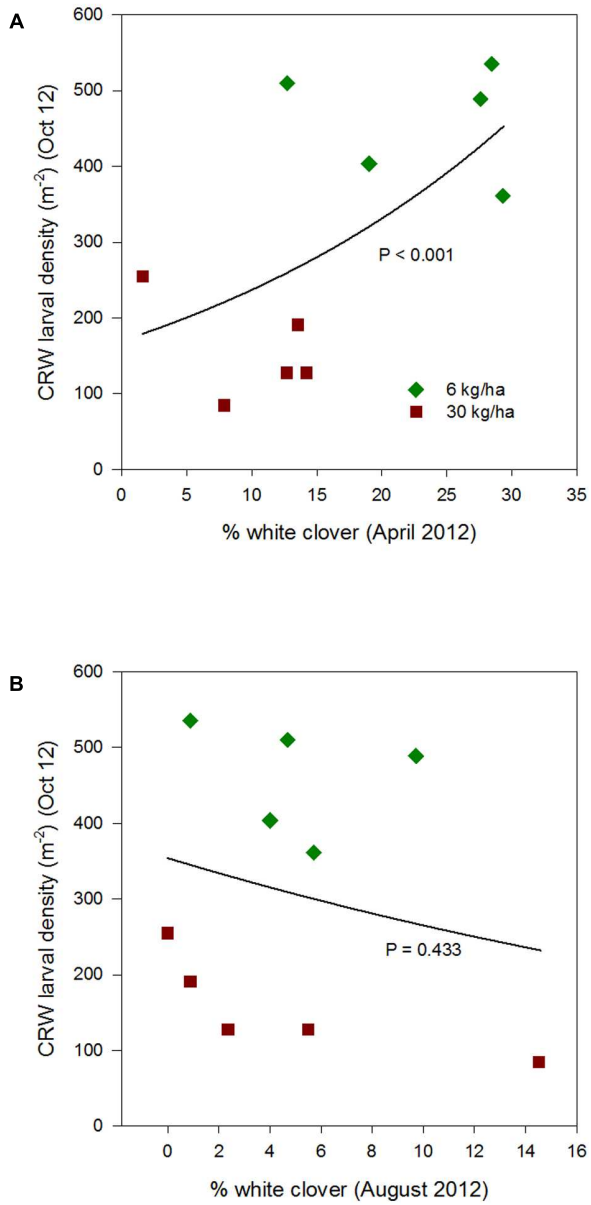


FIGURE 3 | Clover root weevil larval densities measured in October 2012 in relation to % white clover measured in April 2012 (A) and August 2012 (B), from ryegrass plots (cv. Nui) sown at 6 and 30 kg/ha (solid line shows the estimated relationship across all plots, irrespective of ryegrass sowing rate).

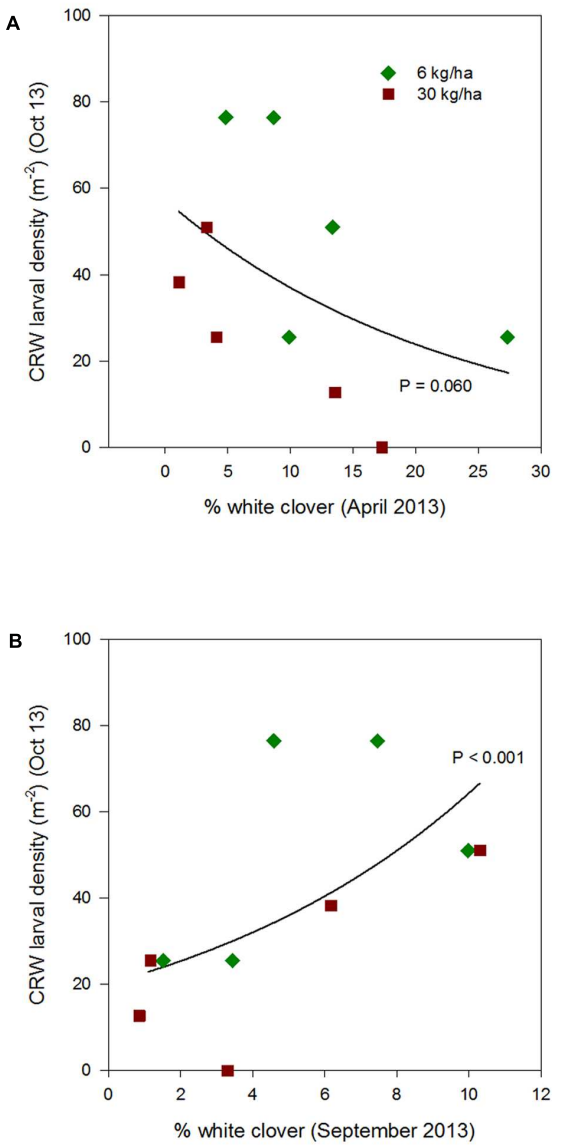


FIGURE 4 | Clover root weevil larval densities measured in October 2013 in relation to % white clover measured in April 2013 (A) and September 2013 (B), from ryegrass plots (cv. Nui) sown at 6 and 30 kg/ha (solid line shows the estimated relationship across all plots, irrespective of ryegrass sowing rate).

Interaction with % White Clover Measured in Autumn

Larval populations measured in October 2012 were significantly higher in plots where ryegrass had been sown at 6 kg/ha compared to plots sown at 30 kg/ha (**Figure 1**). This corresponded with the significantly larger mean % white clover in April 2012 in the 6 kg/ha plots (**Figure 2**). This sowing rate group difference also led to a statistically significant positive correlation with the % white clover overall ($P < 0.001$; **Figure 3A**; **Table 1**).

Larval populations measured in October 2013, 2014, and 2015, had a negative relationships with the autumn % white clover (**Figures 4A**, **5A**, and **6A**, respectively, **Table 1**) with the relationship highly significant for the 2014 data ($P < 0.001$; **Figure 5A**).

Interaction with Percentage White Clover Measured in Spring

The relationship of October CRW larval populations with % white clover measured in spring (late August 2012, September 2013 to 2015) was also mixed. There was no significant larvae – %

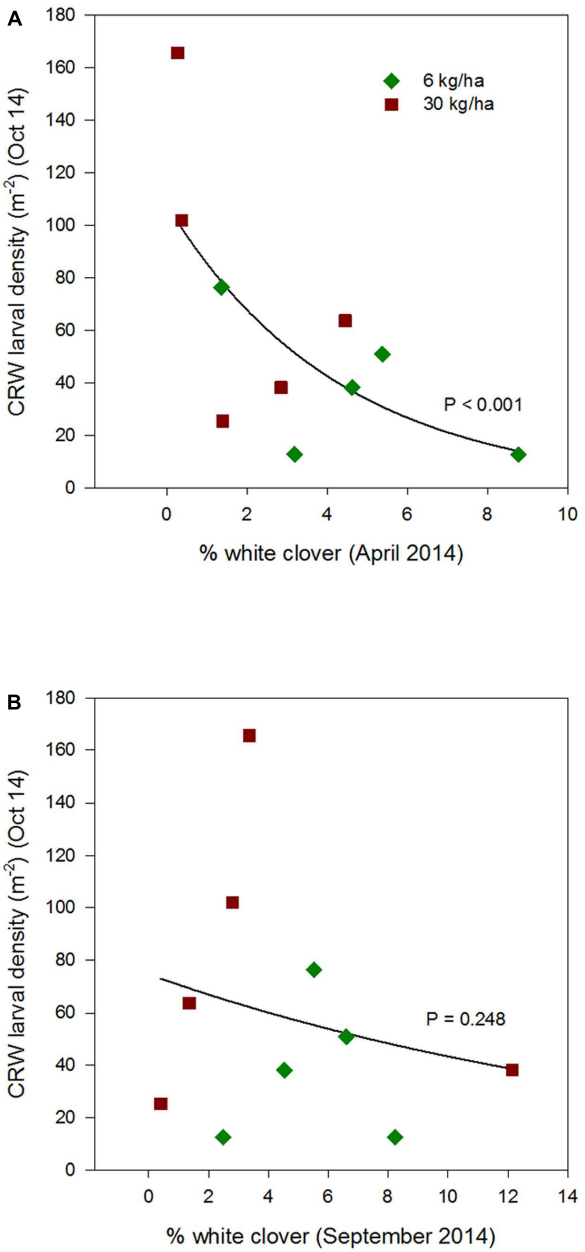


FIGURE 5 | Clover root weevil larval densities measured in October 2014 in relation to % white clover measured in April 2014 (A) and September 2014 (B), from ryegrass plots (cv. Nui) sown at 6 and 30 kg/ha (solid line shows the estimated relationship across all plots, irrespective of ryegrass sowing rate).

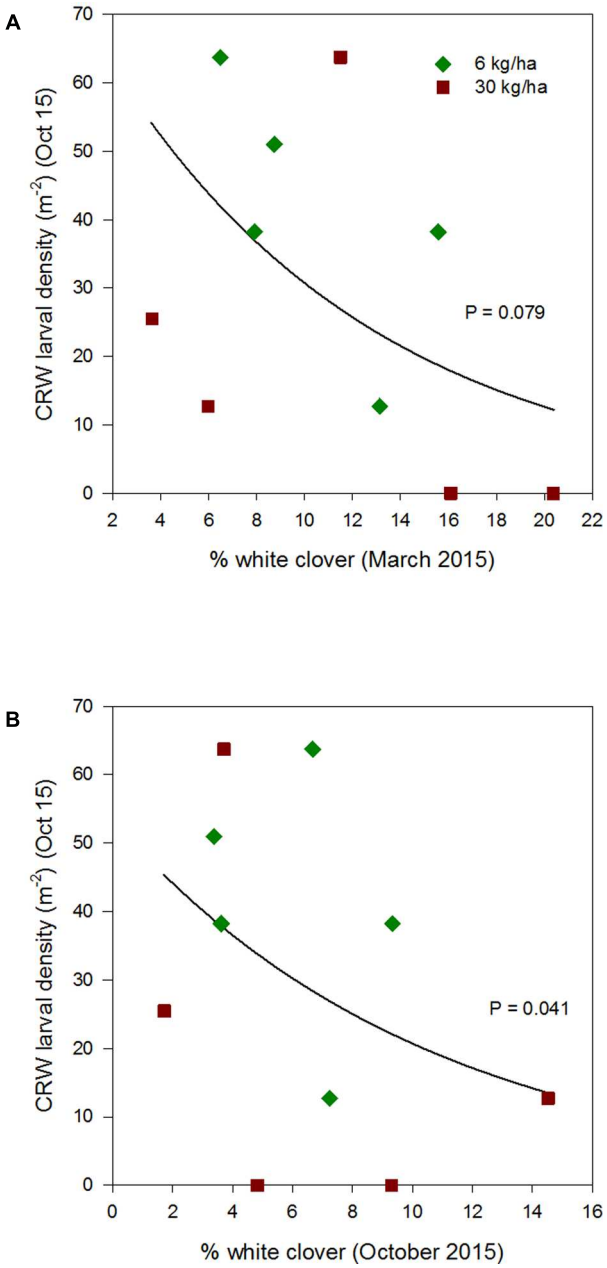


FIGURE 6 | Clover root weevil larval densities measured in October 2015 in relation to % white clover measured in March 2015 (A) and October 2015 (B), from ryegrass plots (cv. Nui) sown at 6 and 30 kg/ha (solid line shows the estimated relationship across all plots, irrespective of ryegrass sowing rate).

white clover interaction measured in August 2012 ($P = 0.433$; **Figure 3B**; **Table 1**). Conversely, in 2013, the October larval population had a significant positive relationship with % white clover measured in September ($P < 0.001$; **Figure 4B**; **Table 1**). In 2014, there was slightly negative, but not significant relationship between the October larval population and % white clover measured in September ($P = 0.248$; **Figure 5B**; **Table 1**). For 2015, the relationship was again negative and significant ($P = 0.041$; **Figure 6B**; **Table 1**).

Larval Density and % White Clover across Years

Repeated measures analysis across all 4 years detected a significant positive relationship between spring CRW larval

TABLE 1 | Equations describing the relationship between CRW larval density and % white clover (% wc) measured from 2012 to 2015.

Larval sample month	% clover sample	Estimated relationship	*P*-value
October 2012	April 2012	Larval density (m^{-2}) = 127.4 × Exp (0.287 + 0.0334*% wc)	<0.001
	August 2012	Larval density (m^{-2}) = 127.4 × Exp (1.0213 − 0.0289*% wc)	0.433
October 2013	April 2013	Larval density (m^{-2}) = 127.4 × Exp (−0.799 − 0.0438*% wc)	0.060
	September 2013	Larval density (m^{-2}) = 127.4 × Exp (−1.844 + 0.1160*% wc)	<0.001
October 2014	April 2014	Larval density (m^{-2}) = 127.4 × Exp (−0.168 − 0.2321*% wc)	<0.001
	September 2014	Larval density (m^{-2}) = 127.4 × Exp (−0.535 − 0.0541*% wc)	0.248
October 2015	March 2015	Larval density (m^{-2}) = 127.4 × Exp (−0.536 − 0.0886*% wc)	0.079
	October 2015	Larval density (m^{-2}) = 127.4 × Exp (−0.871 − 0.0945*% wc)	0.041

density with % white clover measured in autumn ($P = 0.030$; **Figure 7A**; **Table 2**) and spring ($P < 0.001$; **Figure 7B**; **Table 2**). The apparent contradiction with the year results may be due to the small range of % white clover values within years and/or the high leverage data from 2012 before the CRW population collapsed had on the analysis (**Figure 7**). If data from 2012 is omitted, there is no longer evidence of a positive relationship.

If the decline of CRW larvae from 2012 to 2013 followed the decline of % white clover between autumn of 2012 and 2013, it is not unreasonable to consider that the spring larval population had a positive relationship with white clover coverage in the previous autumn (i.e., higher the white clover coverage in autumn, the larger the larval population in spring). However, between 2013 and 2014, the larval population did not follow the further autumn % white clover decline and instead, slightly increased, although the increase was not statistically significant. In addition, the spring larval populations in 2013, 2014, and 2015 had rather negative relationships with the previous autumn clover coverage, with most of these negative relationships found to be statistically significant (**Table 1**). These results are contradictory to a positive correlation expected from the hypothesis.

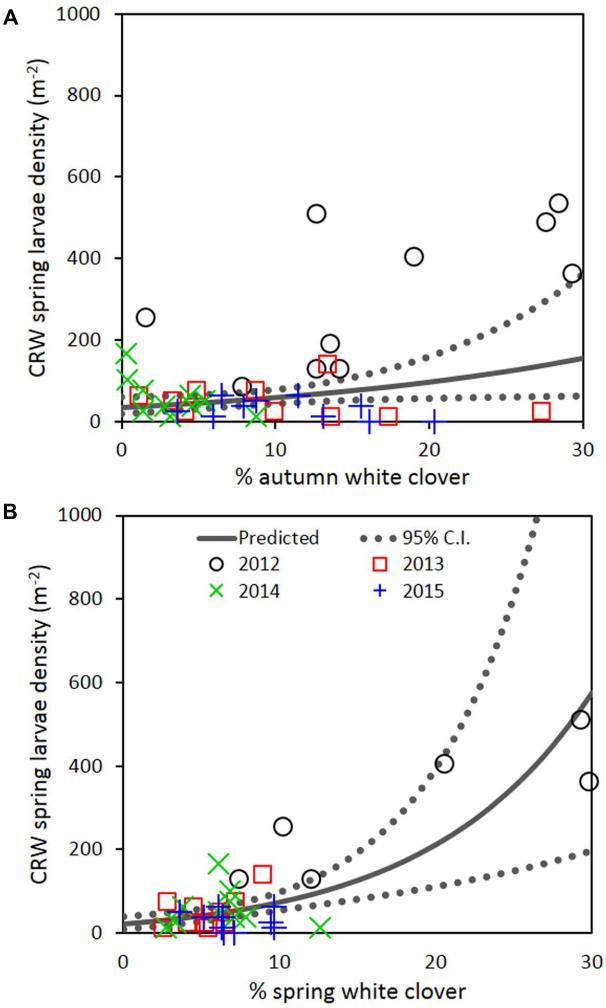


FIGURE 7 | Clover root weevil larval densities measured in spring in relation to % white clover measured in (A) autumn and (B) spring of 2012–2015. The solid line shows predicted density from the repeated measures analysis, with the dotted lines representing 95% confidence intervals of the predicted.

TABLE 2 | Repeated measures analysis of transformed spring CRW larval density against % white clover measured from 2012 to 2015.

Season	Slope estimate for % white clover	SE of slope	df	*P*-value
Autumn	0.0189	0.0083	36	0.030
Spring	0.0422	0.0100	37	<0.001

DISCUSSION

This study observed a rapid colonization of newly established white clover pastures by CRW, which went from no larvae in December 2011 but by May 2012 had increased to a mean of 240 larvae m^{-2}. In those first 18 months following establishment, colonization was greater where there was more clover in the sward and supported the hypothesis that clover content supports higher larval densities. Once the CRW population was established, the autumn clover content declined sharply which supports the idea that the clover decline was driven by the impact of CRW. Thereafter, there was no consistent relationship between spring larval population and % white clover in autumn, or % white clover in spring.

Across all years, relating the 2012 transformed spring larval data with % clover in spring, summer or autumn 2012 indicated that predicted populations for 2013 to 2015 were larger than observed (**Figure 8A**; autumn data only). Conversely, when 2015 spring larval data and % clover was regressed against 2012–2014 larval populations, observed densities tended to be higher

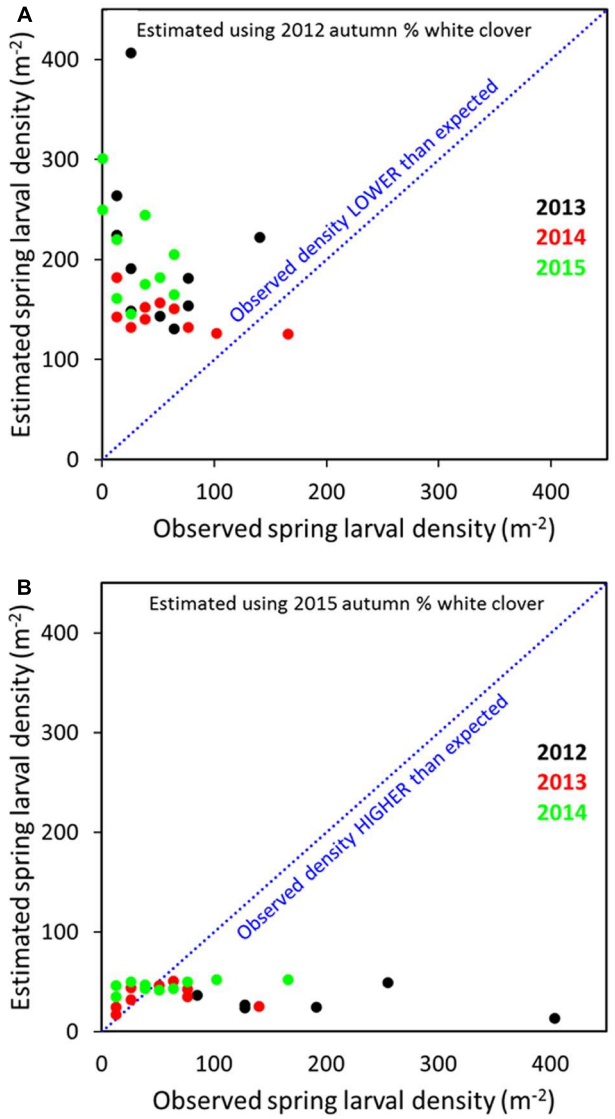


FIGURE 8 | Predicted and observed *S. obsoletus* larval densities for 2013–2015 populations based on 2012 $\log_{10}$ spring larvae data, with % autumn white clover as the explanatory variable (A) and 2012–2014 larval populations based on 2015 spring larval data with % autumn white clover as the explanatory variable (B).

than predicted, but the numbers came closer to predicted for the 2013 and 2014 populations (**Figure 8B**). The presence of the *M. aethiopoides* and its impact on CRW egg laying is considered an important factor in reducing larval populations. Based on dissection data, high levels of parasitism were observed on the site. This meant a significant decline in egg laying potential by parasitized female CRW, which consequently reduced larval recruitment. Root nodule availability is a significant driver to CRW first instar establishment and survival (Gerard, 2001) and feature common to other *Sitona* species (e.g., Quinn and Hower, 1986; Goldson et al., 1988; Lohaus and Vidal, 2010), but even allowing for density dependent larval survival in relation to root nodule availability (e.g., Goldson et al., 1988), parasitism of adult CRW by the *M. aethiopoides* would have contributed to the overall decline in larval populations. Variation in the application of artificial nitrogen over the course of the study, as a basis for larval population changes was considered but disregarded after correlation analysis found no relationship between artificial nitrogen and larval populations (Cave, unpublished data).

Finding precedents to explain the two contradictory relationships observed at the Lincoln site (positive and negative relationships between CRW larval populations and clover coverage) is difficult. Gerard et al. (2007) found a significant positive linear relationship between mean numbers of CRW larvae present and % clover cover. This supports the positive correlation and was observed in 2012 in this study. However, in Gerard et al. (2007), measurements of the two variables (larval number and % clover cover) were taken concurrently, whereas in the work reported here there was at least a 30-day interval between the botanical and larval measurements. Whether this difference is important is debatable.

Feeding by CRW early instar larvae has been shown to have a range of impacts on infested plants, including significant reductions in leaf and root DM, the number of nodules and total N of leaf and root tissue (Murray et al., 2002). Under laboratory conditions, feeding by first instar *S. hispidulus* on *Medicago sativa* L., led to overcompensatory growth of nodules, increased N-fixation and root growth (Quinn and Hall, 1992). The rate of nodulation was highest in plants with low initial nodule biomass and lowest in plants with relatively high initial nodule biomass, suggesting that the rate of compensatory nodulation may increase as feeding by nodule herbivores increases. However, at some point repeated denodulation will lead to significant damage to the root system and yield losses (e.g., Goldson et al., 1988). Quinn and Hower (1986) found that under field conditions first and second -instar larvae of *S. hispidulus* were correlated with small root nodules of *M sativa* and soil moisture, but not taproot biomass. For later instars, there was no correlation with nodules, with only fifth-instar larval numbers showing a correlation with taproot biomass (Quinn and Hower, 1986).

There is a paucity of studies that have described the relationship between white clover foliage and root biomass including nodule dynamics (J Crush, AgResearch, pers. comm.), particularly between above ground foliage and the root system of plants over winter in Canterbury, when white clover leaf biomass becomes reduced in size and in some cases ostensibly disappears from the sward. And the scale of this study did not allow for detailed assessment of the root biomass, particularly root nodules during sampling. For this reason, establishing the exact cause for the inverse density dependent relationship between % white clover and larval populations is problematic. Potentially, if pasture with high clover content supports a commensurate adult and hence larval population, larval competition for root resources and naturally occurring entomopathogenic diseases such as *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metchnikoff) Sorokin, acting on populations

during the winter-early spring period, may explain the observed relationships.

CONCLUSION

Establishment of a white clover- ryegrass pasture, led to rapid colonization by CRW and high larval populations which showed significant declines in the following years. Parasitism of CRW adults can be considered a contributing factor to this decline in larval populations from 2013 onward. Based on % representation in plots, white clover abundance was highest in the first autumn following establishment, but significantly less in the following years. No overall relationship between CRW larval populations and % white clover was found, except when the 2012 data was included. However, within each year there were significantly relationships, often negative particularly, in 2014 and 2015 which indicated high autumn % clover had a detrimental impact on larval densities in spring, i.e., fewer spring larvae in relation to increased % clover in the previous autumn. The reasons for this are uncertain, but may be related to density dependent larval mortality due to loss of root biomass over winter. Irrespective of cause, larval populations by 2015 were significantly less than recorded in 2012, and were independent of % white clover.

AUTHOR CONTRIBUTIONS

MM: Developed the research concept, led and carried out the insect sampling and processing, wrote the paper. CvK and VC: Analyzed the insect and plant data and wrote the paper. DC: Developed the research concept. HH: led and carried out the plant sampling and processing.

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Apparent Acquired Resistance by a Weevil to Its Parasitoid Is Influenced by Host Plant

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Field parasitism rates of the Argentine stem weevil *Listronotus bonariensis* (Kuschel; Coleoptera: Curculionidae) by *Microctonus hyperodae* Loan (Hymenoptera: Braconidae) are known to vary according to different host *Lolium* species that also differ in ploidy. To further investigate this, a laboratory study was conducted to examine parasitism rates on tetraploid Italian *Lolium multiflorum*, diploid *Lolium perenne* and diploid hybrid *L. perenne* × *L. multiflorum*; none of which were infected by *Epichloë* endophyte. At the same time, the opportunity was taken to compare the results of this study with observations made during extensive laboratory-based research and parasitoid-rearing in the 1990s using the same host plant species. This made it possible to determine whether there has been any change in weevil susceptibility to the parasitoid over a 20 year period when in the presence of the tetraploid Italian, diploid perennial and hybrid host grasses that were commonly in use in the 1990's. The incidence of parasitism in cages, in the presence of these three grasses mirrored what has recently been observed in the field. When caged, weevil parasitism rates in the presence of a tetraploid Italian ryegrass host were significantly higher (75%) than rates that occurred in the presence of either the diploid perennial (46%) or the diploid hybrid (52%) grass, which were not significantly different from each other. This is very different to laboratory parasitism rates in the 1990s when in the presence of both of the latter grasses high rates of parasitism (*c*. 75%) were recorded. These high rates are typical of those still found in weevils in the presence of both field and caged tetraploid Italian grasses. In contrast, the abrupt decline in weevil parasitism rates points to the possibility of evolved resistance by the weevil to the parasitoid in the diploid and hybrid grasses, but not so in the tetraploid. The orientation of plants in the laboratory cages had no significant effect on parasitism rates under any treatment conditions suggesting that plant architecture may not be contributing to the underlying mechanism resulting in different rates of parasitism. The evolutionary implications of what appears to be plant-mediated resistance of *L. bonariensis* to parasitism by *M. hyperodae* are discussed.

Keywords: biological control of insect, decline, host plant effect, *Lolium multiflorum*, *Lolium perenne*, natural enemy, parasitism rate, pasture

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INTRODUCTION

Over the last 25 years there has been increasing confidence that the impact of the Argentine stem weevil (*Listronotus bonariensis*) on *Lolium*-based pasture grasses has declined (Goldson et al., 2014a,b, 2015). This has largely been based on the use of selected strains of *Epichloë* endophytes that confer pest resistance in ryegrass (Johnson et al., 2013) combined with the significant impact of the braconid parasitoid biological control agent, *Microctonus hyperodae* (e.g., Barker and Addison, 2006).

Recently, however, there has also been growing field evidence that *M. hyperodae* may be losing its efficacy as a biological control agent of *L. bonariensis*. This declining control has been based on reports of a notable reappearance of *L. bonariensis* damage to pasture (e.g., Popay et al., 2011). In response, and as part of an investigation into the loss of efficacy, research has been focused on comparing current weevil parasitism levels with those in the 1990s (e.g., Goldson et al., 2014a,b). Such data can be very variable due to fluctuations in weevil population dynamics and parasitoid oviposition activity. However, during *L. bonariensis* overwintering diapause and coinciding parasitoid diapause, parasitism rates remain constant due to the hiatus in the insects' development (Goldson and Emberson, 1981; Goldson and McNeill, 1992). Such overwintering stability has therefore permitted meta-analyses of historical datasets, which have shown that parasitism rates have declined notably in *Lolium*-based pastures since the parasitoid's initial establishment and equilibration in the first 6 years of its release (e.g., Goldson et al., 2014a,b).

It is tempting to attribute this downward trend in parasitism to weevil resistance arising from continuous and high parasitoid selection pressure over the last *c.* 20 years as has been discussed recently by Goldson et al. (2015). The prospect of resistance is supported by the fact that the parasitoid undergoes parthenogenic (thelytokous) reproduction while the weevil reproduces sexually. This situation is what is sometimes described as an 'unequal evolutionary arms-race'. However, other mechanisms could also contribute to the decline, such as changed farming practice, climate change, and the use of novel endophytes. These possibilities have been variously investigated, thus far without identifying any clear causative reason for the parasitism decline (e.g., Goldson et al., 2015); thus the acquisition of rapidly evolved resistance remains a possibility.

Rapid evolution in insect biocontrol has been known to occur elsewhere. In a study of field crickets (*Teleogryllus oceanicus*) on the Hawaiian islands, Pascoal et al. (2014) showed that genetically based resistance in this species occurred twice and involved separate genetic changes on different islands within the archipelago. On both occasions the crickets stopped stridulating (after about 24 generations) because such activity attracted the parasitic fly (*Ormia ochracea*) and this species exerted negative selection pressure. *Listronotus bonariensis* may similarly have developed genetically based resistance as it has undergone *c.* 50 generations since the first releases of *M. hyperodae*.

It is possible that plant species used in pastures may play a part in the observed reduction in parasitism. Goldson et al. (2015) noted in the field that parasitism of *L. bonariensis* by *M. hyperodae* was significantly higher in tetraploid *L. multiflorum* (Italian) ryegrass paddocks than in diploid perennial (*L. perenne*) ryegrass paddocks. These perennial paddocks were exposed to the same *L. bonariensis* and *M. hyperodae* populations as the Italian paddocks. In order to see if such differences could also be detected in the laboratory, preliminary observations were made in 2014 and these suggested that weevils maintained on the Italian or on perennial plants had different parasitism rates (Goldson and Tomasetto, unpublished data).

Of the 23 different species of pasture plants now commercially available to farmers in New Zealand (Charlton and Belgrave, 1992; Charlton and Stewart, 1999), *Lolium perenne*, *L. multiflorum*, and the hybrid *L. perenne* × *L. multiflorum* (*L. boucheanum* syn. *Hybridum*) are the most common. A description of these three pasture plants can be found in Langer's (1973) textbook.

This contribution describes a systematic laboratory study using grasses similar to those studied in the field by Goldson et al. (2015) to determine whether similar plant-associated differences in parasitism rates occurred in the controlled and very different environment of cages. At the same time, this also permitted direct comparison with those data obtained during similar and extensive laboratory-based parasitoid research and rearing throughout the 1990s (e.g., Goldson et al., 1993; McNeill et al., 1999, 2002). Through such comparison it was possible to determine whether, in the intervening years, there has been a reduction in laboratory weevil susceptibility to *M. hyperodae* similar to that which has been found in the recently collated extensive field parasitism data (e.g., Goldson et al., 2014a,b).

MATERIALS AND METHODS

Grass Type and Parasitism Rate

The *Lolium* grasses used in this study were Italian tetraploid *L. multiflorum* (cv. Grasslands Tama), diploid *L. perenne* (cv. Grasslands Samson) and diploid hybrid *L. perenne* × *L. multiflorum* (cv. Grasslands Manawa). For clarity and brevity these grasses are referred to as 'Italian' grass, 'perennial' grass and 'hybrid' grass, respectively; all were endophyte free. Endophytes were excluded because there is now a wide range of differently acting novel endophyte strains in use in New Zealand pasture grasses. Also endophytes may have subtle effects on parasitoid behavior although this has not shown up in a recent field study (e.g., Goldson et al., 2015). Finally endophytes often do not perform very well in the tetraploid *L. multiflorum* varieties, so in general it seemed prudent to exclude the endophyte variable from the experiment. The grass types that were chosen represent the typical pasture types used in New Zealand farming since the release of the parasitoid.

All experimental work was conducted at ambient laboratory temperatures (23 ± 2°C) and 16:8 L:D photoperiod. Weevil adults were collected from mid-Canterbury ryegrass pastures using a modified leaf blowing machine (Goldson et al., 2000) between January 11, 2016 and January 22, 2016. They were then purged of egg and larval parasitoids by storing them for

a minimum of 40 days and a maximum of 55 days with the remaining unparasitised population used for the experiment. The *M. hyperodae* pupae that emerged from these weevils were reared to obtain adult parasitoids for this study. Overall as detailed below, the experiment comprised three main treatments (grass types) with two subtreatments (plant positioning). These were replicated four times. There we also four grass-free control cages making 28 cages in total.

The experiment was established on March 17, 2016 using 305 mm × 205 mm × 130 mm translucent plastic cages with gauze lids. The four grass main treatment replicates were the minimum required to deal with pseudoreplication (Johnson et al., 2016). All cages were stocked with 23 *L. bonariensis* and two *M. hyperodae*. Each cage contained one of the three grass species treatments in the form of two 150 mm long bouquets with their moistened roots and associated soil in tightly sealed small polythene bags at the base of the plants. This resulted in at least 40 tillers per box (Supplementary Figure S1). Each grass treatment comprised two subtreatments, in separate cages, whereby the bouquets were positioned either horizontally or vertically; thus the cages were, respectively, positioned either on one end, or lying flat (Supplementary Figure S1). This different positioning of the plants was specifically to gain an initial indication of whether departure from vertical plant architecture had an effect on weevil parasitism in any of the three grass types. Gerard (2000) noted that weevils tend to leave the upright foliage in the presence of the parasitoid and Phillips (2002) suggested that plant orientation in a cage may influence parasitoid efficacy. There was also a control treatment comprising four cages (two horizontal and two vertical) containing 23 *L. bonariensis*, two *M. hyperodae* and two water-soaked dental wicks to maintain humidity. All paired treatments were placed randomly in the laboratory (Supplementary Figure S1). Parasitoids were removed from the cages after 48 h. Thereafter the weevils were maintained in the same ambient conditions for another 3 days until March 22, 2016 when they were frozen at −20°C prior to being dissected to assess parasitism rates (i.e., number of parasitized weevils per total number of weevils dissected).

Comparative Rates of Parasitism between the 1990s and 2016

In this study, the opportunity was taken to use the same *Lolium* grass types as were used throughout the 1990s during general research into *M. hyperodae* including the parasitoid's mass-rearing for release (Goldson et al., 1993; McNeill et al., 1999, 2002). This allowed us to directly compare the results obtained from this experiment with both published and unpublished work conducted in the 1990s. Notably, while the exposure periods of the weevils in some of the comparator experiments were sometimes longer than 48 h this was of minor importance as Phillips et al. (1996) have shown that parasitoid ovipositional effort declines rapidly after the first 48 h.

Statistical Analysis

To test for statistical significance between parasitism rates in the treatments and control, non-parametric complete random permutation tests (n cycles = 10000) were run for a one-way analysis of variance (ANOVA) via the package "*lmPem*" (Wheeler, 2010) and subsequently we tested the statistical significance via *post hoc* Tukey's HSD pairwise permutation tests embedded in the package "*stats*" in R 3.2.1 (R Development Core Team, 2016). This approach implements the methods for permutation tests described by Kabacoff (2011).

RESULTS

Grass Types and Parasitism Rates Within Foliage Positioning Subtreatments

The rate of parasitism of *L. bonariensis* by *M. hyperodae* in the presence of the experimental grass types and in the controls are presented in Supplementary Table S1 and the effects of grass type in **Figure 1**. In addition, **Tables 1–3** show these results in the context of other studies yielding parasitism rates both in the 1990s and recently, in the presence of the Italian, diploid, and hybrid grasses, respectively. For purposes of comparison, all

TABLE 1 | Summary table presenting the results of this study and other published and unpublished laboratory work on *Microctonus hyperodae* parasitism rates (% shown in bold) in caged *Listronotus bonariensis* populations in the presence of tetraploid *Lolium multiflorum* (cv. Grasslands Tama) plants.

Grass type and subtreatments	N° weevils	N° parasitoids	Duration (h)	Parasitism (%)	Reference	Date of experiment
L. multiflorum						
Horizontal treatment	30	1	216	**72**	McNeill et al., 1999	1999
Horizontal treatment	12	1	72	**67**	Goldson et al., 2004	2004
Horizontal treatment	10	1	48	**76**	Goldson and Tomasetto, unpublished data	2014
Horizontal treatment	12	1	48	**73**	This study	2016
Vertical treatment	10	1	48	**81**	Goldson and Tomasetto, unpublished data	2014
Vertical treatment	10	1	48	**77**	This study	2016

Notably the 1990s data have been normalized as the number of weevils (N° weevils) per parasitoid (N° parasitoids). Also presented is the duration (hours) of the various laboratory experiments (Duration). The parasitism data used in this table comprised those from the both the horizontal and vertical grass subtreatments as the different orientations did not lead to significantly different results. Attack rates measured in the 1990s in cages with the tetraploid L. multiflorum plants did not differ significantly from more recent results.

TABLE 2 | Summary table presenting the results of this study and other published and unpublished laboratory work on *M. hyperodae* parasitism rates (% shown in bold) in caged *L. bonariensis* populations in the presence of diploid *Lolium perenne* (cv. Grasslands Samson) plants.

Grass type and subtreatments	N° weevils	N° parasitoids	Duration (h)	Parasitism (%)	Reference	Date of experiment
L. perenne						
Horizontal treatment	23	1	96	**68**	(Barker and Addison, 1996)	1992–3
Horizontal treatment	23	1	96	**80**	(Barker and Addison, 1996)	1992–3
Horizontal treatment	15	1	96	**73**	(Barker and Addison, 1997)	1994
Horizontal treatment	21	1	72	**94**	(Bultman et al., 2003)	2003
Horizontal treatment	10	1	48	**33**	Goldson and Tomasetto, unpublished data	2014
Horizontal treatment	12	1	48	**45**	This study	2016
Vertical treatment	10	1	48	**48**	Goldson and Tomasetto, unpublished data	2014
Vertical treatment	12	1	48	**45**	This study	2016

Notably the 1990s data have been normalized as the number of weevils (No. weevils) per parasitoid. Also presented is the duration of the various laboratory experiments (Duration). The parasitism data used in this table comprised those from the both the horizontal and vertical grass subtreatments as the different orientations did not lead to significantly different results. Attack rates measured in the 1990s in cages with the diploid L. perenne plants were significantly higher than those from recent studies indicating the probability of acquired resistance by the weevils.

TABLE 3 | Summary table presenting the results of this study and other published and unpublished laboratory work on *M. hyperodae* parasitism rates (% shown in bold) in caged *L. bonariensis* populations in the presence of diploid hybrid *L. perenne* × *L. multiflorum* (cv. Grasslands Manawa) plants.

Grass type and subtreatments	N° weevils	N° parasitoids	Duration (h)	Parasitism (%)	Reference	Date of experiment
L. perenne* × *L. multiflorum						
Horizontal treatment	23	1	72	**78**	(Barker and Addison, 1996)	1992–3
Horizontal treatment	7	1	48	**68**	(Barratt et al., 1996)	1994
Horizontal treatment	12	1	48	**45**	This study	2016
Vertical treatment	12	1	48	**58**	This study	2016

Notably the 1990s data have been normalized as the number of weevils (No. weevils) per parasitoid. Also presented is the duration of the various laboratory experiments (Duration). The parasitism data used in this table comprised those from the both the horizontal and vertical grass subtreatments as the different orientations did not lead to significantly different results. Attack rates measured in the 1990s in cages with the diploid L. perenne plants were significantly higher than those from recent studies indicating the probability of acquired resistance by the weevils.

data have been normalized to be expressed as the effect of one parasitoid per population of weevils.

Horizontal and Vertical Treatments Combined

The rate of parasitism in the presence of the Italian grass (75 ± 4%) was significantly higher than in either of the other grass treatments ($P < 0.001$). There was no significant difference in parasitism rates between cages containing perennial grass (46 ± 5%) and hybrid grass (52 ± 4%; $P = 0.8$). Parasitism rate in the control cages was 33 ± 7% and was significantly less than that found in cages containing grass ($P < 0.01$).

Horizontal Treatments

In the horizontal subtreatments, the rate of parasitism that occurred in the presence of Italian grass (73 ± 8%) was significantly higher ($P < 0.001$) than in either of the other horizontal subtreatments (**Figure 1**). There was no significant difference in parasitism rates between cages containing perennial grass (45 ± 5%) and hybrid grass (45 ± 5%, $P = 0.08$; **Figure 1**). Parasitism rate in the empty controls was 33 ± 18% which was not significantly different from the perennial and hybrid treatments but significantly less than in the Italian grass ($P < 0.05$; **Figure 1**).

Vertical Treatments

In the vertical subtreatments, the rate of parasitism that occurred in the presence of Italian grass (77 ± 3%) was significantly higher ($P < 0.001$) than in either of the other upright treatments (**Figure 1**). Again, there was no significant difference in parasitism rates in the cages containing the perennial grass (48 ± 8%) and the hybrid grass (58 ± 4%, $P = 0.08$; **Figure 1**). Parasitism in the empty control was 34 ± 1% which was not significantly different from the hybrid and perennial treatments ($P = 0.5$; **Figure 1**).

Horizontal versus Vertical Treatments

Horizontal versus vertical positioning of grass bouquets within the cages resulted in no significant differences in the rates of *L. bonariensis* parasitism by *M. hyperodae* across all of the grass types (Italian grass, $P = 0.8$; perennial grass $P = 0.1$, and hybrid grass = 0.8, respectively).

Comparative Parasitism Rates between the 1990s and 2016

In the 1990s experiments, only horizontal treatments were used, therefore only the data from the horizontal subtreatments in this study were used for direct comparisons.

Descriptive analysis of the horizontal data in **Tables 1–3** show that in the 1990s, the mean parasitism rate in rearing cages containing perennial grass was 74 ± 4% as opposed to 39 ± 5% in the current study. Similarly the parasitism rate associated with the hybrid grass was 73 ± 6% in the 1990s compared with 45 ± 5%

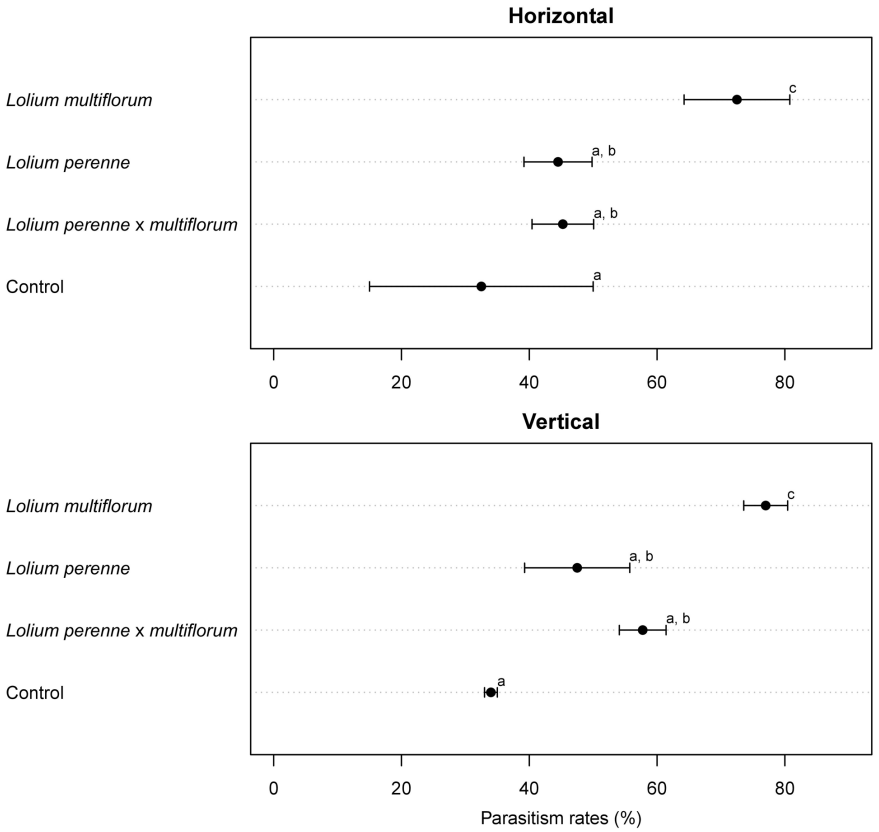


FIGURE 1 | Cleveland dotplot for *Microctonus hyperodae* mean parasitism rates (%) as measured in *Listronotus bonariensis* in cages containing Italian tetraploid *L. multiflorum* (cv. Grasslands Tama), diploid *L. perenne* (cv. Grasslands Samson) and diploid hybrid *L. perenne* × *L. multiflorum* (cv. Grasslands Manawa) and in cages containing no *Lolium* spp (Control). The horizontal and vertical orientations (i.e., subtreatments) are shown here. Error bars represent SEM. Means with different letters were significantly different in pairwise comparisons.

in this study. Conversely, the 73 ± 8% parasitism found in the presence of the Italian grass is very similar to that in the 1990s (70 ± 5%).

DISCUSSION

An emphasis of this study was to determine whether there have been significant changes in parasitism rates of Argentine stem weevil by *M. hyperodae* on typical pasture grasses since the 1990s rather than it being a definitive study of grass type effect on parasitism levels. Such direct comparison with historical data was possible because the same tetraploid Italian and hybrid cultivars were used in this study as throughout the 1990s. Thus any varietal genetic uncertainty is controlled for. The diploid *L. perenne* does not have complex genetic origins thus the direct comparison of cv. Samson in this study to cv. Nui in the 1990s is legitimate as both were derived from old New Zealand perennial pasture.

Grass Types and Parasitism Rates

The observed 42% decline in *M. hyperodae* parasitism observed in this cage study in the diploid and hybrid grasses (**Tables 2** and **3**), compared to 1990s laboratory data, conforms to the findings of recent field-based studies that have indicated a similar *c.* 50% decline in parasitism rates in diploid grasses since the 1990s (Goldson et al., 2015). That such a reduction in parasitism did not occur in the presence of tetraploid Italian grass either in this study (**Table 1**) or in the field (Goldson et al., 2015) suggesting that whatever factor(s) reduced parasitism rates in the perennial and hybrid grasses (**Tables 2** and **3**) did not occur in the presence of Italian grass (**Table 1**). It is also significant that the laboratory parasitism rates in the Italian grasses in this study were typical of those previously occurring in both the perennial and hybrid grass types in the 1990s (**Tables 2** and **3**).

Insignificant Plant Orientation Effects

Horizontal versus vertical positioning of grass bouquets within the cages resulted in no significant differences in the rates of parasitism of *L. bonariensis* across all of the grass treatments (**Table 1**; **Figure 1**). Such result indicates that, at least in the absence of soil or detritus, the orientation of plant material does not affect parasitism rates. This is contrary to Phillips (2002) suggestion that plant orientation in a cage may influence parasitoid efficacy. Further, the lower attack rates in the diploid and hybrid grasses were unlikely to have been based on the

avoidance of parasitism by the weevils abandoning the foliage in the presence of the parasitoid as discussed by Gerard (2000). In the horizontal treatment, the grass leaves were broadly spread across the floor of the cage obviating the ability of *L. bonariensis* to drop off. The results of this study also point to the probable incorrectness of the contention of Goldson et al. (2015) who suggested that the higher levels of parasitism in the tetraploid *L. multiflorum* could have resulted from a difference in the architecture of the tetraploid versus diploid and hybrid perennial plants. This architecture hypothesis would seem to have been possible when considering vertical plants. However, the horizontal plant placement was a gross departure from the natural growth habit, yet there were no differences in the levels of parasitism between plant positioning subtreatments and the upright plants. This suggests that plant architecture was unlikely to be the underlying cause of the observed differences in parasitism between the grass types.

Finally, all treatments in the cages comprised grass bouquets that were bundled at their stem bases where the roots entered the polythene bags thereby providing limited scope for the weevils to 'hide' from the parasitoids. This is clearly different from the growth habit of the plants in the field.

Ecological Implications

The lack of any notable difference in *L. bonariensis* parasitism rates in the cages containing diploid and endophyte-free hybrid grasses is significant ecologically. At the time of the first parasitoid releases, and in order to expedite its establishment by using areas with plentiful weevils, the work was conducted in either pure hybrid pastures or pastures comprising a mix of diploid and hybrid ryegrass (Goldson et al., 1998; Barker and Addison, 2006) as the hybrid is known to be preferred to the perennial as a host plant of the weevil (Goldson, 1982). As a consequence, some of the early parasitism field data were collected from these hybrid sites. This study has shown no differences in parasitism rates in the perennial and the hybrid grasses (Gaynor and Hunt, 1982; Goldson et al., 1998; Barker and Addison, 2006). This eliminates the prospect of any bias having occurred through possibly higher measured parasitism rates occurring in the limited and very early sampling in the hybrid grasses. This is consistent with the observation that high parasitism rates were typically found in the diploid pasture that surrounded the original release sites during investigation into the parasitoid's lateral dispersal from the release sites (e.g., Barker and Addison, 1997; McNeill and Goldson, unpublished data).

Mechanisms for the Measured Differences in Attack Rates in the Laboratory and the Field

The attack rates measured in this caging study were very similar to those currently observed in the field (Goldson et al., 2015). This is surprising given the obvious environmental differences between the field and laboratory cages (e.g., no soil or detritus).

It can be hypothesized that the underlying mechanism for the observed general decline in parasitism rates since the 1990s (e.g., Goldson et al., 2014a,b) could have been based on the adoption of novel endophytes. However, none of the grasses in this laboratory study were infected with endophytes. Additionally, Goldson et al. (2015) in a 5-month summer field study, showed no significant field effects of endophyte on *L. bonariensis* parasitism rates in the mix of *Lolium* varieties and endophytes.

Contrary to the findings here, the data collected in the 1990s indicated no differences in parasitism rates, irrespective of grass type. At that time weevil parasitism rates in the hybrid and perennial grasses were comparable to those now only found in the tetraploid Italian plants (**Tables 1–3**).

Barker (1989) observed much higher rates of *L. bonariensis* feeding and oviposition in the leaves of tetraploid Italian grasses than in the perennial grasses. Related to this Phillips (2002) showed that weevil feeding, walking, grooming defecating, or mating predisposes it to higher levels of parasitoid attack and this therefore could be the reason for higher parasitism rates on the Italian grass. Conversely, Barker (1989) also showed that hybrid ryegrass (cv. Grasslands Manawa) is equally favored as a host by *L. bonariensis* as the Italian grass. In spite of this, the results here showed significantly less parasitism in the hybrid grasses than in the Italian grasses. This observation suggests that the intensity of weevil feeding and oviposition *per se* may not entirely be the reason for varied parasitism rates. Significantly, the growth habit of the hybrid grass is much closer to that of the diploids and neither of these ryegrass types support the same levels of leaf-feeding and oviposition as found in the Italian plants (Barker, 1989). It is also of interest that parasitism rates in grass-free control cages, while usually lower than in the cages with the grasses present, still showed substantial parasitism indicating that *L. bonariensis* remains susceptible to parasitism when not feeding or ovipositing.

The decline in parasitism in the hybrid and diploid grasses since that 1990s has not coincided with any sign of physiological resistance in the weevils. In spite of 1000s of weevils having been dissected by numerous workers since the introduction of the parasitoid, there has never been any observation of *M. hyperodae* early stages being encapsulated in *L. bonariensis* (e.g., Goldson et al., 2015).

Adaptive Implications

In general, the results in this study support the contention of Goldson et al. (2015) that if selection pressure has led to an enhancement of some kind of parasitoid-avoiding behaviors amongst *L. bonariensis*, then such evolution would most likely to have occurred in the country's extensive diploid pastures rather than in the rare tetraploid Italian *L. multiflorum* pastures (B.R. Belgrave, Grasslanz Technology Ltd., pers. comm.).

CONCLUSION

It has been confirmed that different patterns of parasitism associated with different *Lolium* species and ploidy observed in the field also occurred in the laboratory experiments. At the same time, it has been demonstrated in the laboratory that diploid *L. perenne* and the diploid hybrid *L. perenne* × *L. multiflorum* no

longer support the levels of attack that were found in the 1990s. This is consistent with the contention that the weevil has evolved resistance to the parasitoid. The cause and mechanisms of this have yet to be determined; for example it is not known if there is a species or a ploidy effect, although field work has shown that parasitism levels in tetraploid *L. perenne* are no different from those in diploid *L. perenne* (Goldson et al., 2015). The possibility that resistance to a biological agent is dependent on plant type would seem to be unique in the literature.

There is now the prospect genetic and genomic analysis of both the weevils and parasitoid to explore further the underpinning of the observations in this contribution. By combining various approaches, the understanding of the reasons for success and failure in biological control must continue to develop (Mills and Kean, 2010).

AUTHOR CONTRIBUTIONS

SG and FT conceived and designed the experiment. FT performed the analysis. SG and FT wrote the article with significant intellectual input from both authors. FT and SG conducted the experimental work described in the article with SG overseeing collection of further data used in **Tables 1–3**. Both authors contributed to the discussion and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01259

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Is the Invasive Species *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae) (Argentine Stem Weevil) a Threat to New Zealand Natural Grassland Ecosystems?

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Listronotus bonariensis (Argentine stem weevil) is a stem-boring weevil that has become a major pasture pest in New Zealand, and cool climate turf grass in Australia. This species is also frequently found in native tussock grassland in New Zealand. Laboratory and field trials were established to determine the risk posed to both seedlings and established plants of three native grass species compared to what happens with a common host of this species, hybrid ryegrass (*L. perenne* X *L. multiflorum*). Adult weevil feeding damage scores were higher on *Poa colensoi* and *Festuca novae-zelandiae* than *Chionochloa rigida*. Oviposition was lower on *P. colensoi* than hybrid ryegrass, and no eggs were laid on *F. novae-zelandiae*. In field trials using the same four species established as spaced plants *L. bonariensis* laid more eggs per tiller in ryegrass in a low altitude pasture site than in ryegrass in a higher altitude site. No eggs were found on the three native grass species at the tussock sites, and only low numbers were found on other grasses at the low altitude pasture site. Despite this, numbers of adult weevils were extracted from the plants in the field trials. These may have comprised survivors of the original weevils added to the plants, together with new generation weevils that had emerged during the experiment. Irrespective, higher numbers were recovered from the tussock site plants than from those from the pasture site. It was concluded that *L. bonariensis* is likely to have little overall impact, but a greater impact on native grass seedling survival than on established plants.

Keywords: ***Listronotus bonariensis*** **(Kuschel), invasive species, native grasses,** ***Chionochloa rigida*****,** ***Festuca novae-zelandiae*****,** ***Poa colensoi*****,** ***Lolium perenne***

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INTRODUCTION

Environmental impacts of invasive alien species have been shown to be varied, including native species extinction, changes in species richness and abundance, and alterations to food web interactions etc. (Blackburn et al., 2014). However, in some cases exotic species have minimal demonstrated impacts in new environments, or have indirect impacts that may not be immediately apparent (Brockerhoff et al., 2010). In general, but with some exceptions, exotic invertebrates have shown low impact on plants in New Zealand's natural ecosystems (Brockerhoff et al., 2010) possibly

as a result of the high level of endemism of New Zealand native plants and their phylogenetic distance from host plants of many invasive plant pests.

Native grasslands in New Zealand provide a number of ecosystem services depending on their degree of modification (Mark et al., 2013). These include pollination, biological control, water, and soil conservation. They also provide the basis for education, ecotourism, and recreational services. For example, it has been demonstrated that water collection and retention by tall tussock species from fog can yield more fresh water than any other land use measured (Mark and Dickinson, 2008). Disturbance to natural grasslands such as burning, grazing, intensification of land use and weed invasion threaten their ability to provide ecosystem services, but little is known about the threat from invasive exotic invertebrates.

Several exotic species of Curculionidae have been recorded in New Zealand native grasslands (Barratt et al., 1998; Brockerhoff et al., 2010; Mark et al., 2013), but few have been recorded to be feeding or breeding on New Zealand native plants. Their presence in native grasslands may often be simply a case of vagrance, for example the lucerne weevil, *Sitona discoideus* Gyllenhal is a strong flyer with an annual dispersal phase (Goldson et al., 1984). This species has been collected at 1300 m altitude in the Waikaia Ecological Region (Dickinson et al., 1998), and at 2800 m altitude on the Inland Kaikoura Ranges (Phillips, unpublished). However, its hosts are restricted to species of *Medicago* spp. and *Trifolium* spp. (Vink and Phillips, 2007) and it is unlikely to have host plants in New Zealand's native flora. In contrast, a flightless, polyphagous, European weevil, *Otiorhynchus ovatus* L. which occurs in tussock grasslands in Central Otago (Barratt et al., 2009) might feed on some New Zealand native plants (Brockerhoff and Bain, 2000), but as yet there are no published records of this. Furthermore, this species, and three other *Otiorhynchus* spp. that are established in New Zealand, were not recorded on native plants sampled by Kuschel (1990).

The invasive weevil species that is perhaps most likely to have host plants amongst New Zealand's native grassland flora is the "Argentine stem weevil" (*Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae) (Barratt et al., 2007). This species was first reported in New Zealand in the late 1927 (Marshall, 1937) but its introduction is likely to have been earlier in the twentieth century (Kuschel, 1972) and there may have been more than one introduction. The weevil has become so abundant throughout New Zealand that it has been long recognized as an agricultural pest (Kelsey, 1958). Further, it is a dispersive flier (Goldson et al., 1999), and is the most frequently found exotic species in tussock grassland (Barratt et al., 2007). It has been collected in Otago from remnant native shrubland (Derraik et al., 2001), tussock grasslands (Murray et al., 2003), and up to 1640 m on Coronet Peak, Otago. In New Zealand's predominantly ryegrass pastures the species can reach adult densities of 700 per m^2) (Barker and Addison, 1993) which is vastly higher than in the "vega" or "mallines" type valleys which Lloyd (1966) has suggested is its center-of-origin. These alpine ecosystems are high-fertility, moist valley areas south of 39°S in the Andes (Squeo et al., 2006; Stewart, 2014) and are the habitat equivalent of New Zealand alpine grasslands, herbfields and cushion bogs (Wardle et al., 2001). Typical native species of the "mallines" alongside the *Juncaceae* and *Cyperacae* are grasses such as *Festuca pallescens, Poa lanuginose*, and *Hordeum comosom* (Gaitán et al., 2010). These malline grasses are in the same genera as some of New Zealand's introduced Gramineae including common cereals and pasture grasses (Morrison, 1938; Jacques, 1940; Doull, 1954; Kain and Barker, 1966). *L. bonarensis* can frequently be found in association with these plants. Whether there are any *Lolium* spp. native to South America seems uncertain, but it has been suggested that *Lolium rigidum lepturoides* could be. Although closely related to cosmopolitan *Festuca* spp., *Lolium* spp. was not introduced into New Zealand until the arrival of the early European pastoralists, probably in the early 1900s (Hunt and Easton, 1989). *L. bonariensis* is also a major pest of cool climate turf grasses in Australia (Hardy et al., 1979). On the balance though, it seems that *L. bonariensis* has been highly adventive and has acquired host plants that it did not evolve with, at least at the species level.

Having become established throughout New Zealand, by the early 1990s *L. bonariensis* was estimated to be causing damage to the pastoral sector amounting to NZ$78–251M annually (Prestidge et al., 1991). These estimated losses have been considerably offset by the successful introduction of the parasitoid, *Microctonus hyperodae* Loan (Hymenoptera: Braconidae) in 1992 (Goldson et al., 1993) combined with the widespread adoption of endophytic ryegrass cultivars which provide resistance to *L. bonariensis* (Popay and Wyatt, 1995). Recent research, however, has indicated that *M. hyperodae* is becoming less effective as a biological control agent for reasons that are not entirely understood (Popay et al., 2011; Goldson et al., 2014).

While the impact of *L. bonariensis* in intensive pastoral ecosystems is well understood, little is known of the potential risk posed by this species to New Zealand's natural grassland ecosystems, despite the species being so commonly encountered in this environment (Barratt et al., 2009; Brockerhoff et al., 2010). In this study, our objective was to determine the impact of *L. bonariensis* on tussock grasslands in Otago, New Zealand by focusing specifically on the effect of adult and larval feeding on seedling tussock plants both in the laboratory and amongst established plants in the field. Three endemic grass species, which are amongst the most common and widespread members of tussock grassland plant communities New Zealand Plant Conservation Network (2016), were selected for the study on the basis that any substantial reduction in vigor or distribution of these species would change the plant communities in which such changes occurred. Hybrid ryegrass, (*L. perenne* X *L. multiflorum*), known to support high densities of *L. bonariensis*, was included in the study for purposes of comparison with the tussock plants. In order to determine whether the impact of *L. bonariensis* was dependent upon environmental conditions, a field trials using the same plant species were carried out in both an elevated altitude tussock grassland environment, and in a lowland pastoral ecosystem.

MATERIALS AND METHODS

The tussock grass species used in this weevil impact study were, snow tussock (*Chionochloa rigida*), fescue tussock (*Festuca*

novae-zelandiae), and blue tussock (*Poa colensoi*). The attack rates and damage on these plants were compared to the impacts on the introduced pasture species and known host, the hybrid ryegrass cv. "Grasslands Manawa" (*L. perenne* X *L. multiflorum*). This cultivar was selected because it is endophyte-free and is known to be susceptible to *L. bonariensis* damage (Kelsey, 1958; Goldson, 1982; Gaynor and Hunt, 1983; Barker, 1989). Henceforth, for simplicity, this will be referred to as hybrid ryegrass. For laboratory seedling survival studies and weevil feeding/oviposition experiments, the plant species were greenhouse grown from local seed. For the field experiments, mature plants obtained from a commercial native plant nursery were used.

L. bonariensis adults were collected from lowland pasture near Mosgiel, New Zealand using a suction sampler (modified BlowerVac®). These were maintained in groups of 30–50 in laboratory cages (160 × 180 × 75 mm deep) with a fine gauze lid. They were provided with hybrid ryegrass grown in commercial potting mix in cell trays, each cell containing 6–8 plants. When the plants were 50–100 mm high, they were transferred with roots and soil intact into a small plastic bag sealed abound the base of the plants with a cable clip. Two such plant "packets" were placed in each cage. Water was supplied in the form of 4 soaked dental wicks placed in the cage. Weevils were kept for up to a week until required for experiments.

LABORATORY EXPERIMENTS

Laboratory Experiment (Seedling Survival)

Seed of *C. rigida*, *P. colensoi*, *F. novae-zelandiae* and hybrid ryegrass were sown in cell trays containing potting mix. After germination individual seedlings were transferred to 50 ml specimen containers (38 mm diameter × 65 mm high) filled with potting mix (**Figure 1**). When seedlings were 30–40 mm high three *L. bonariensis* adults were added to each container, which was then covered with another inverted 50 ml pot held in pace with tape. The lower container was provided with drainage holes and the upper container was closed at the top with fine mesh to prevent the escape of the weevils and reduce condensation. Thirty replicates of each grass species (15 for *C. rigida*) were established and they were exposed to the weevils for 18–20 days. An equivalent number of replicate plants of each species were also established as controls without weevils. Containers were spatially randomized in a screen house exposed to ambient outdoor temperatures. At the end of the exposure period plant survival was assessed and a feeding damage score was recorded on a scale of 1–5, where 1 was undamaged or very superficial feeding damage, and 5 was heavily damaged or almost completely consumed by weevil feeding. Plants were considered not to have survived if damage caused the plants to become desiccated and collapsed, or if feedings was so severe that no foliage remained.

It was not possible to conduct all experiments simultaneously because of different plant germination and growth rates. Thus experiments were carried out in both the summers of 2011–2012 and 2012–2013 in a screen house. Hybrid ryegrass was included in experiments carried out in both years. Control plants were left unexposed to weevils.

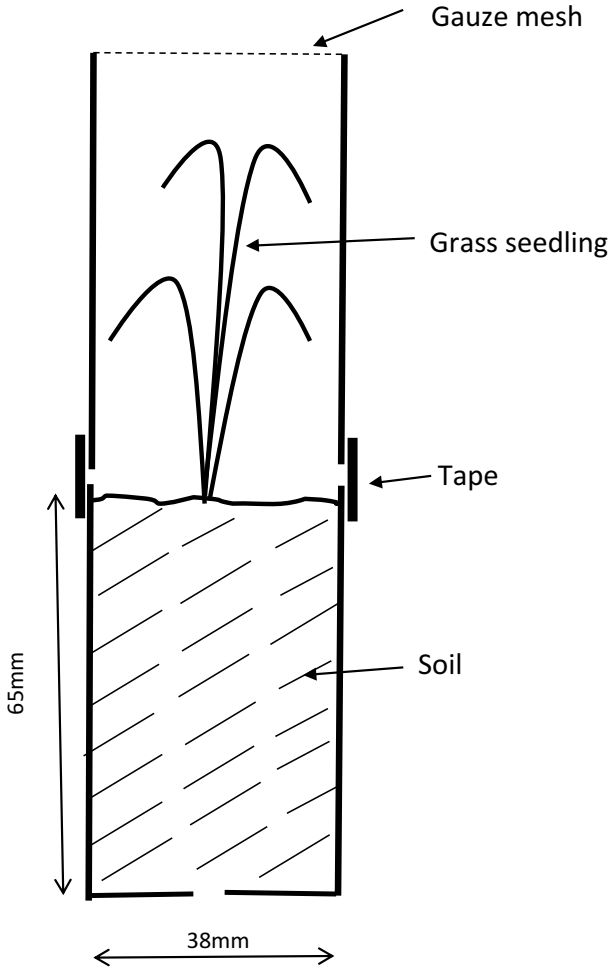


FIGURE 1 | Diagram to show container used for seedling damage experiment.

Laboratory Experiment (Weevil Oviposition)

Seeds of the four grass species were grown as above until they were approximately 120 mm high with 2–3 tillers, depending upon the species. Plants were potted up individually into 38 mm diameter × 65 mm high containers filled with potting mix as above. Petri dishes were prepared with 85 mm diameter holes cut in their centers across which, fine nylon gauze was glued. Each pot's seedling leaves were then poked through the mesh and the Petri dishes gently lowered to the base of the plants such that the dish rested on top of the pots at soil level. This arrangement thus prevented weevils from moving into the soil while allowing the tillers to continue to grow through the mesh and allowing access for the weevils to the tiller bases. A transparent plastic cylinder (65 mm diameter; 140 mm high) with a fine mesh lid was placed over the plants and attached to the Petri dish base with Blutac®. Three *L. bonariensis* adults then were introduced to each container. All were supplied with a water-soaked dental wick, and left for 18–20 days in a screen house as above. At the end of this period the weevils were removed and dissected in order to determine gender. Replicates with no females were

excluded from the analysis, leaving between 28 and 33 replicates of hybrid ryegrass, *P. colensoi* and *F. novae-zelandiae*, and 15 for *C. rigida*. Plant tillers were counted, the basal tiller diameters measured and then all tillers were examined under a binocular microscope for weevil eggs and larvae. As for the seedling survival study, this experiment was run over two seasons.

FIELD TRIALS

Two field study sites were used. The first was in a tussock grassland ecosystem on the East Otago Plateau between the Rock and Pillar Range and the Lammermoor Range along the Old Dunstan Road (−45.490, 169.964) and the second was in pasture on the Taieri Plain near Mosgiel (−45.862, 170.378). These are referred to as the "tussock grassland site," and the "lowland pasture site" respectively.

Both sites were initially treated with glyphosate to prepare them for planting. At the tussock grassland site the foliage of any surviving *C. rigida* plants was trimmed to 50 mm. At both locations spaced mature plants of *C. rigida*, *P. colensoi*, *F. novae-zelandiae* and hybrid ryegrass were planted in rows 1m apart with 700 mm between the plants. These were arranged in four blocks each comprising 30 replicates of plant species randomly arranged. The site characteristics and treatment details are given in **Table 1**. The test plants were transplanted in the autumn of 2012 and were left to become established until the following early summer, when 10 *L. bonariensis* adults were introduced to each plant. At this time the plants were enclosed in gauze bags pushed into the soil with a metal cylinder.

Five weeks after weevil introduction in the lowland pasture site and after 7 weeks in the tussock grassland site (**Table 1**), 5–10 tillers were selected from different parts of each test plant and cut near the tiller base. A longer period was allowed for oviposition and larval emergence at the cooler higher altitude tussock grassland site. The excised tillers were taken to the laboratory for eggs and larval counts. Based on a technique developed by Goldson (1978) larval counts were carried out by placing the tillers on a wire mesh over a 5l container containing glycerol into which the larvae dropped while escaping from the desiccating tillers. The glycerol was thereafter filtered through a fine mesh and the larvae counted. After 2 weeks when no further larvae were extracted, the tillers were stored in a deep freeze pending microscopic examination for eggs and any remaining larvae.

In late March 2013 (lowland pasture site) and early May 2013 (tussock grassland site) the entire plants were excavated to a depth of approximately 100 mm. In the laboratory, the foliage from the plants was cut from the base of each plant and both the foliar and basal parts of the plants (which included the root material) were placed in modified Tullgren funnels (Crook et al., 2004) for 7 days to extract adult and larval *L. bonariensis*. The funnels used heat from 4 × 150 W light bulbs positioned approximately 300 mm above each sample to extract all invertebrates, which were collected below the funnels into monopropylene glycol. After extraction, the collected material was washed in water through a fine sieve to retain the invertebrates which were stored in 70% ethanol, before examination under a binocular microscope to identify and count adult and larval stages of *L. bonariensis*. Adults were examined to determine whether they were the original weevils used in the experiment, or part of a new generation that had emerged during the experiment. This was assessed based on the degree of wear of scales on the elytra, the condition of hairs on the elytral surface and the presence of any teneral individuals.

TABLE 1 | Field trial site description and sample dates.

Site	Taieri Plain (lowland pasture site)	East Otago Plateau (tussock grassland site)
Altitude above sea level	20 m	900 m
Mean annual rainfall	684 mm	630 mm
Soil type	Wingatui silt loam	Teviot silt loam
Vegetation	pasture	*Chionochloa rigida* grassland
Site prepared	Jan 2012	Dec 2011
Tussocks planted	3 Apr 2012	8 Mar 2012
L. bonariensis adults added	7 Dec 2013	12 Dec 2013
Tiller samples taken for eggs and larvae	15 Jan 2013	1 Feb 2013
Plants excavated	27 Mar 2013	2 May 2013

DATA ANALYSIS

Laboratory and field data were analyzed using Genstat 16.2 (VSN International, 2013). Seedling survival data were analyzed using a binomial GLM with a logit link function to compare the effects of plant species. ANOVAs were carried out on the laboratory seedling damage scores, and field adult and larval density data to ascertain the significance of site and plant species treatments effects. For oviposition data a simple linear comparison of means was carried out and standard errors calculated. Comparisons for the laboratory data were made within but not between years. For the field data, the proportion of the adults extracted from the tussock plants at the end of the experiment were analyzed using a binomial GLM with a logit link function to compare the effects of plant species.

RESULTS

Laboratory Experiments—Seedling Survival and Weevil Oviposition

The results of the laboratory seedling experiments are shown in **Table 2**. All control seedlings (not exposed to *L. bonariensis* adults) survived, and scored 1 (undamaged) for feeding damage so these data have not been included in **Table 2**.

In the experiments carried out in 2011 hybrid ryegrass seedling survival after 18–20 days exposure to adult *L. bonariensis* was significantly higher than for *P. colensoi* ($P < 0.001$), but in 2012 survival of hybrid ryegrass and *F. novae-zelandiae* survival were not significant (**Table 2**). The plant damage scores were accordingly higher for *P. colensoi* than hybrid ryegrass ($P < 0.001$) in 2011, but not different in the 2012 comparison

TABLE 2 | Survival and damage scores of seedling grasses exposed to *L. bonariensis* adults in the laboratory.

Plant seedling survival					
Plant species	**Date exposed**	**Date assessed**	**No plants**	**Mean plant damage score (1–5)**	**Mean proportion of plants surviving (SE)**
Hybrid ryegrass	23 Dec 11	10 Jan 12	30	2.27 (0.31)	0.97 (0.03)
P. colensoi	23 Dec 11	10 Jan 12	30	4.3 (0.20)	0.43 (0.09)
Hybrid ryegrass	13 Dec 12	2 Jan 13	30	4.3 (0.24)	0.47 (0.09)
F. novae-zelandiae	13 Dec 12	2 Jan 13	30	4.3 (0.09)	0.43 (0.09)
C. rigida	13 Feb 12	2 Mar 12	15	2.53 (0.44)	0.8 (0.1)

Weevil Oviposition						
Plant species	**Date exposed**	**Date assessed**	**No plants**	**Mean no. eggs plus larvae per female (SE)**	**Mean no. tillers per plant (SE)**	**Mean diam of tillers (SE)**
Hybrid ryegrass	23 Dec 11	11 Jan 12	28	9.89 (2.19)	3.1 (0.26)	1.36 (0.08)
P. colensoi	23 Dec 11	11 Jan 12	33	0.53 (0.19)	1.27 (0.09)	0.76 (0.05)
Hybrid ryegrass	13 Dec 12	2 Jan 13	30	2.47 (0.87)	2.37 (0.22)	1.6 (0.09)
F. novae-zelandiae	13 Dec 12	2 Jan 13	30	0	1.67 (0.15)	0.8 (0.04)
C. rigida	17 Feb 12	2 Mar 12	15	0.13 (0.13)	1.6 (0.25)	1.39 (0.11)

Weevil oviposition on seedling grasses is also shown in relation to mean tiller numbers and diameter. The means for seedling damage and survival are those predicted by the regression model; standard errors are shown in brackets.

between hybrid ryegrass and *F. novae-zelandiae* (**Table 2**). *C. rigida* exposed to *L. bonariensis* in February 2012 sustained a relatively low damage score and consequently survival was about 80%.

Oviposition was higher on hybrid ryegrass than on *P. colensoi* ($P < 0.001$) and *F. novae-zelandiae* ($P < 0.01$) in the 2011 and 2012 comparisons respectively. Small numbers of eggs were laid on *P. colensoi* but none were laid on *F. novae-zelandiae* (**Table 2**). Oviposition on *C. rigida* was not compared directly with hybrid ryegrass, but the number of eggs laid on this species was also very low. The hybrid ryegrass plants had more tillers than both *P. colensoi* and *F. novae-zelandiae* ($P < 0.001$) in both 2011 and 2012 experiments, and the mean tiller diameter of hybrid ryegrass was about twice that of both *P. colensoi* and *F. novae-zelandiae* ($P < 0.001$) when measured in 2011 and 2012 comparisons. *C. rigida* had a similar number of tillers to *F. novae-zelandiae*, but tiller width was more similar to hybrid ryegrass.

FIELD TRIALS

At both the lowland pasture site and the tussock grassland site, no eggs at all were found in tillers of the three native grass species. However, in hybrid ryegrass, eggs were found in tillers at both sites, with significantly more at the lowland pasture site ($P < 0.001$) (**Table 3**). Larvae were found in tillers of all species at the lowland pasture site, but numbers were very low in the native grass species. No larvae were found in tillers of the native grass species at the tussock grassland site. In hybrid ryegrass significantly more larvae were found at the lowland pasture site than the tussock grassland site ($P < 0.001$) (**Table 3**). An average of 2 immature stages (eggs plus larvae) per tiller was found in the hybrid ryegrass at the lowland pasture site whereas in the native grass species 0.01–0.03 immature stages per tiller were found in the native grass species (**Table 3**).

When the plants were excavated and heat-extracted in the laboratory to recover adult weevils, some larvae were also collected. These comprised approximately 10 and 5% of the total numbers at the lowland pasture and tussock grassland sites respectively. The larval numbers were combined with adult counts for the analysis. There was a significant site effect with more adults and larvae recovered from the tussock grassland site ($P < 0.001$), and a significant plant species effect with more weevils recovered from hybrid ryegrass than the native grass species at both sites ($P < 0.001$). There was no significant site $\times$ plant species interaction ($P = 0.461$).

The assessment of adult weevils extracted to determine whether they were the original weevils added to the plants in the field, or new generation weevils that had developed through their life cycle during the experiment, showed that development was faster at the lowland pasture site as would be expected. Over 60% of the weevils collected from ryegrass here were new generation compared with 14% collected from ryegrass at the tussock grassland site (**Table 3**). This was true also for the other grass species except for *P. colensoi* where only 7–8% of weevils were new generation at both sites. Interestingly, despite no immature stages being found in native species at the tussock site, a very small number of apparently new generation weevils were extracted from these plants.

DISCUSSION

This study was designed to determine whether the invasive pest species, *L. bonariensis*is is likely to be having an ongoing impact on native grasses in natural grassland ecosystems, where it is commonly found. The data suggest that under current conditions, it is unlikely that this invasive weevil is posing a threat to the tussock species investigated, especially to mature plants.

TABLE 3 | Mean numbers of eggs and larvae in tiller samples, and mean number of immatures per tiller taken from the field trials at the lowland pasture and tussock grassland sites.

	Plant species							
	Lowland pasture site				Tussock grassland site			
	Chionochloa rigida	*Festuva novae-zelandica*	*Poa colensoi*	*Lolium perenne*	*Chionochloa rigida*	*Festuva novae-zelandica*	*Poa colensoi*	*Lolium perenne*
Mean no. eggs in tillers	0	0	0	14.6 (2.4)	0	0	0	5.43 (0.96)
Mean no. larvae in tillers	0.1 (0.06)	0.3 (0.12)	0.13 (0.08)	5.47 (0.81)	0	0	0	0.69 (0.21)
Mean no. immatures per tiller	0.01 (0.01)	0.03 (0.01)	0.013 (0.01)	2 (0.28)	0	0	0	0.61 (0.11)
Mean no. adult weevils per plant	1.2 (0.27)	1.07 (0.34)	0.83 (0.22)	3.03 (0.46)	2.7 (0.42)	2.53 (0.31)	2.87 (0.34)	5.57 (0.58)
Proportion of new generation adults	0.30	0.15	0.08	0.64	0.11	0.06	0.07	0.14

The mean number of adult weevils, and proportion of them which were "new" generation, extracted from plants at the end of the trials is also shown. Standard errors are given in brackets.

Laboratory experiments which examined the survival of seedling stage native plants exposed to weevils indeed found that that two of the native grass species tested, *P. colensoi* and *F. novae-zelandiae,* were equally or more susceptible to feeding damage and mortality as hybrid ryegrass seedlings. Conversely snow tussock seedlings (*C. rigida*), were significantly less susceptible (**Table 2**). The difference between the hybrid ryegrass results for the two seasons is unclear, but the seedlings used in the second season might have been slightly smaller than those used in the first season. This is indicated by the smaller number of tillers present in the second season.

It was noted by Goldson (1982) that some grass species showing resistance to insect feeding, could have been the result of elevated alkaloid levels. This was later found to be associated with endophyte, which is now well known as conferring insect resistance to some ryegrasses (but absent from "Grasslands Manawa" used in this study) and some other pasture grass species (Rowan and Gaynor, 1986). Very little is known about endophytes in native grass species but a survey of 24 species (including several samples of *P. colensoi*, one of *F. novae-zelandiae* but not *Chionochloa*) revealed no endophytic associations (Rolston et al., 2002).

Listronotus bonariensis eggs were found to be absent in the native grasses at both sites, but presence of larvae in the tillers in the native grass species at the lowland pasture site (**Table 3**) indicated that any eggs laid had already hatched by the time of sampling. Furthermore, since the weevils that were recovered from the tussocks at both sites, comprised at least some new generation adults this indicated that some full development had occurred on the native plants. In contrast, at the time of sampling, eggs were still being found in hybrid ryegrass plants at both sites. This could indicate that a longer period of oviposition was associated with the more suitable host plant or that the scarcity of eggs being laid in the native plants was below the detection threshold. *L. bonariensis* prepares to oviposit by chewing a hole in the leaf sheath of a tiller and then via the hole, insert eggs between the upper and lower epidermis of the leaf sheath. When the larvae hatch they typically bore into the tiller and move downwards toward the base (Barker and Addison, 1990).This study has shown that *L. bonariensis* oviposition was significantly lower on native grasses compared with hybrid ryegrass. In *F. novae-zelandiae* there was no oviposition recorded in the laboratory or in the field, although in a preliminary laboratory study Lister (2006) found some oviposition on *F. novae-zelandiae*, but none on *C. rigida*. Goldson and Penman (1979) noted that females require a tiller diameter of about 1 mm for oviposition. Mean tiller diameter of the *P. colensoi* and *F. novae-zelandiae* were both found to be <1 mm in this study, which could have contributed to the comparatively low (or lack of) oviposition in these species.

Goldson (1982) further hypothesized that, as well as a minimum tiller diameter, ovipositional preference and the typically low and highly variable levels of first to second instar survival (6–23%)were inversely proportional to plant cellulose levels. In this study cellulose levels were not measured in the native grasses, but Bailey and Connor (1972) measured cellulose levels of 30–32% of dry weight in *Chionochloa rigida*. In hybrid ryegrass, depending upon the cultivar, cellulose levels were recorded in the region of 10–15% of dry weight (Bailey, 1965), although Lancashire and Ulyatt (1975) measured levels between 18 and 27% depending upon cultivar and season. Native grasses also often accumulate dead tissue at the base of tillers, which might also impede weevil access to the tillers for oviposition. Conversely, tussock burning which is practiced to remove litter, and promote new palatable regrowth in tussock grasses for grazing animals, could possibly render the plants more palatable to *L. bonariensis*, and thereby facilitate oviposition. It needs to be recognized also that the weevils collected for the field experiment were sourced of necessity from lowland pasture, and hence feeding/oviposition preferences as well as general fitness for a native grassland environment might have been influenced by such conditioning. In future work it would be of interest to determine whether tussock grassland-sourced populations of *L. bonariensis* might have become adapted to native grass species and the environment in general.

Given the uncertainty around the taxonomic affiliations of the genera *Lolium* and *Festuca* spp., it is likely that the only new association with the native grass species in this study was *Chionochloa*; however, this was not found to be a highly susceptible to *L. bonariensis*, probably as a result of the tough stem epidermis.

The density of *L. bonariensis* used in this study's field sites was deliberately higher that normally encountered in native tussock grassland at 900 m so that a "worse-case scenario" could be investigated. Previous work has shown that *L. bonariensis* can reach densities of up to 40/m^2 in lower altitude tussock grassland at 360 to 640 m, with a maximum density of 13/m^2 at 450 to 850 m (Barratt et al., 2007). An intensively studied tussock grassland site at 1100 m yielded a January maximum of 5.5/m^2 (Barratt et al., 2009). Climatic conditions are likely to be a factor in the fitness and performance of *L. bonariensis* in the tussock grassland environment, and almost certainly its phenology. Barker (1988) found that there was no egg or larval development at <10°C in the laboratory. Temperature was not measured during this study but to provide some context, at a site at 460 m on the Lower Otago Plateau, 35 km from the tussock grassland site, the 30-year mean monthly temperature was 12–13°C in December and January with mean monthly mimina of 7–8°C respectively. This indicates development of *L. bonariensis* immature stages at the tussock grassland site would be more protracted, and might explain the higher number of adult weevils remaining at the tussock grassland site at the end of the field study and smaller proportion of teneral and new generation adults.

CONCLUSIONS

This study has indicated that *L. bonariensis* is unlikely to be a significant threat in tussock grassland, at least to the very common species included in this investigation. If development times at this elevation are indeed a factor, then this might also apply to other native grass species occurring in this environment. For rare or endangered native grass species, the potential risk from this invasive weevil might be greater. Should the density of *L. bonariensis* increase, adult feeding could play a role in reducing seedling establishment for some native species. The impact of moderate animal grazing pressure on *C. rigida* seedling survival (Lee et al., 1993) is known to be significant (Lee et al., 1993). The relatively minor mortality rates found in *C. rigida* seedlings exposed to *L. bonariensis* for 2–3 weeks is trivial in comparison. It is, therefore, unlikely that *L. bonariensis* would add substantially to the overall impact of grazing animals. However, the other grass species tested could possibly be at more risk than *C. rigida* based on indications of greater feeding damage and reduced plant survival. Irrespective though the climatic conditions in higher altitude tussock grasslands, combined with the relative unsuitability of native grass species to *L. bonariensis* all point to little overall impact.

AUTHOR CONTRIBUTIONS

BB led the project design, data collection and analysis, writing up and manuscript submission. DB assisted with planning the project, carried out a large proportion of the field and laboratory work and assembled data for analysis. BP carried out a large proportion of the field and laboratory work and assisted with assembly of data for analysis. CF assisted with planning the project led the field site preparation for the trial, and field work data collection. SG provided background information on the biology and ecology of Argentine stem weevil, both in South America and in New Zealand, and had a major input into data interpretation and writing the manuscript.

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Evolution of Specialization of *Cassida rubiginosa* on *Cirsium arvense* (Compositae, Cardueae)

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The majority of herbivorous insects are specialized feeders restricted to a plant family, genus, or species. The evolution of specialized insect–plant interactions is generally considered to be a result of trade-offs in fitness between possible hosts. Through the course of natural selection, host plants that maximize insect fitness should result in optimal, specialized, insect–plant associations. However, the extent to which insects are tracking plant phylogeny or key plant traits that act as herbivore resistance or acceptance characters is uncertain. Thus, with regard to the evolution of host plant specialization, we tested if insect performance is explained by phylogenetic relatedness of potential host plants, or key plant traits that are not phylogenetically related. We tested the survival (naive first instar to adult) of the oligophagous leaf-feeding beetle, *Cassida rubiginosa*, on 16 selected representatives of the Cardueae tribe (thistles and knapweeds), including some of the worst weeds in temperate grasslands of the world in terms of the economic impacts caused by lost productivity. Leaf traits (specific leaf area, leaf pubescence, flavonoid concentration, carbon and nitrogen content) were measured as explanatory variables and tested in relation to survival of the beetle, and the phylogenetic signal of the traits were examined. The survival of *C. rubiginosa* decreased with increasing phylogenetic distance from the known primary host plant, *C. arvense*, suggesting that specialization is a conserved character, and that insect host range, to a large degree is constrained by evolutionary history. The only trait measured that clearly offered some explanatory value for the survival of *C. rubiginosa* was specific leaf area. This trait was not phylogenetically dependant, and when combined with phylogenetic distance from *C. arvense* gave the best model explaining *C. rubiginosa* survival. We conclude that the specialization of the beetle is explained by a combination of adaptation to an optimal host plant over evolutionary time, and key plant traits such as specific leaf area that can restrict or broaden host utilization within the Cardueae lineage. The phylogenetic pattern of *C. rubiginosa* fitness will aid in predicting the ability of this biocontrol agent to control multiple Cardueae weeds.

Keywords: Cardueae, thistles, weeds, biological control, *Cassida rubiginosa*, *Cirsium arvense*, host specificity

INTRODUCTION

The majority of phytophagous insects are specialized feeders with host plant ranges restricted to a single plant family or lower taxonomic group (Strong et al., 1984; Bernays and Chapman, 1994). Since specialization is the predominant feeding strategy of phytophagous insects, it is often considered a mechanism for speciation and generation of biological diversity (Mitter et al., 1988; Janz et al., 2006), yet the ecological and evolutionary forces that promote specialization are still poorly understood (Forister et al., 2012). The evolution of specialization is generally framed in the theory of fitness trade-offs, whereby utilization of one host in a particular environment comes at the expense of utilizing an alternative host (Futuyma and Moreno, 1988; Joshi and Thompson, 1995; Roff and Fairbairn, 2007). Through the course of natural selection, host plants that maximize insect fitness should result in optimal, specialized, insect–plant associations. The ecological selection pressures promoting specializing in natural communities are multiple and varied, and include factors such as time to locate and develop on suitable hosts (Doak et al., 2006), competition for resources (Svanbäck and Bolnick, 2007), and enemy-free space (Bernays, 1989). While these contemporary ecological pressures can be important factors promoting specialization they are undoubtedly constrained to some degree by evolutionary histories that also play a role in shaping specialized insect–plant associations (Winkler and Mitter, 2008; Futuyma, 2010). Most insect–plant associations share long evolutionary histories that can be traced millions of years, often to the Paleogene, 30–60 mya (Farrell, 1998; Currano et al., 2008). These long evolutionary associations have allowed phytophagous insects to adapt to the chemical and physical defensive traits of particular plant lineages and may explain the commonly observed pattern of insect host ranges being phylogenetically conserved. However, even specialized insects have limits to the concentration of chemical defenses or magnitude of physical resistance traits that they can contend with (Ali and Agrawal, 2012). Therefore, even though closely related plants often share the same defensive traits, the traits themselves can vary quantitatively, which may be more important in determining insect performance than phylogenetic relatedness.

Within the host ranges of specialized phytophagous insects, performance hierarchies are common, but the plant traits and the phylogenetic relationships of the host plants that might explain the differences in insect performance arey seldom identified. The increasing availability of comprehensive resolved phylogenies is permitting more explicit tests of hypotheses concerning the evolution of specialization, and could permit better prediction of insect host ranges, and the degree of host plant specialization (Ødegaard et al., 2005). If specialized phytophagous insects exhibit a phylogenetically conserved pattern of performance across their potential host range it would suggest that insects are tracking overall plant trait similarity to an optimal host and indicate that host plant specialization is largely governed by evolutionary history. Alternatively, if phylogeny is not a good predictor of insect performance it would suggest that specialization, although broadly bound by evolutionary history, is labile within a plant lineage, and that insects track key plant traits that may exist through convergent or parallel evolution.

Previously, the fitness of the leaf-feeding beetle, *Cassida rubiginosa*, was tested in relation to plant defensive traits in a phylogenetically controlled experiment with three *Cirsium* species (Cripps et al., 2015). The beetle is classified as an oligophagous feeder restricted to plants in the tribe Cardueae (thistles and knapweeds), but has a well-known primary host plant, *Cirsium arvense* (Zwölfer and Eichhorn, 1966). The herbivore defensive traits of Cardueae species have not been well characterized, but some key traits that we identified as plausibly providing defense against specialized herbivores were leaf flavonoid concentration and leaf structural defenses, which were measured as specific leaf area, and the proportion of leaf pubescence. Flavonoids are known to act as feeding deterrents or stimulants in many insect–plant systems, depending on their concentration (Harborne and Grayer, 1993; Simmonds, 2001), and are the predominant secondary chemical group in the Cardueae (Wagner, 1977; Jordon-Thaden and Louda, 2003). Similarly, leaf pubescence and toughness are well documented as herbivore resistance traits (Hanley et al., 2007), and were identified as key defensive traits reducing *Cassida* fitness on congeners of *C. arvense* (Cripps et al., 2015). This raised the question of whether or not host plant utilization within the Cardueae was driven by key defensive traits (i.e., any given Cardueae species could be equally suitable, depending on key traits), or if there was a broad phylogenetic pattern explaining host plant specialization (i.e., adaptation to an optimal host over evolutionary time, independent of defensive traits). We hypothesized that both defensive leaf traits and phylogenetic relationship would affect the survival of the beetle. To determine the component effects of plant phylogeny and defensive traits on *Cassida* survival we measured the phylogenetic distance of each Cardueae test species from the primary host, *C. arvense*, and measured several leaf traits (specific leaf area, leaf pubescence, flavonoid concentration, carbon and nitrogen content) that might explain differences in insect survival.

MATERIALS AND METHODS

Study System

The Cardueae is one of the largest tribes of the Compositae family comprised of approximately 2400 species in five subtribes (Susanna and Garcia-Jacas, 2007), and are considered a monophyletic group with a nearly completely resolved phylogeny (Susanna et al., 1995, 2006; Barres et al., 2013). The tribe originated during the mid-Eocene, and subtribal diversification events occurred throughout the Oligocene-Miocene period (Barres et al., 2013). The current native distribution is almost entirely in the northern hemisphere, and all of the Cardueae test species used in this study are native to Eurasia, and were introduced to New Zealand (NZ) either inadvertently, or deliberately as agricultural or ornamental plants (Webb et al., 1988). All of the inadvertently introduced Cardueae species in NZ are considered agricultural weeds that vary from minor to extreme economic importance (Cripps et al., 2013). The

Cardueae plants selected for this study span three of the five subtribes and include representatives from widespread, species rich genera (e.g., *Cirsium* and *Centaurea*), and species poor genera with narrow geographic ranges (e.g., *Cynara*, *Ptilostemon*), and therefore provide a good representation of the tribe (Susanna and Garcia-Jacas, 2007; Vilatersana et al., 2010).

The tortoise beetle, *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae), is native to the Palearctic region where it is one of the most common insect herbivores on *C. arvense* (Zwölfer and Eichhorn, 1966). It was deliberately introduced to NZ in 2007 as part of the biological control program against *C. arvense* (Cripps et al., 2011b). Although the beetle is oligophagous, with many host plants in the Cardueae tribe, its release as a biological control agent was considered safe since there are no native Cardueae plants in NZ (Webb et al., 1988), and potential damage to economic plants (e.g., artichoke) was considered acceptable in cost-benefit analyses (Barratt et al., 2010). In its native range, at least 21 Cardueae species have been recorded as host plants, most belonging to the subtribe Carduinae (true thistles); however, the beetle shows a marked preference for *C. arvense*, which is considered its primary host (Zwölfer and Eichhorn, 1966). The beetle is univoltine and completes its entire life cycle (egg, 5 larval instars, pupa, and adult) on the foliage of host plants. Both adults and larvae are leaf feeders. Adults overwinter under leaf litter debris in hedge rows, or forest margins. Upon emergence in spring adults seek out host plants where they deposit their egg masses (oothecae), typically on the underside of leaves. Larvae are confined to the host plant where their eggs are laid, or adjacent overlapping shoots, but cannot move long distances along the ground to a new host plant (Tipping, 1993).

Collection and Preparation of Test Plants

All of the test plants were grown from seed, except *Onopordum acanthium* and *Ptilostemon afer,* which were collected as rosettes from natural field sites in October 2013 and directly transplanted into 12-l pots. Seeds were either collected from the field (2009–2013) or purchased from a commercial supplier (King Seeds NZ Ltd.). Seeds of each species were sown on 21 August 2013. Seedlings were grown in a glasshouse at AgResearch until 17 October, when all plants were transplanted into 12-l plastic pots, and shifted outside to the experimental location in a fenced compound area on the campus of AgResearch, Lincoln (S 43°38′20.54″; E 172°28′28.2″). The plants were arranged in a randomized complete block design with five replicate blocks (80 plants total). The plants were grown in a standard potting mix (54% aged bark, 45% sand, 1% nutrients, by weight) containing added nutrients as Osmocote® 17–11–10 (N–P–K), lime, superphosphate, sulfate of potash, and calcium nitrate. Watering of potted plants occurred four times daily (every 6 h) through an automatic irrigation system.

Collection and Application of *Cassida*

Adult *Cassida* beetles were collected on 4 and 5 November 2013 from two field sites (Winton and Manapouri) in Southland NZ, where they had been released as biocontrol agents in 2007 and 2008, respectively. All adult beetles were collected from *C. arvense*. Adult beetles were kept in 2.2-l ventilated plastic boxes and fed *C. arvense* shoot clippings. The beetle colony was maintained in a laboratory at AgResearch Lincoln at a constant room temperature (*ca*. 20°C). Egg masses that were laid on the leaves of the clippings were removed and placed on moist filter paper in Petri dishes.

From 15 to 21 November, 12 naïve first instar larvae (hatched within 12 h) were placed on new fully expanded leaves of each plant in the experiment (total of 960 larvae). Larvae were applied one block at a time (1 or 2 days per block). A polyester mesh bag (50-cm × 125-cm) supported by wire struts was placed over each potted plant. At the time of beetle larvae placement, plants in the experiment were either large rosettes (biennial species), or bolting (annual species). On 27 January 2014 each plant was cut at the base and inspected in the laboratory for adult beetles. All surviving individuals were adults by this time (no other growth stages, i.e., larvae or pupae, were found), and the number of individuals per plant surviving to the adult stage was recorded. The mean temperature over the duration of the experiment (15 November to 27 January) was 16.3°C (range 4.4–34.8°C), based on hourly temperature readings recorded in the compound area from a Tinytag® (Gemini Data Loggers Ltd) data logger.

Specific Leaf Area, Leaf Pubescence, Flavonoids, Carbon, and Nitrogen Analyses

Three leaves from each plant in the experiment (representative of the size and age of leaves that the beetles were feeding on) were taken on 3 December. A 1.0-cm diameter cork-borer was used to cut a leaf disk from each of the three leaves, avoiding the midvein of each leaf. The three disks from each replicate plant were dried in an oven at 50°C for 48 h and the dry weights were recorded to the nearest 0.1 mg. The specific leaf area (SLA) was calculated as the ratio of the leaf area to dry weight (mm^2/mg) of the three disks. The remainder of the leaves were also dried and then ground in a Retsch® centrifugal mill through a 1.0-mm sieve. After milling, tomentous leaf pubescence (tightly adhering, matted leaf hairs) was separated from the dry leaf tissue material (re-milled two or three times as necessary to separate all leaf pubescence material) and weighed separately. The proportion of tomentous pubescence of the total leaf dry weight was recorded.

Total flavonoids were determined according to Zhu et al. (2010) but volumes were adapted for 96-well microplates. Beetle larvae were still feeding on the plants at the time of sample collection (3 December), and it is possible that this could have induced specific flavonoids, and thereby altered concentrations relative to plants in an undamaged state. However, since herbivore induction typically triggers species-specific, and specialized responses (e.g., single compounds) (Karban and Baldwin, 1997) the comparisons between plant species were considered to represent constitutive flavonoid concentrations, and any induction effects were assumed to be minor. Dried leaf powder (100 mg) was weighed into a 25-ml screw cap bottle and extracted with 7 ml ethanol (70% v/v) in a sonicator bath for 30 min. The extract was filtered (LBS 0001.090, Labserv, NZ) and an aliquot of 100 μl was transferred to a 2-ml microreaction tube. For the colorimetric reaction, 100 μl $NaNO_2$

(5%, w/v) and 100 µl $Al(NO_3)_3$ (10% w/v) were added. After each step, the solution was vortex mixed and left to react for 6 min. Finally, 1 ml NaOH (4% w/v) and 600 µl aqua dest was added to the reaction tube, which was then vortexed, and left for 15 min. The absorbance of the extract (175 µl) was read at 500 nm against the uncoloured sample solution blank using a spectrophotometer (Multiskan GO, Thermo Scientific, USA). Rutin (Sigma–Aldrich, Australia) was used as a standard. Each sample was measured in triplicate. The total carbon and nitrogen concentrations (%) of the leaf samples were analyzed using an Elementar Vario-Max CN elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany).

Phylogenetic Data and Analyses

The phylogeny of the 16 Cardueae test species was pruned from a comprehensive phylogeny of the Cardueae tribe based on nuclear ribosomal DNA and chloroplast DNA markers (Barres et al., 2013). Phylogenetic distance (in millions of years, my) was calculated from the total branch lengths separating each species from *C. arvense* using the function cophenetic.phylo (R package *ape*) (**Figure 1**).

Since survival was recorded as the number of *Cassida* adults from a group of 12 larvae that either survived or not, the percent survival values were analyzed using generalised linear models (GLMs), assuming binomial distributions through a logit link function (Faraway, 2005). The first seven GLM analyses examined if mean percent survival values were related to each of the seven explanatory variables alone (phylogenetic distance, SLA, flavonoids, percent pubescence, C, N, and C/N), i.e., the relationship of each individual variable to survival was examined independently from other variables. This was followed by further binomial GLM analyses that systematically examined if mean percent survival values were related to any combination of the seven explanatory variables using analysis of deviance. The sets of variables identified as statistically significant in the analysis of deviance were then compared based on their Akaike Information Criterion (AIC) values (Claeskens and Hjort, 2008).

The phylogenetic signal of all plant traits plus *Cassida* survival was estimated using Pagel's lambda (λ) (Freckleton et al., 2002). Maximum likelihood estimates of the best λ value were compared to model estimates where λ was set at 0 (no phylogenetic signal, i.e., phylogenetic independence), or 1 (strong phylogenetic signal indicating the trait co-varies directly with shared evolutionary history).

We aimed to also test whether *Cassida* survival was related to plant traits while accounting for possible dependence among trait values due to shared evolutionary history. In recent years several methods have been developed to conduct phylogenetic regression analyses for continuous and binary data (for a review on methods for each type of data see Ives and Garland, 2014; and Symonds and Blomberg, 2014); however, to date there are no methods available for proportional data, such as survival percentages. Therefore, we applied arcsine square root transformations to survival proportions and performed phylogenetic generalised least squares (PGLS) regression (R packages *nlme*, Pinheiro et al., 2013, and *ape*, Paradis et al., 2004). Moreover, a recent study confirms that linear models applied to transformed data provide robust statistical tests for significance over a wide range of conditions (Ives, 2015). PGLS identifies from the phylogeny the amount of expected correlation between species based on their shared evolutionary history, and weights for this in the generalized least squares regression calculation using Pagel's λ (it should be noted that in PGLS the assumptions regarding phylogenetic non-independence refer to the residual errors of the regression model, not the traits themselves). The phylogenetic structure might affect the covariance in trait values across taxa in different ways. Therefore, we compared two types of trait evolution model for each trait: Brownian motion models (BM), in which trait covariance between any pair of taxa decreases linearly with time since their divergence (three models diverging in lambda value were compared: fixed to 1, estimated from data, and fixed to 0); and Ornstein–Uhlenbeck models (Martins and Hansen, 1997), where the expected covariance decreases exponentially, as governed by the parameter alpha (values ranging from 0.5 to 10 were tested). PGLS was performed on all traits versus survival, each trait was first tested independently and then for all trait combinations in relation to *Cassida* survival.

RESULTS

Mean *Cassida* survival (naïve first instar to adult) on the 16 Cardueae host plants ranged from 0 to 85% (**Figure 2**). The survival rates of *Cassida* on *Carduus tenuiforus*, *Cirsium palustre*, *Cirsium vulgare*, and *Arctium lappa* were statistically equivalent to *C. arvense*. Survival rates on the remaining 11 test plant species were significantly lower than on *C. arvense* (Duncan's multiple comparison procedure, $P < 0.05$).

Of the traits measured, strong phylogenetic signal (Pagel's λ) was detected for *Cassida* survival and leaf pubescence (**Table 1**). The strong phylogenetic signal of *Cassida* survival is a good indicator that phylogeny is a good predictor of *Cassida* performance, and that it can be used as a surrogate for plant traits influencing fitness of this species. Although leaf pubescence showed a phylogenetic signal (**Table 1**) it did not contribute significantly to *Cassida* survival.

Based on the AIC values of significant GLMs, the most appropriate single-variable model predicting *Cassida* survival was phylogenetic distance, followed by SLA (**Table 2**). Survival of *Cassida* decreased with increasing phylogenetic distance from the primary host, *C. arvense* (**Figure 2**; **Table 2**), and this relationship remained significant after controlling for phylogenetic non-independence in the residuals (PGLS), indicating that phylogenetic distance itself (i.e., total branch length separation from *C. arvense*) is a good proxy for survival rates. Survival of *Cassida* increased with increasing SLA (**Figure 3**; **Table 2**), and this also remained significant after correcting for phylogenetic non-independence. From the single-variable GLMs, phylogenetic distance explained 58.4% of the variation in survival, and SLA explained 50.1% (i.e., the model correctly classified these percentages of individuals into survival/death groups). Similarly, based on the AIC values of significant multi-variable GLMs the most appropriate model was that of phylogenetic distance combined with SLA (**Table 2**). The

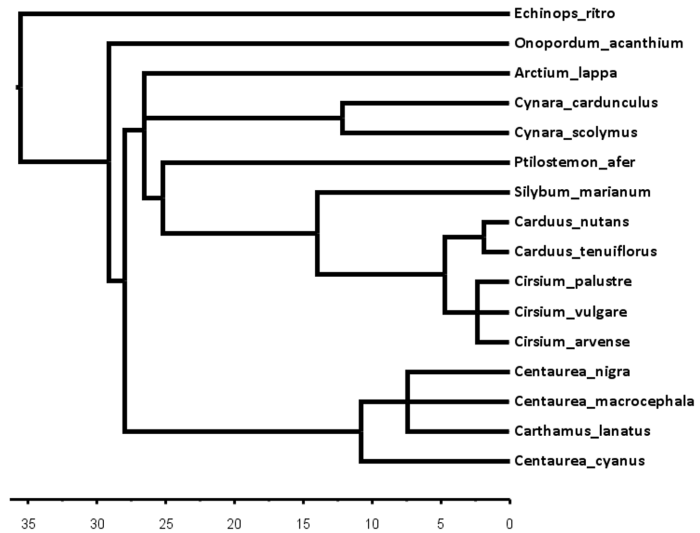


FIGURE 1 | Chronogram of the 16 Cardueae test species pruned from a comprehensive phylogeny of the tribe (Barres et al., 2013). Branch lengths depict phylogenetic distances in millions of years (my).

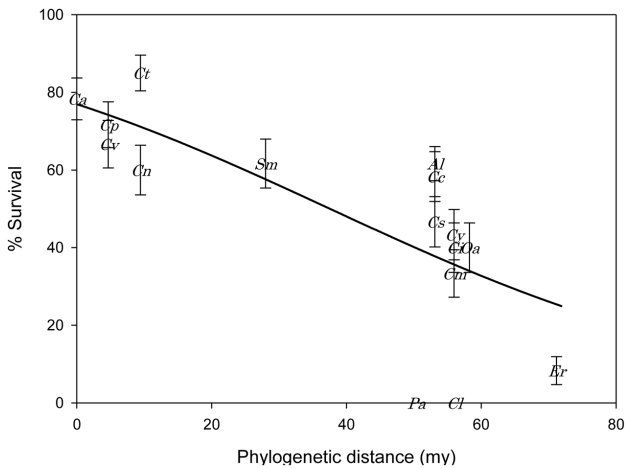


FIGURE 2 | Mean (±SE) percent survival of *Cassida rubiginosa* in relation to phylogenetic distance (million years, my) of each Cardueae test species from the primary host plant, *Cirsium arvense*. The relationship between % survival and phylogenetic distance (PD) is given by the equation, % survival = 100/[1 + exp(−1.206 + 0.0321 × PD)]. Ct, *Carduus tenuiflorus*; Ca, *Cirsium arvense*; Cp = *Cirsium palustre*; Cv, *Cirsium vulgare*; Sm, *Silybum marianum*; Al, *Arctium lappa*; Cn, *Carduus nutans*; Cc, *Cynara cardunculus*; Cs, *Cynara scolymus*; Cy, *Centaurea cyanus*; Ci, *Centaurea nigra*; Oa, *Onoporodum acanthium*; Cm, *Centaurea macrocephala*; Er, *Echinops ritro*; Pa, *Ptilostemon afer*; Cl, *Carthamus lanatus*.

GLM of phylogenetic distance + SLA explained 66.7% of the variation in *Cassida* survival. None of the other traits that were measured significantly explained survival of *Cassida* across the 16 test plants. In particular, total flavonoid concentrations did not explain *Cassida* survival ($P = 0.413$). The plant species with the highest survival rate (85%, *C. tenuiflorus*), and the species with the lowest survival rates (0%, *P. afer* and *C. lanatus*), had similarly low flavonoid concentrations (mean flavonoid concentrations for *C. tenuifolorus* = 0.2 mg/gDW; *P. afer* = 0.2 mg/gDW; and *C. lanatus* = 0.6 mg/gDW) (**Figure 4**).

In PGLS analyses, the most appropriate single-variable model predicting *Cassida* survival was SLA, followed by PD (phylogenetic distance), and C content (**Table 3**). According to the AIC values of significant multi-variable PGLS models, the most appropriate models are SLA + Percent pubescence, SLA + N content, SLA + flavonoids, and SLA + C:N ratio. All these models obtained very similar AIC values (**Table 3**). Only one of these four models (SLA + N) was significantly improved (according to likelihood ratio-test) by the addition of other variables: SLA + N + PD + C:N + Percent pubescence (**Table 3**). In this more complex model, survival decreases with phylogenetic distance, and increases with SLA, N content, C:N, and pubescence, although the final contributing variable (pubescence) was not statistically significant on its own.

DISCUSSION

In accordance with our hypothesis, this study demonstrated that the survival of *Cassida* decreased with increasing phylogenetic distance from the primary host plant, *C. arvense*. This indicates that specialization is a conserved character, and that insect host range, to a large degree is constrained by evolutionary history. While the phylogenetically conserved pattern of insect performance has been long recognized (Wapshere, 1974), the novelty of the present work is that is uses a quantitative measure of evolutionary separation between hosts to predict insect herbivore performance across its host range (Pearse and Hipp, 2009; Rasmann and Agrawal, 2011). Phylogenetic distance is in essence a composite measure of trait similarity among the Cardueae test species, and is ultimately a good

predictor of *Cassida* survival. However, the particular traits (chemical/physical) that account for the phylogenetic effect are still uncertain.

The only trait measured that clearly offered some explanatory value for the survival of *Cassida* across the 16 Cardueae test plants was SLA. This trait was independent of phylogeny, and when combined with phylogenetic distance from *C. arvense* was the best model explaining *Cassida* survival. This also conferred with our hypothesis that defensive traits, in addition to phylogenetic relationship, affect insect survival. Plant species with high SLA have many features conducive to herbivore feeding, such as lower dry matter, faster growth rate, and reduced leaf toughness (Salgado-Luarte and Gianoli, 2010), and this trait was shown to be an important factor explaining survival of *Cassida* on plants congeneric to *C. arvense* (Cripps et al., 2015). The only plant trait found to have a clear phylogenetic signal was leaf pubescence, but this trait was not related to *Cassida* survival. The fact that leaf pubescence showed a phylogenetic signal is likely an artifact of low sample size since this trait is common in plant species across the Cardueae subtribes (Susanna and Garcia-Jacas, 2007), and is therefore unlikely to be part of the underlying phylogenetic explanation for *Cassida* survival. Leaf pubescence is known to provide resistance against most types of insect herbivores, including both generalist and specialist feeders (Hanley et al., 2007), and previously trichome density was found to be an important factor determining survival rates of *Cassida* on plants congeneric to *C. arvense* (Cripps et al., 2015). In the present study, leaf pubescence was measured as a proportion of total leaf dry weight and specific pubescence structures that might differently affect *Cassida* survival were not distinguished. On *Cirsium* species, where the density of pubescence is important for *Cassida* survival, leaves are villose below and hirsute-hispidule above, and both types of hairs can create a barrier to the leaf surface. However, some other Cardueae species (e.g., *Cynara* and *Onopordum* spp.) have a relatively high proportion of leaf pubescence, but it is tightly adhering tomentous pubescence that does not appear to inhibit access to the leaf surface, and therefore does not affect *Cassida* survival.

Total flavonoid concentration did not show a phylogenetic signal, and did not explain *Cassida* survival. Interestingly, the host plant with the highest survival rate (85%, *C. tenuiflorus*) had a similarly low flavonoid concentration as the two non-host species (*P. afer* and *C. lanatus*). From other studies, there is mixed evidence with regard to the importance of flavonoids on insect performance, with inhibitory effects on some herbivore species, but no effects on others (Simmonds, 2001), regardless of being a generalist or specialist (Bi et al., 1997). While total flavonoids did not explain *Cassida* survival in

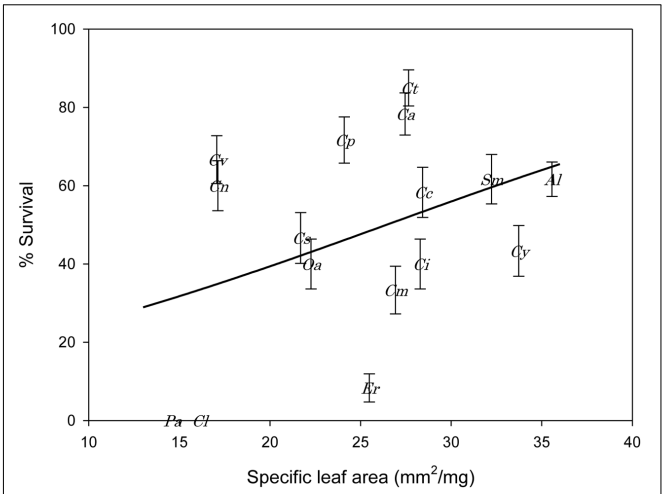


FIGURE 3 | Mean (±SE) percent survival of *Cassida rubiginosa* in relation to specific leaf area of the 16 Cardueae test species. The relationship between % survival and specific leaf area (SLA) is given by the equation, % survival = 100/[1 + exp(1.770 – 0.0670 × SLA)]. Ct, *Carduus tenuiflorus*; Ca, *Cirsium arvense*; Cp, *Cirsium palustre*; Cv, *Cirsium vulgare*; Sm, *Silybum marianum*; Al, *Arctium lappa*; Cn, *Carduus nutans*; Cc, *Cynara cardunculus*; Cs, *Cynara scolymus*; Cy, *Centaurea cyanus*; Ci, *Centaurea nigra*; Oa, *Onoporodum acanthium*; Cm, *Centaurea macrocephala*; Er, *Echinops ritro*; Pa, *Ptilostemon afer*; Cl, *Carthamus lanatus*.

TABLE 1 | Phylogenetic signal (Pagel's λ) for plant traits and survival of *Cassida rubiginosa*.

Trait	$\lambda_{estimated}$	Significantly different from $\lambda = 0$	Significantly different from $\lambda = 1$
Cassida survival (%)	0.85	0.01	0.14
Leaf pubescence (%)	0.95	0.01	0.44
Flavonoid concentration (mg/g)	0	1	2.97×10^{-4}
Specific leaf area (mm^2/mg)	0	1	2.97×10^{-4}
C (%)	0	1	1.36×10^{-5}
N (%)	0	1	1.48×10^{-3}
C:N ratio	0	1	6.40×10^{-3}

Values of λ close to 1 indicate a strong phylogenetic signal (resulting from shared evolutionary history), whereas values close to 0 indicate that phylogenetic signal is weak or null.

TABLE 2 | Plant traits predicting survival of *Cassida rubiginosa* resulting from the binomial generalised linear model (GLM) analyses.

GLM	Relationship to survival (%)	Significance	AIC
Phylogenetic distance (PD)	100/[1 + exp (−1.206 + 0.0321 × PD)]	<0.001	1158
Specific leaf area (SLA)	100/[1 + exp (1.770 – 0.0670 × SLA)]	<0.001	1216
PD + SLA	100/[1 + exp(0.973 + 0.0404 × PD – 0.0978 × SLA)]	<0.001	1105

Lower Akaike Information Criterion (AIC) values indicate a closer fit to the data.

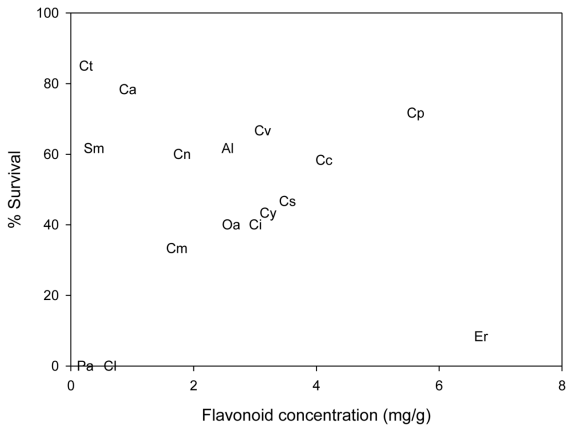


FIGURE 4 | Mean flavonoid concentration and percent survival of *Cassida rubiginosa* on 16 Cardueae test species. The relationship between % survival and flavonoid concentration is not significant. Ct, *Carduus tenuiflorus*; Ca, *Cirsium arvense*; Cp, *Cirsium palustre*; Cv, *Cirsium vulgare*; Sm, *Silybum marianum*; Al, *Arctium lappa*; Cn, *Carduus nutans*; Cc, *Cynara cardunculus*; Cs, *Cynara scolymus*; Cy, *Centaurea cyanus*; Ci, *Centaurea nigra*; Oa, *Onoporodum acanthium*; Cm, *Centaurea macrocephala*; Er, *Echinops ritro*; Pa, *Ptilostemon afer*; Cl, *Carthamus lanatus*.

this study it is likely that the specific flavonoid composition is important. In a related study, *Cassida* was shown to be tolerant to double the concentration of flavonoids on plants congeneric to *C. arvense*, which suggested that *Cassida* might be adapted to specific flavonoids that were common to plants closely related to *C. arvense* (Cripps et al., 2015). Although flavonoids are the predominant group of secondary chemicals in the Cardueae, other chemicals such as terpenoids and alkaloids have also been identified from Cardueae species, and could also play a role in resistance to herbivores (Wagner, 1977). Cardueae species share many secondary chemical compounds, but also have unique profiles, and it is possible that the underlying phylogenetic effect is explained by overall chemical similarity to the primary host (Jordon-Thaden and Louda, 2003). Much attention is often given to the role of defensive secondary chemistry; however, it is also possible that non-defensive chemicals might explain the phylogenetic effect on *Cassida* performance (Jermy, 1993). Insects often require sufficient stimuli for host recognition and sustained feeding that can be obtained through chemical attributes of the leaf surface, such as volatile organic compounds, epicuticular waxes, and primary metabolite exudates such as sugars and amino acids (Müller and Riederer, 2005).

Both phylogenetic relatedness and putative resistance traits independent of phylogeny (i.e., low SLA) contributed to the survival of *Cassida*, a conclusion also reached by similar studies (Becerra, 1997; Rasmann and Agrawal, 2011; Gilbert et al., 2015). These data indicate that specialization of *Cassida* on *C. arvense* has likely arisen through a combination of the beetle tracking phylogenetically conserved traits and responding to plant resistance characters that impose selection pressures for or against host utilization. This is in contrast to the conclusion reached by Slotta et al. (2012) who suggested that specialized herbivores on *Cirsium* and *Carduus* species do not follow a phylogenetic pattern, and that phylogeny does not predict host specificity. However, it should be noted that their conclusion was based only on literature records of known host associations and therefore the insect–plant associations for the bulk of species in their phylogeny are uncertain. Furthermore, the fact that a plant is recorded as a host does not mean it is an equivalent host in terms of insect fitness.

The potential to utilize other, more distantly related Cardueae plants, raises the question of what conditions might promote shifts in primary host use, and whether or not these could be sustained. The question is particularly pertinent in novel ranges such as NZ, where altered selection pressures are likely to exist, and may result in changes in the pattern of host plant use. Resistance characters might act to channel host plant selection and utilization without eliminating the innate ability of the insect to utilize other host plants, but whether or not resistance traits have evolved in response to herbivory is unclear. Traits such as low SLA (thick, tough leaves) and leaf pubescence are typically considered adaptations to xeric environments that primarily function to regulate temperature and conserve water (Johnson, 1975; Niinemets, 2001), and therefore these traits

TABLE 3 | Plant traits predicting survival of *Cassida rubiginosa* resulting from phylogenetic least squares regression (PGLS) to account for possible dependence among trait values due to shared evolutionary history.

GLM	Relationship to survival (%)	λ	Significance	AIC
Phylogenetic distance (PD)	$[\sin (1.0836 - 0.0093xPD)]^2$	0	<0.0001	−1.13
Specific leaf area (SLA)	$[\sin (-0.1126 + 0.0272xSLA)]^2$	1	<0.0001	7.73
C content	$[\sin (2.0847 - 0.0377xC)]^2$	1	<0.0001	12.75
SLA + % pubescence (PP)	$[\sin (-0.0806 + 0.0211xSLA + 0.0067xPP)]^2$	1	<0.0001	−21.64
SLA + flavonoids	$[\sin (-0.0974 + 0.0268xSLA - 0.0030x \text{ flavonoids})]^2$	1	<0.0001	−20.45
SLA + N	$[\sin (0.1215 + 0.0242xSLA - 0.0499xN)]^2$	1	<0.0001	−21.62
SLA + C:N	$[\sin (-0.2996 + 0.0245xSLA - 0.0194xC{:}N)]^2$	1	<0.0001	−21.35
SLA + N + PD	$[\sin (0.2729 + 0.0268xSLA + 0.0277xN - 0.0087xPD)]^2$	1	0.0001	−4.6
SLA + N + PD + C:N	$[\sin (-5.0978 + 0.0364xSLA + 0.7693xN - 0.0121xPD + 0.2252x\ CN)]^2$	1	0.0001	−15.61
SLA + N + PD + C:N + PP	$[\sin (-4.9092 + 0.0341xSLA + 0.7611xN - 0.013xPD + 0.2157xC{:}N + 0.0036xPP)]^2$	1	0.0358	−18.02

Lower Akaike Information Criterion (AIC) values indicate a closer fit to the data. Survival values were arc-sine square-root transformed. Phylogenetic signal associated with the regression was estimated with λ (values near 1 indicate strong phylogenetic signal of the residuals, values near 0 indicate null or weak phylogenetic signal).

could be retained even in the absence of herbivory. However, if *Cassida* herbivory has fitness consequences for the plant, this could impose selection pressure for increased defensive traits (resistance and/or tolerance). This would depend on the degree of variation in defensive traits of thistles in NZ, but could result in reduced performance of *Cassida* over time. In the novel range of NZ, most Cardueae plants have experienced over a century of release from specialized insect herbivores (Cripps et al., 2011a), which may have allowed for evolution of reduced defenses (Bossdorf et al., 2005) that could, at least temporarily, enable increased fitness in *Cassida* on *C. arvense* and other Cardueae hosts.

Part of the reason for successful classical biological control is that insect biocontrol agents are released in novel, relatively benign environments, compared to their native ranges, and can achieve greater population numbers (Myers, 1987). The primary reason for this is release from the regulating influence of specialized predators and parasitoids. In NZ, *Cassida* experiences relaxed natural enemy pressure that allows population numbers of the beetle to increase far greater than observed in the native range (Cripps, 2013). Large numbers of the beetle might result in competition for the food resource and promote selection for alternative hosts, where competition is reduced, and survival rates are greater. Changes in the relative abundance of host plants due to successful biological control of one species, or land management changes that favor particular species, could also promote a shift in primary host plant utilization. Even with fitness trade-offs on alternative hosts (e.g., due to resistance traits), a more abundant resource might contribute more to beetle population numbers, and therefore selection could act to favor the more abundant plant (Futuyma, 2010). In fact, regional differences in Cardueae species abundance are thought to have led to biotype development of specialized capitulum-feeding insects, and was suggested as a mechanism for sympatric or parapatric speciation over evolutionary time (Zwölfer and Romstöck-Völkl, 1991). Given that other Cardueae plants are suitable hosts for *Cassida*, and four species other than the primary host were determined to be equivalent hosts for larval survival, there is potential for an altered pattern of host use in NZ (Van Klinken and Edwards, 2002). This will largely depend on the ecological selection pressures acting on host use, and how these are balanced against possible fitness consequences of using an alternative host. Determining the ecological selection pressures (e.g., enemy-free space) that influence the realized host range of *Cassida* in NZ will be the subject of future investigation, and will reveal the potential for contemporary evolution in novel environments, and also aid in predicting the success of an oligophagous biocontrol agent for controlling multiple thistle weeds.

AUTHOR CONTRIBUTIONS

The study was conceived by MC, MR, and GB. The experimental work was carried out by MC, SJ, and MR. Phylogenetic data was provided AS, and analyses were carried out by CR and CvK. MC wrote the manuscript with contributions from all authors.

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frontiers in Plant Science

REVIEW
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Get Tough, Get Toxic, or Get a Bodyguard: Identifying Candidate Traits Conferring Belowground Resistance to Herbivores in Grasses

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Grasses (Poaceae) are the fifth-largest plant family by species and their uses for crops, forage, fiber, and fuel make them the most economically important. In grasslands, which broadly-defined cover 40% of the Earth's terrestrial surface outside of Greenland and Antarctica, 40–60% of net primary productivity and 70–98% of invertebrate biomass occurs belowground, providing extensive scope for interactions between roots and rhizosphere invertebrates. Grasses invest 50–70% of fixed carbon into root construction, which suggests roots are high value tissues that should be defended from herbivores, but we know relatively little about such defenses. In this article, we identify candidate grass root defenses, including physical (tough) and chemical (toxic) resistance traits, together with indirect defenses involving recruitment of root herbivores' natural enemies. We draw on relevant literature to establish whether these defenses are present in grasses, and specifically in grass roots, and which herbivores of grasses are affected by these defenses. Physical defenses could include structural macro-molecules such as lignin, cellulose, suberin, and callose in addition to silica and calcium oxalate. Root hairs and rhizosheaths, a structural adaptation unique to grasses, might also play defensive roles. To date, only lignin and silica have been shown to negatively affect root herbivores. In terms of chemical resistance traits, nitrate, oxalic acid, terpenoids, alkaloids, amino acids, cyanogenic glycosides, benzoxazinoids, phenolics, and proteinase inhibitors have the potential to negatively affect grass root herbivores. Several good examples demonstrate the existence of indirect defenses in grass roots, including maize, which can recruit entomopathogenic nematodes (EPNs) via emission of (E)-β-caryophyllene, and similar defenses are likely to be common. In producing this review, we aimed to equip researchers with candidate root defenses for further research.

Keywords: root defense, Poaceae, plant secondary metabolites, silica, physical defence, benzoxazinoids, indirect defence, root herbivory

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INTRODUCTION

Grasses (the family Poaceae) evolved 66 million years ago (Piperno and Sues, 2005) and have been exploited by humans for around 12,500 years (Baker, 2009). In fact, just three grass species (wheat, rice, and maize) provide 50% of the World's food (Varshney et al., 2012) and other species are important sources of forage, fuel, and fiber (Blair et al., 2014). Grasslands also represent

crucial ecosystems, storing up to a third of global climate stocks and account for up to 40% of terrestrial land mass (Gibson, 2009). In grasslands, between 40 and 60% of net primary productivity occurs belowground (Coleman, 1976) and between 70 and 98% of invertebrate biomass is located in the soil (Curry, 1994). Interactions between grass roots and invertebrates must therefore be extensive, yet there are key gaps in our knowledge about these interactions, particularly in terms of plant defenses and root herbivory. Here, we identify candidate grass root traits that assist in resisting herbivory, including physical and chemical defenses and indirect defenses (i.e., herbivore natural enemy recruitment). Where information is available, we describe the efficacy of defenses (sometimes in above-ground tissues or against vertebrate herbivores where that is the only information available), their occurrence in grasses and their documented or likely occurrence in grass roots. It should be noted that in plants generally, including grasses, secondary metabolites found in aboveground tissues of plants are commonly also found in their roots (Rasmann and Agrawal, 2008).

HERBIVORES OF GRASS ROOTS

Apart from a few mammals, such as pocket gophers, grass roots are principally attacked by plant parasitic nematodes and herbivorous insects (Andersen, 1987). Plant parasitic nematodes can consume as much net primary productivity as do cattle, and are probably the biggest single group of root feeders in grasses (Seastedt and Murray, 2008). Nematode herbivores are ubiquitous root feeders in grasslands, whereas insect herbivores appear to show particular geographical distributions. In North America and Australasia, scarab larvae are regarded as the most important belowground herbivores in grasslands (Seastedt and Murray, 2008; Frew et al., 2016a), whereas leatherjackets (Tipulidae) and wireworms (Elateridae) are the dominant root-feeding insects in European grasslands (Blackshaw and Kerry, 2008; Seastedt and Murray, 2008). Less well-recognized is the ability of Collembola to act as root herbivores under some circumstances (Endlweber et al., 2009).

While root herbivores are undoubtedly less diverse than shoot herbivores (Johnson et al., 2016b), up to 21 insect species may feed on a the roots of a single plant species (van Dam, 2009) and they show varying degrees of host specificity (Van Der Putten, 2003). With specialists more common in agricultural (Van Der Putten, 2003) and natural (Van der Putten and Van der Stoel, 1998) grass monocultures. Western corn root-worm (*Diabrotica virgifera virgifera*) is a highly specialized feeder on maize (*Zea mays*) and its historical relatives (e.g., teosinte) and has evolved counteradaptations to that plant's root defenses (Robert et al., 2012). African black beetle (*Heteronychus arator*) is a generalist, feeding on more than 190 species of grasses from 33 genera in Australia alone (Hangay and Zborowski, 2010) as well as numerous other monocots and dicots (Frew et al., 2016a). Many grass root herbivores readily switch between hosts, illustrated by the grayback canegrub (*Dermolepida albohirtum*) which was originally a feeder of Australian native grasses, but switched grass species to become a highly destructive pest of sugarcane when it began to be cultivated in Queensland in the early twentieth century (Allsopp, 2010; Frew et al., 2016a). The economic status of these particular grass root feeders has most likely biased research efforts toward certain groups, especially those that chew roots and potentially induce different types of defensive response than fluid-feeding groups. The latter do exist, however, and include sporadically damaging pests such as the mealy grass root aphid (*Aploneura lentisci*), rice root aphid (*Rhopalosiphum rufiabdominale*), and pasture mealybug (*Balanococcus poaea*). Most of our knowledge about grass root defenses, however, appears to come from attack by chewing root herbivores.

Even minor root herbivory can damage plants and alter their physiology by (i) decreasing nutrient and water uptake, (ii) causing disproportionate resource losses by severing roots, (iii) diverting assimilates away from shoot growth for root re-growth belowground, (iv) imposing leaf water deficits, and (v) aggravating pathogen infection (Zvereva and Kozlov, 2006; Johnson and Murray, 2008). From a global agricultural perspective, root herbivores are amongst the most economically damaging, persistent and difficult to detect and control (Johnson et al., 2016a).

WHY WOULD GRASSES DEFEND THEIR ROOTS?

Plant defense can be divided into two main strategies, tolerance of, and resistance to herbivory (Strauss and Agrawal, 1999), and plants often invest in both of these strategies simultaneously (Núñez-Farfán et al., 2007). Grasses commonly invest 50–70% of fixed C in root construction (De Deyn et al., 2003) and as roots are essential for water and nutrient uptake, it seems likely that grasses defend them (Rasmann and Agrawal, 2008; van Dam, 2009). Most grasses have adventitious, dense root systems with many fine, fibrous axes (Ciamporová et al., 1998), and relatively low nitrogen (N) concentrations compared with forb roots (Tjoelker et al., 2005), although roots can contain significant starch reserves. Generally poor nutritional quality may sometimes lower the risk of herbivory and reduce the need for explicit defense. Plants generally are less tolerant of root herbivory than of shoot herbivory (Zvereva and Kozlov, 2012), although tolerance remains an important component of belowground defense (Rasmann et al., 2011) and herbaceous plants may be better able to compensate for root herbivory than woody plants (Massad, 2013). Chemical defense of roots is also common (van Dam, 2009), although relative concentrations of defense compounds found in above- and below-ground plant parts varies among plants (Rasmann and Agrawal, 2008).

Because so little is known of below-ground defense in grasses, it is worthwhile considering what is known of above-ground grass parts. Many grasses are extremely tolerant of herbivory, particularly when abundant resources are available for regrowth (McNaughton, 1979; Hamilton et al., 1998; Hawkes and Sullivan, 2001), mostly because their growth and regrowth occurs at basal intercalary meristems that are protected by hard leaf sheaths that allow regrowth after herbivory to occur almost immediately (Haukioja and Koricheva, 2000).

As a consequence of this architecture and the usual lack of abscission of grass leaves, grasses often show overcompensatory above-ground growth and overcompensatory photosynthesis after above-ground herbivory as the sward is reduced and more light reaches the meristems (Alward and Joern, 1993; Rosenthal and Kotanen, 1994). In contrast, simulated defoliation of trees often reduces growth (Heichel and Turner, 1984), although a recent metanalysis emphasized the diversity of responses within growth forms (Massad, 2013). Although grasses produce roots with apical meristems at the root tip, they differ from most eudicots in developing roots from multiple sites above and belowground (Sebastian et al., 2016) and this greater degree of modularization may also limit damage from root herbivory and facilitate compensatory growth.

The most obvious aboveground herbivore-resistance traits of grasses are physical, and include the deposition of silica phytoliths (Hartley and DeGabriel, 2016) and high proportions of cellulose and lignin, while chemical resistance traits are generally viewed as less significant (with notable exceptions, e.g., Vicari and Bazely, 1993). However, the apparent general lack of chemical defense in grasses may reflect a lack of investigation and a focus on a few economically important species (Kellogg, 2015). This knowledge gap is magnified still further when attention is turned to grass roots, as these are almost always ignored, even when aboveground defenses are investigated.

Investment in resistance traits can require resources that plants could otherwise direct toward growth and reproduction and thus, optimal defense theory (ODT) predicts that allocation of resources to these traits will be driven by the relative costs and benefits of this investment. Investment costs are influenced by the biosynthetic cost and composition of chemical defense, as well as the opportunity cost of forgone growth and reproduction, and the benefits of investment are determined by the vulnerability of plants and plant parts to herbivory and the value of these plant parts to the plant (Zangerl and Rutledge, 1996). The application of ODT to roots lags well-behind its application to above-ground plant parts, largely due to difficulties in determining these costs, values, and degrees of vulnerability (van Dam, 2009). If ODT is applied simply to the question of whether grasses should invest more in the root or shoot resistance to herbivores, however, the apparent reliance on tolerance over resistance for above-ground defense and the comparatively lower tolerance of roots combined with their value to the plant, suggests that chemical defense may be at least, if not more, important for roots.

If herbivore attack is rare or unpredictable, plants can often defer and potentially avoid defense costs by inducing defenses in response to herbivory (Karban and Baldwin, 1997), and this strategy is likely to be as common below-ground as above (Rasmann and Agrawal, 2008). This strategy is observed in all three types of defense discussed below.

GET TOUGH—PHYSICAL DEFENSES

Physical defense is a first line of defense against herbivores (Hanley et al., 2007), and in shrub and tree leaves can explain more variation in chewing herbivory than chemical defense (Caldwell et al., 2016). It can prevent or discourage attack by chewing and piercing herbivores, and make nutrients inaccessible or indigestible. Obvious physical defenses such as thorns and trichomes do not occur belowground, though root hairs are the developmental equivalent of leaf trichomes and the product of neofunctionalization arising from a gene duplication event (Kellogg, 2001). There has been some speculation that root hairs may offer some protection by preventing very small herbivores (e.g., neonates) from reaching and penetrating the root epidermis or may possibly provide refugia for natural enemies of herbivores such as entomopathogenic nematodes (Johnson et al., 2016a). Although not strictly a physical defense, root hairs may also increase the root surface available for colonization by beneficial soil microbes, which in turn can sometimes confer resistance to insect and nematode herbivores of grasses (Piskiewicz et al., 2009; Santos et al., 2014). Notably, root hairs can be induced, for example by plant-parasitic nematodes in barley (Haase et al., 2007).

More important belowground, both for defense against root herbivores and for protection against inadvertent uprooting by grazing ungulates, is the resistance of roots to shearing, puncturing, and tearing. This is a product of the architecture and physico-chemical composition of roots. Crystalline deposits of silica and calcium oxalate may play important roles (discussed below), but variation in the proportion of cortex and stele, and corresponding differences in cellulose, lignin, callose, and suberin composition all contribute to root toughness (Gregory, 2006), for example the strength of turfgrass rhizomes and stolons can be best explained by lignin concentrations (Lulli et al., 2011). To date, general patterns of structural chemical composition that confer greater root strength have not been sought or identified, and we suggest that this would be a worthwhile research aim.

The roots of annual and perennial grasses also differ in key attributes including specific root length, root tissue density, modal root diameter and root nitrogen concentration (Roumet et al., 2006), traits associated with nutrient and water acquisitiveness, root lifespan, and relative growth rate (Perez-Harguindeguy et al., 2013). Differences in specific root length can result from either low tissue density or low diameter (Perez-Harguindeguy et al., 2013), with thin roots exerting less penetrative force on soil and transporting less water, and denser roots showing longer longevity. In leaves, toughness per density makes a greater contribution to mechanical strength than lamina thickness and tissue density combined (Onoda et al., 2011), and the same may be true for root diameter and density. In both grasses and trees, the tensile strength of roots decreases with root diameter (i.e., thinner roots are stronger) and this can be explained partly by a scaling effect commonly seen in fracture mechanics and partly by the higher cellulose concentrations observed in fine roots (Genet et al., 2005; Teerawattanasuk et al., 2014).

To our knowledge, only one study has directly investigated the effects of root toughness on root herbivory (Johnson et al., 2010). It found a positive correlation between fracture toughness and root penetration time by *Agriotes* spp. wireworms (Coleoptera: Elateridae), mediated by lignin concentration and composition,

suggesting that root toughness could be an effective barrier to root herbivory.

Many, if not most, grasses form rhizosheaths along much of their root length (Goodchild and Myers, 1987; Kellogg, 2015). This casing comprises mineral earth, root hairs and living cap cells, held together by mucilage and is especially well-developed in mesophytic and xerophytic grasses (McCully, 1995, 2005). Particularly when allowed to dry, the rhizosheath forms an integral part of the root, to which it adheres firmly and shows a degree of strength when excavated (Watt et al., 1994). Furthermore, the distribution of soil particle sizes in rhizosheaths is shifted significantly toward smaller particles, relative to the surrounding soil (Ma et al., 2011). As the movement of both nematode and insect herbivores is substantially retarded by increasing soil density (Johnson et al., 2004; Barnett and Johnson, 2013), it may be possible that rhizosheaths afford some degree of protection from root herbivores.

Silica

In grasses, a major component of physical resistance to aboveground herbivory is via deposition of silica (SiO_2), a defense that, unusually, may be used more extensively by grasses than by other plants (Hodson et al., 2005). Silica has been linked to drought resistance, structural strength, disease resistance and defense against a range of insect herbivores, the latter via reductions in digestibility and mouthpart wear (Hartley and DeGabriel, 2016). Silica is taken up by roots in the form of monosilicic acid, before being transported to the site of concentration and deposition. There it polymerises as opaline silica, either as a varnish or as morphologically-diverse phytoliths. In many grass species, silica deposition in grass leaves and stems is induced by above-ground herbivory, particularly by vertebrates (Hartley and DeGabriel, 2016), and the same above-ground response was seen in two grasses after root herbivory by scarab beetle larvae (Power et al., 2016), although root silica was not measured in that study.

Silica was first reported from sorghum roots in 1924 (Parry and Kelso, 1975) and its distribution in roots has subsequently been described for several species (**Table 1**). Total concentrations of silica in wild grass roots can sometimes substantially exceed those observed aboveground (McNaughton et al., 1985; Seastedt et al., 1989) but this varies among species; for example the roots of *Phragmites* possess negligible silica, despite its abundance above ground (Schaller et al., 2013) whilst the thick, long-lived cord roots of *Molinia* (also from the tribe Molinieae in the subfamily Arundinoideae) deposits extracellular silica in all root tissues including epidermal, schlerenchyma, and xylem vessels, forming an almost complete cylinder (Parry and Kelso, 1975). Although the anatomical distribution of silica in roots has only been described in detail for a few grasses (**Figure 1**), mostly crops, the most common pattern among those species is of deposition on the inner transverse cell walls (and sometimes more extensively) of the endoderm (Parry et al., 1984). This pattern does not seem to be ideal for defense of the root cortex, even though most root nutrients, particularly stored carbohydrates, are to be found there. More in line with the predictions of the ODH, several studies have reported greater silica concentrations in basal proximal) than in apical (distal) roots (Parry and Kelso, 1975; Hodson and Sangster, 1989).

Regardless of where it is localized in roots, silica may contribute to the overall toughness of roots and might play a significant defense role in grasses where it is deposited in the root epidermis and throughout the root. Direct evidence that silica is involved in defense against grass root herbivores comes from a study by Frew et al. (2016b), which found that the relative consumption by and subsequent mass gain of root-feeding grayback canegrub (*D. albohirtum*) feeding on sugarcane roots was negatively correlated with silicon concentrations. It has also been suggested that silica in roots may also play an important role in resisting penetration by the huastoria of parasitic plants such as *Striga* (Hodson and Sangster, 1989).

Calcium Oxalate

Calcium oxalate (CaC_2O_4) is another mineral deposit that can serve as an inducible (Molano-Flores, 2001) anti-herbivore defense in many plant tissues, in addition to playing roles in structural support, Ca^{2+} regulation, protection against heavy metal toxicity, and drought tolerance (Franceschi and Nakata, 2005; Polley et al., 2013). In common with silica, calcium oxalate deposits are morphologically diverse, and include raphides, druses, and sands (Franceschi and Nakata, 2005). The deposits are hard and can abrade insect mouthparts (Korth et al., 2006) and reduce the digestibility of food by lepidopteran insects via a physical action (Park et al., 2009).

Crystalline calcium oxalate is reported from the shoots of many forage grasses from the large tribe Paniceae as well as from rice and bamboo, but may be absent from grasses more generally (**Table 1**, Libert and Franceschi, 1987; Cheeke, 1995; Prychid and Rudall, 1999; Rahman and Kawamura, 2011). Little published work has quantified calcium oxalate in grass roots, but Rahman et al. (2010) reported greater concentrations of soluble oxalate (discussed below) and similar concentrations of insoluble oxalate (calcium oxalate) in the shoots compared to the roots of *Pennisetum purpureum.* Raphides are concentrated in the root apical meristem in some other plants, apparently offering a defense against herbivores (e.g., Osuji, 2013). Hodson and Sangster (1989) observed concentrations of Ca in the outer cortical and epidermal cell walls of the subapical zone of *Sorghum* roots, and these might also be associated with calcium oxalate deposits. Some mycorrhizal fungal symbionts are also capable of synthesizing oxalic acid, which forms calcium oxalate in the presence of Ca^{2+} (Malajczuk and Cromack, 1982), and this might contribute to grass root defense due to its intimate association with the root.

GET TOXIC—CHEMICAL DEFENSE

Grasses are less defended by toxic plant secondary metabolites (PSMs) or digestibility-reducing PSMs such as tannins than woody plants, but a variety of defenses have been cataloged from across the Poaceae, at least in the shoots (**Table 1**, Vicari and Bazely, 1993; Cheeke, 1995). Some defenses in grass roots are

TABLE 1 | Reported defenses in grass shoots and roots.

	Subfamily Tribe	Genera	Tannins	SiO_2	CaC_2O_4 $H_2C_2O_4$	NO_3^-	BXs	Volatile terpenes	Saponins	Plant alkaloids	Endophyte alkaloids	Cyanogenic glucosides
BOP CLADE	**Anomochloideae**											
	Pharoideae											
	Puelioideae											
	Ehrhartoidea											
	Erharteae	*Microlaena*	S_1									
	Oryzeae	*Oryza*		SS_4, RRR_5	SSS_{12}		~~S~~ $_{21}$				S_{16}	
	Bambusoideae											
	Bambuseae	*Sasa*	~~S~~ $_1$	R_5							S_{16}	
		Chusquea					S_{22}					
	Pooideae											
	Stipeae	*Stipa, Oryzopsis*	~~S~~ $_1$	S_4							$S_{16, 31}$	
	Brachypodieae	*Brachypodium*		SS_4								
	Poeae	*Avena*		S_4, RRR_5		SSS_{13}			S_{13}		**S – common in tribe$_{16}$**	
		Phalaris		SS_4			~~S~~ $_{22}$			$S_{13, 31}$		
		Briza		S_4								
		Agrostis		SSS_4							S_{17}	
		Poa		S_4			~~S~~ $_{22}$				S_{17}	
		Festuca		SS_4						S_{13}	SSS, RRR_{19}	
		Lolium	S_2	SS_4		SSS_{13}	~~S~~ $_{22}$		S_{13}	S_{29}	SSS_{18}	
		Dactylis		S_4			~~S~~ $_{22}$					
		Deschampsia		S_4								
		Holcus	S_{32}									
		Puccinellia	S_{33}									
	Meliceae	*Glyceria*									S_{16}	SSS_{13}
	Bromeae	*Bromus*		S_4			~~S~~ $_{22}$					
	Triticeae	*Elymus*					S_{22}					
		Hordeum		S_4			S_{23}			S_{13}	**S – common in tribe$_{16}$**	
		Secale		S_4			S_{24}, R_{25}					
		Triticum, Aegilops, Agropyron		SS_4, RR_5			R_{26}					
PACMAD CLADE	**Aristidoideae**											
	Panicoideae											
	Andropogoneae	*Sorghum*	**S – common tribe$_{1,3}$**	R_6		SSS_{13}	~~S~~ $_{22}$			S_{13}	S_{16}	SSS_{13}

(Continued)

TABLE 1 | Continued

	Subfamily Tribe	Genera	Tannins	SiO_2	CaC_2O_4 $H_2C_2O_4$	NO_3^-	BXs	Volatile terpenes	Saponins	Plant alkaloids	Endophyte alkaloids	Cyanogenic glucosides
PACMAD	Andropogoneae	*Zea*	**S – common in tribe$_{1,3}$**	S_4		SSS_{13}	R_{27}					SSS_{13}
CLADE	Continued	*Saccharum*		RRR_7			S_{28}					
		Cymbopogon		S_4				S_{30}				
		Chrysopogon						S_{30}				
		Bothriochloa						S_{30}				
		Andropogon		RRR_8								
	Paniceae	*Megathyrsus*	**S – v.rare in tribe$_1$**		S_{13}						S_{16}	
		Urochloa				SSS_{13}						
		Brachiaria			SS_{13}				$S_{13,31}$			
		Pennisetum		S_4	$SSS_{13,14,15,31}$, RRR_{14}	SSS_{13}					S_{20}	
		Panicum		SS_4, $RRR_{8,9}$	SS_{14}				$S_{13,31}$	S_{13}		
		Setaria		S_4	$SSS_{12,14,31}$							
		Cenchrum			SS_{12}							
	Chloridoideae		**S – v.rare in subfam$_1$**	RR_9							S_{16}	
	Eragrostideae	*Eleusine*										
	Cynodonteae	*Dactyloctenium*				SSS_{13}						
		Brachyachne/ Cynodon		S_4			~~S~~ $_{22}$		S_{31}			$SSS_{13,31}$
		Bouteloua		S_4								
		Eustachys/ Chloris		RRR_8								
	Danthonioideae										S_{16}	
	Arundinoideae		**S – v.rare in subfam$_1$**									
	Molineae	*Molinia*		RRR_{10}								
		Phragmites		~~R~~ $_{11}$								
		Arundo		S_4			S_{22}					
		Arundinaria		SSS_4								
	Micrairoideae											

Table indicates the reported presence of tannins, silica (SiO_2), oxalic acid, or calcium oxalate ($H_2C_2O_4$, CaC_2O_4), nitrate (NO_3-), benzoxazinoids (BXs), stored volatile terpenes such as monoterpenes, saponins, alkaloids of plant origin, alkaloids of endophytic fungal origin and cyanogenic glucosides. ~~S~~ , reported absence in shoots; S, reported presence in shoots (SS and SSS indicate more substantial concentrations); ~~R~~ , reported absence in roots; R, reported presence in roots (RR and RRR indicate more substantial concentrations).

References: 1, Ellis, 1990; 2, Jackson et al., 1996; 3, Awika and Rooney, 2004; 4, Hodson et al., 2005; 5, Sangster, 1978; 6, Hodson and Sangster, 1989; 7, Parry and Kelso, 1977; 8,McNaughton et al., 1985; 9,Geis, 1978; 10, Parry and Kelso, 1975; 11, Schaller et al., 2013; 12, Rahman and Kawamura, 2011; 13, McKenzie, 2012; 14, Rahman et al., 2010; 15, Marais et al., 1997; 16, Clay, 1990; 17, Crawford et al., 2010; 18, Hennessy et al., 2016; 19, Patchett et al., 2008; 20, Ryley et al., 2007; 21, Kato-Noguchi and Peters, 2013; 22, Zuniga et al., 1983; 23, Barria et al., 1992; 24, Rice et al., 2005; 25, Copaja et al., 2006; 26, Lu et al., 2012; 27, Robert et al., 2012; 28, Singh et al., 2003; 29, Koulman et al., 2008; 30, Kaul and Vats, 1998; 31, Cheeke, 1995; 32, Iason et al., 1995; 33, Volz and Clausen, 2001.

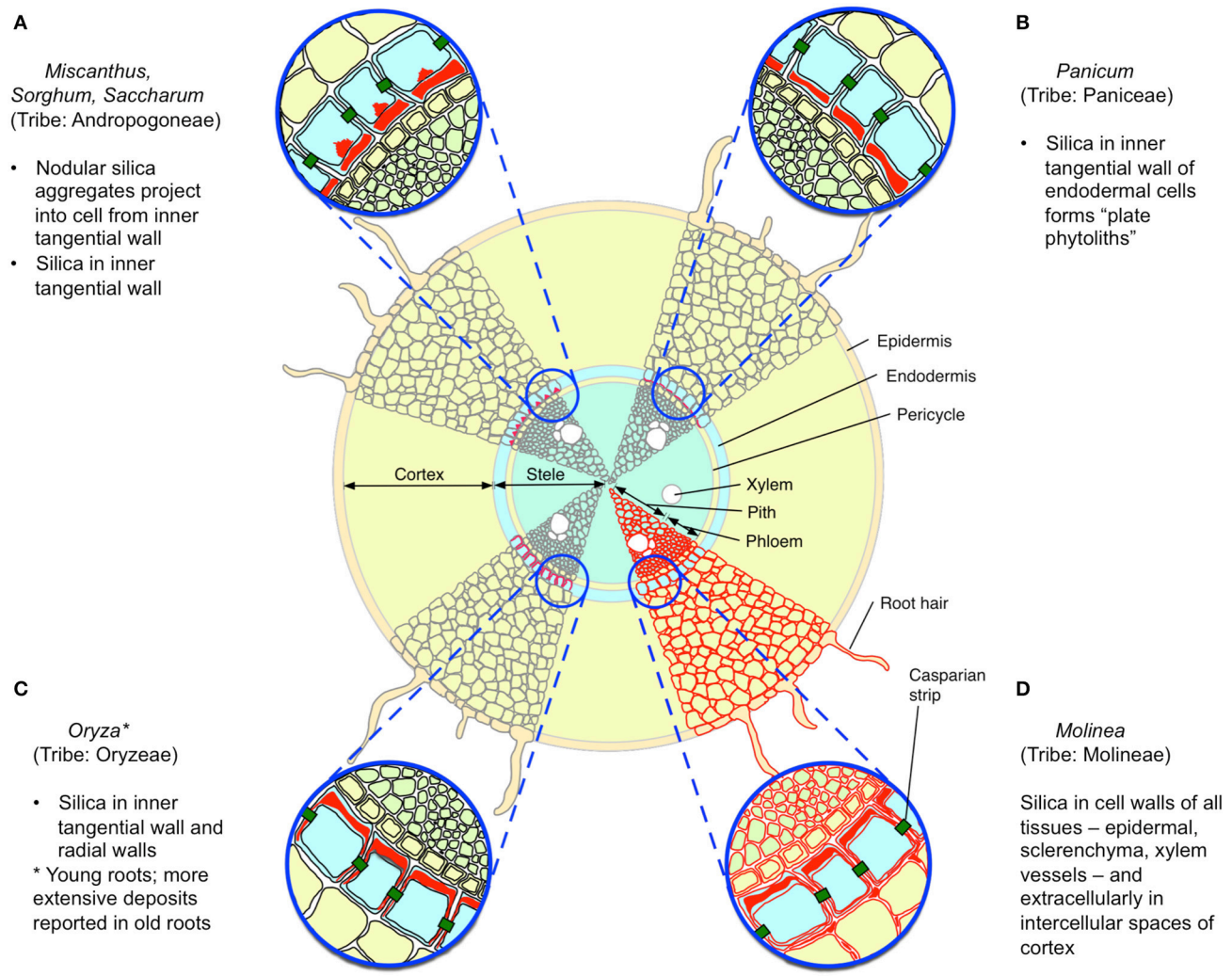


FIGURE 1 | Reported patterns of silica deposition in grass roots. Four patterns are illustrated, with silica indicated in red, based upon descriptions from Sangster (1978) and Parry et al. (1984). **(A–C)** Silica is deposited in the endodermis, particularly in the inner tangential wall; in **(A)**, nodular aggregates are also observed inside the cell. **(D)** Silica is found in the cell walls of all root tissues, and extracellularly in the root cortex. Other reported patterns include deposition in all endodermal cell walls (e.g., some wheat and barley cultivars) and no apparent deposition (e.g., *Phragmites australis*).

likely to be induced by herbivory, and although the oxylipin plant hormone, jasmonic acid (JA), is intimately involved in the initiation of induced defense responses, surprisingly little is known about the mechanisms of its action in grasses (Shyu and Brutnell, 2015). The limited evidence available suggests that the JA burst in response to attack, and the degree of localized induction, tend to be milder in plant roots than in shoots (Erb et al., 2012), although Erb et al. (2012) intriguingly postulate the existence of additional, unknown signals that may induce root defenses in the absence of a JA burst.

The toxicity of any PSM and its role in herbivore resistance is not absolute. Toxicity can be herbivore-specific and herbivore resistance is influenced by the intrinsic vulnerability and nutritional value of a plant tissue to herbivores, both negatively via compensatory feeding and positively via decreased palatability in different situations (Behmer, 2009; Erb et al., 2013; Johnson et al., 2014), as well as by the type and amount of PSMs and the interaction of nutrient and PSM levels (Behmer, 2009; Couture et al., 2016). Thus, the high fiber and low nutrient concentrations typical of most (but not all e.g., Tilman and Wedin, 1991) roots relative to leaves, may mean that herbivores can be deterred by a lower relative investment in chemical defense. Furthermore, the intrinsic chemical properties of some defense compounds makes them more effective in a below-ground environment than above-ground (van Dam et al., 2009), making direct comparisons between above- and below-ground levels of defense complicated. As is the case for above-ground herbivores (Bernays and Chapman, 1994), specialist root herbivores can also overcome chemical defenses and in some

cases even use them as feeding cues (Johnson et al., 2011; Robert et al., 2012), however this does not preclude a role for these compounds in resistance against generalists.

Benzoxazinoids

One well-studied chemical class almost entirely restricted to grasses (but occasionally reported from individual species from several dicotyledenous families (Adhikari et al., 2015) is the benzoxazinoids (BX), which includes the benzoxazolinone [e.g., benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA)], lactam [e.g., 2-hydroxy-1,4-benzoxazin-3-one (HBOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA)], and hydroxamic acid [e.g., 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)] subclasses. These are widely reported from grass roots and root exudates, and appear to occur naturally both as glycosides and aglycones (Niemeyer, 2009), with the former stored in vacuoles in the roots (Copaja et al., 2006). Benzoxazinoids have been best studied for their roles in allelopathy and defense in the cereal crops maize, rye, and wheat, but also occur in many wild grasses (Zuniga et al., 1983). In root tissue, biosynthesis of BXs can be induced by competition (Rice et al., 2005; Lu et al., 2012) and by jasmonic acid or herbivory by *D. virgifera* (Robert et al., 2012). Allocation to roots relative to shoots can also increase in response to defoliation stress. Robert et al. (2012) show that allocation of DIMBOA to belowground parts of maize matches the predictions of optimal defense theory, with the greatest concentrations in the most nutritious crown roots. DIMBOA is deterrent to generalist root herbivores, and although several studies have reported positive correlations between BX concentrations and resistance to the specialist root herbivore *D. virgifera*, Robert et al. (2012) showed most recently that it is unaffected by DIMBOA and even uses high concentrations as a cue to locate its preferred (and most nutritious) crown roots. Nematodes appear to be relatively unaffected by BXs, with root knot nematodes *Meloidogyne incognita* in rye suppressed only at extremely high concentrations (Meyer et al., 2009) and reproduction of the stubby-root nematode *Paratrichodorus minor* unrelated to BX concentrations in maize. BXs are strongly involved in the resistance of some grasses to aboveground-feeding aphids, but this has not been demonstrated for root-feeding aphids (Niemeyer, 2009).

Nitrate

Although a primary, rather than a secondary metabolite, excess nitrate in plant tissues presents a well-known risk of toxicity to grazing mammalian herbivores (Cheeke, 1998). Leaves of some grass genera, including *Sorghum*, *Avena, Lolium*, *Zea*, *Dactyloctenium*, and *Urochloa* can accumulate toxic nitrate levels in nitrogen-rich soils and after rain following dry periods, and this can be directly caustic to the gut lining (McKenzie, 2012). This is particularly damaging for monogastric vertebrates but might affect invertebrates as well. In vertebrates, nitrate toxicity is associated with the conversion of hemoglobin to methaemoglobin which cannot carry oxygen, and nitrate similarly reduces the affinity of haemocyanins for oxygen (Hazes et al., 1996; Cheng and Chen, 2002). The importance of these oxygen-binding proteins in insects is poorly understood (Hankeln et al., 2002), but may be underestimated, particularly for belowground herbivores. Nitrate is also a potent inhibitor of the midgut potassium pump in tobacco hornworm (*Manduca sexta*; Schirmanns and Zeiske, 1994) and its associated ATPase (Wieczorek et al., 1986). Although Hatcher et al. (1997b) showed that high nitrate levels in leaves of *Rumex obtusifolia* (Polygonaceae) were deterrent to chrysomelid beetles and Soucek and Dickinson (2012) demonstrated the toxicity of nitrate to aquatic insects, nitrate is not commonly considered as a plant defense. For now, data about root nitrate concentrations are scarce (Roumet et al., 2006), although substantial concentrations (>6% DM) are known to accumulate in the roots of wild-type *Arabidopsis* (Segonzac et al., 2007).

Oxalic Acid

Another primary metabolite involved in grass defense against herbivores is oxalic acid. The physical defense role of crystalline calcium oxalate has been discussed earlier, however much of the oxalate in grasses and particularly in roots may be in soluble form. Oxalate can inhibit feeding by homopteran insects (Yoshihara et al., 1980), and reduce larval growth rates in cotton bollworm (Yoshida et al., 1995). When free oxalic acid is consumed by herbivores, it can also form the insoluble salt, calcium oxalate, *in vivo*—potentially leading to nephrolithiasis (kidney stones) in both vertebrates and invertebrates (Hirata et al., 2012). This same process can reduce the bioavailablity of Ca^{2+}, and oxalate thus acts as an antinutrient, potentially leading to hypocalcemia. In vertebrates, especially horses, this is a leading cause of "big head" syndrome (Cheeke, 1998) however any consequences for invertebrates are unknown.

High oxalate concentrations occur, usually along with calcium oxalate, in some tropical grasses and particularly in their roots (see above; also Rahman et al., 2010). Experimental evidence shows that oxalate (and calcium oxalate) synthesis increases in plants including spinach, rice and Napier grass (*Pennesetum purpureum*) with high availability of nitrate, but not of ammonium (Hatcher et al., 1997a; Rahman et al., 2010).

Terpenoids

Only a small number of tropical aromatic grasses including *Cymbopogon, Bothriochloa, Vetiveria* and *Chrysopogon* (Kaul and Vats, 1998) possess specialized storage cells and accumulate significant concentrations of mono- and sesquiter penes in their leaves and stems (Lewinsohn et al., 1998). Evidence is currently lacking either for or against volatile terpene accumulation in grass roots. Other terpenoid products synthesized by grass roots but apparently not investigated as herbivore defenses include iridoid glycosides from maize (*Z. mays*) (Rengasamy et al., 2015) and diterpene momilactones from rice (*Oryza sativa*) (Kato-Noguchi and Peters, 2013).

Some grasses also produce steroidal saponins and sapogenins (saponin aglycones) that are derived from terpene precursors. Many of these are strongly molluscicidal (reviewed by Francis et al., 2002). Some triterpene saponins act against insects via their action as phytoecdysteroids, meaning that they mimic

insect molting hormones. However, Dinan (1995) reported phytoecdysteroid activity in only five grass species (from the genera *Avena*, *Briza*, and *Festuca*) out of 45 tested, and then only from the seeds (the only part he tested). Although the genomes of these plants thus possess the capacity for phytoecdysteroid biosynthesis, this is not evidence of their presence in roots. Previously, similar activity had been reported from root extracts from the grass *Coix lachrymal-jobi* (Matsuoka et al., 1969 cited by Dinan, 1995), although more recent reports suggest that ecdysteroid activity of saponins may be attributable to increased membrane permeation rather than to direct effects on ecdysteroid receptors (De Geyter et al., 2012). While not universal, insect deterrence and toxicity have been observed for steroid and triterpene saponins from a variety of dicots, both above- and below-ground (Sutherland et al., 1982; De Geyter et al., 2010) although only antimicrobial actions have been reported for avenacins, triterpene saponins that accumulate in the roots of oats (Mylona et al., 2008).

Steroidal saponins, either alone or in synergy with other hepatotoxins, are associated with secondary (hepatogeneous) photosensitization in livestock feeding on many warm-climate grasses, including *Panicum* and *Cynodon* (Cheeke, 1995). These damage the liver, which is no longer able to remove the chlorophyll metabolite, phylloerythrin, to the bile for excretion. Sunlight then interacts with accumulated phylloerythrin, causing skin lesions, dermatitis, and photophobia. Invertebrate root herbivores are not exposed to sunlight or dietary chlorophyll, but may experience equivalent damage to systems for the elimination of toxic metabolites, such as ATP-binding cassette transporters (Robey et al., 2006).

Alkaloids

Alkaloids are basic PSMs that contain nitrogen and are widespread plant defenses, both above- and below-ground. Endogenous alkaloids produced by grasses include hordenine, a phenylethylamine alkaloid from barley, sorghum, millet, and *Phalaris aquatica*. Hordenine is deterrent to *Heliothis* caterpillars (Bernays et al., 2000), grasshoppers (Harley and Thorsteinson, 1967), and ruminants (Marten et al., 1976). Indole alkaloids including gramine and perloline occur in barley, *P. aquatic*, and *Festuca arundinacea* (McKenzie, 2012). Gramine is toxic to aphids (Corcuera, 1984) and causes staggers and death in livestock (Binder et al., 2010). Perloline and hordenine are both most concentrated in roots, particularly soon after germination (Mann and Mudd, 1963; Gentry et al., 1969). Pyrrolizidine alkaloids were reported from a grass (*Lolium perenne*) for the first time relatively recently (Koulman et al., 2008).

Alkaloids of endophytic fungal origin present in grasses have been widely reviewed because of their detrimental effects on livestock (Clay, 1990; Saikkonen et al., 2013; Schardl et al., 2013), although some specifically affect insects, including root herbivores (Popay et al., 2004; Hennessy et al., 2016). They include lolines, peramine, ergot alkaloids, and indole diterpenes including epoxy-janthitrems produced by endophytes from the genera *Epichloë* and *Neotyphodium*, most prominently in the grass genera *Festuca* and *Lolium*, but also in native North American grasses (Crawford et al., 2010) and numerous cool-climate southern hemisphere grasses (Moon et al., 2002, 2007). However, the conferral of herbivore resistance by endophytes of native grasses is generally weaker and less consistent than in agronomic grasses (Faeth and Fagan, 2002). Although endophytes and their alkaloids are usually not detected when grass roots are analyzed (Clay, 1990; Elmi et al., 2000) endophyte infection appears to be highly detrimental to root knot nematodes of *Festuca* (Elmi et al., 2000) and to deter the root herbivore *Costelytra zealandica* (Rostás et al., 2015), although deterrence in the latter case might also be explained by altered volatile emissions. Evidence that endophyte infection is detrimental to sap-feeding aphids (Wilkinson et al., 2000; Popay et al., 2004) is consistent with observations that lolines can be transported in phloem to grass roots (Burhan, 1984; Patchett et al., 2008; Omacini et al., 2012).

Other grass endophytes may also produce steroidal toxins such as wortmannin, which may be responsible for kikuyu staggers in cattle (Ryley et al., 2007), however, their relevance to root defense and to invertebrate herbivores is unknown.

Amino Acids

Non-proteinogenic amino acids can be toxic and sometimes afford effective defense. *M*-tyrosine can reach concentrations of up to 43% of root exudate dry matter in some *Festuca* species, is allelopathic (Bertin et al., 2007) and can reduce cabbage looper (*Trichoplusia ni*) growth rates when expressed in *Arabidopsis thaliana* (Huang, 2010). Another tyrosine isomer, β-tyrosine, is inducible, and abundant in the roots and root exudates of some rice cultivars, but despite also being strongly allelopathic has no detectable effects on hemipteran or lepidopteran herbivores in bioassays (Yan et al., 2015).

Cyanogenic Glucosides

In common with most plant families, some grasses e.g., *Brachyachne*, *Cynodon*, *Glyceria*, *Zea*, and *Sorghum*, (particularly Johnson grass, *S. halapense* and Sudan grass, *S.* × *drummondii*) produce cyanogenic glucosides including limarin and dhurrin, and these too have been well-studied in some species because they impact livestock (Cheeke, 1998; McKenzie, 2012). Cyanogenic glucosides are deterrent to most generalist insects (Gleadow and Woodrow, 2002) but can be tolerated or avoided by some specialists (Engler et al., 2000). Cyanogenic glucosides are present in roots of *Cynodon dactylon* where an allelopathic role has been proposed (Mahmoodzadeh, 2010) and provide nematocidal benefits in the root epidermis of several *Sorghum* species (Curto et al., 2012). Cyanogenic glucoside concentrations are generally highest in young plants and plants exposed to drought or N-rich soils (Gleadow et al., 2016).

Tannins and Other Phenolics

Tannins are large polyphenolic compounds found widely in shoots and roots generally and best-known for their ability to form insoluble (and thus indigestible to vertebrates) complexes with dietary protein. It should be noted that for most insects, the potential pro-oxidative activity of tannins is more biologically important than protein-precipitating effects, which

do not occur in typically-alkaline insect guts (Appel, 1993; Salminen and Karonen, 2011). Tannins are very rare and/or in extremely low concentrations in grasses, although some grasses, including sorghum, barley, rice, wheat, red finger millet (*Eleusine coracana*), *Festuca arundinacea* and *Lolium perenne*, produce condensed tannins in their caryopsis (grain) (McCallum and Walker, 1990; Gu et al., 2003; Awika and Rooney, 2004; Dykes and Rooney, 2007; Fraser et al., 2016). To our knowledge, grass roots have not been investigated for tannins.

Based on a histological survey, Ellis (1990) and Chesselet et al. (1992) reported "tannin-like substances" from the leaf epidermis of 39 genera (of 1,104 species in 290 genera inspected) of South African grasses, mostly in C4 species growing in poor soils, suggesting that tannins may occur more widely among grasses than is commonly recognized. The presence in leaves of ellagitannins in the arctic grass *Puccinellia artica* (Volz and Clausen, 2001) and condensed tannins in *Holcus lanatus* (Iason et al., 1995) and in *Lolium perenne* and *Digiteria sanguinalis* (Jackson et al., 1996) has also been reported on the basis of colorimetric tests, however more recent LC-MS analysis failed to detect tannins in *L. perenne* and *F. arundinace* leaves (Fraser et al., 2016). In all cases, tannin concentrations in grass vegetative tissue are low and seem unlikely to afford substantial herbivore resistance. Only weak correlative links have been presented between grass tannins and herbivore feeding preferences (Capinera et al., 1983; Volz and Clausen, 2001), while Mole and Joern (1994) concluded that condensed tannins were ineffective against grasshoppers. Simple monomeric phenolics have been little investigated in grass roots, although chlorogenic acid has been implicated in the resistance of maize roots to herbivory (Nuessly et al., 2007; Robert et al., 2012).

Phenolic compounds including tannins, phenolic acids, flavonoids, and anthocyanins are almost ubiquitous in plants, but whether they play any role in species-species interactions is unpredictable and highly structure-dependent (Lane et al., 1985; Barbehenn and Constabel, 2011; Moore et al., 2014). The absolute quantification of "total phenolics" using standard assays is highly problematic (Appel et al., 2001) and without detailed compound identification, useful conclusions about biological activity are not possible. For example, Parker et al. (2012) detected similar concentrations of "total phenolics" in roots and shoots of *Oenothera biennis*, but the allocation of particular phenolics, with differing biological activities, to roots vs. shoots differed.

In recent years, the capacity to identify and quantify individual phenolics, phenolic glycosides and polyphenolics including ellagitannins, has improved dramatically (Salminen et al., 2011) and lead to valuable insights into the role of phenolics in plant defense (e.g., Agrawal et al., 2012). Another useful approach is to implement assays that estimate putative chemical mechanisms of these compounds, such as oxidative activity measured at a pH comparable to the midgut lumen pH of insects (Barbehenn et al., 2006; Salminen and Karonen, 2011). These detailed chemical and mechanistic approaches offer the most promising way forward but have yet to be applied to grass roots.

Proteinase Inhibitors

Proteinase inhibitors (PI) are important defenses against herbivory in many plants, including Solanaceous and Leguminous crops, where they harm herbivores by inhibiting the action of digestive proteinases such as trypsin and chymotrypsin (Farmer, 2014). Most published examples concern aboveground herbivory and relatively few reports exist from grasses, although the presence and induction by JA of subtilisin/chymotrypsin-inhibiting-type PIs have been reported from the leaves of *Brachypodium distachyon* and from wheat endosperm (Mur et al., 2004; Tedeschi et al., 2012). A cysteine protease from maize, which is expressed in all tissues, has been shown to serve in insect resistance by damaging to the peritrophic matrix of lepidopteran larvae (Pechan et al., 2002), and the gene encoding it is also highly expressed in sugarcane roots (Falco et al., 2001). Root herbivory by the southern corn rootworm and western corn rootworm and by experimental application of JA induces the transcription of proteinase inhibitor genes in maize roots (Lawrence et al., 2012; Robert et al., 2012).

GET A BODYGUARD—RECRUITMENT OF NATURAL ENEMIES

All grasses tested to date have been shown to emit volatiles following aboveground herbivory (Degenhardt, 2009) and these often recruit the natural enemies of herbivores, but the situation belowground is relatively less explored. Maize releases a sesquiterpene, (E)-β-caryophyllene, from its roots following attack by the western corn rootworm, attracting entomopathogenic nematodes (EPNs, Rasmann et al., 2005). EPNs infect the bodies of root-feeding insects and cause septicaemia by releasing bacteria while reproducing in the dying insect. Similarly, northern white cedar (*Thuja occidentalis*), and *Citrus* release chemical signals (a C_{12} terpene in the citrus) that attract entomopathogenic nematodes when their roots are attacked by weevils (*Otiorhynchus sulcatus* and *Diaprepes abbreviatus*, van Tol et al., 2001; Ali et al., 2010) and roots of *Panicum bisulcatum* treated with JA (inducing a defensive response) attracted EPNs in soil olfactometers, suggesting this grass species was emitting VOCs that recruited these natural enemies of root herbivores (Hiltpold et al., 2016). The induced emission of volatile terpenes by the grasses *F. arundinacea* and *Poa pratensis* following root herbivory by beetles attracts *Tiphia* parasitoid wasps from aboveground, that subsequently burrow into the soil to attack the grubs (Obeysekara et al., 2014). There is therefore good reason to believe these mechanisms are just as common belowground as aboveground (Turlings et al., 2012).

Microbes and mycorrhizal fungi that interact with grass roots might also play roles as bodyguards, or otherwise alter plant-herbivore interactions. The plant parasitic nematode *Tylenchorhynchus ventralis* is strongly controlled by soil microbes in a coastal foredune grassland (Piskiewicz et al., 2009) and colonization of maize by the rhizobacterium *Azospirillum brasilense* can deter, and reduce the performance of, western corn rootworm (Santos et al., 2014). However, another study of three plant species, including the grass *Holcus lanatus*,

found no effect of the soil microbial community on defense against nematodes (Wurst et al., 2009). Alkaloid-producing endophytes were discussed above, and can also be considered to be bodyguards. However, endophyte infection has also been shown to alter volatile emissions from grass roots (Rostás et al., 2015), and may interfere with the recruitment of natural enemies.

CONCLUSIONS

Most of the grass defenses described above are known from limited sections of the Poaceae, and primarily from crop and pasture species. While economically and often ecologically important, these grass species are not representative of the phylogenetic, morphological, and ecological diversity present in this large and cosmopolitan plant family. Most of these species have been subject to artificial selection through the process of domestication, and this process can alter plant-herbivore interactions and often cause the diminution or loss of plant defenses (Rosenthal and Dirzo, 1997; Kollner et al., 2008; Turcotte et al., 2014; Chen et al., 2015). Furthermore, many crop species experience very little above-ground herbivory by mammalian grazers and may consequently differ from wild grasses that have evolved alongside ungulate herbivores and which may differ in their relative reliance on the resistance and tolerance elements of defense. It has been suggested that grasslands supporting populations of large grazing vertebrates such as ungulates and macropods are more tolerant of grazing than ungrazed grasslands (Rosenthal and Kotanen, 1994). However, tolerance traits such as protected meristems, compensatory growth and compensatory photosynthesis may sometimes be adaptations to fire and drought, rather than, or as well as, adaptations to herbivory (Rosenthal and Kotanen, 1994).

These observations highlight the need for systematic surveys of defense throughout the family. As well as shining a light on phylogenetic patterns of defense, this approach may enable the identification of defense syndromes and/or defense tradeoffs where they exist. Plant defense theories offer many predictions about differential patterns of defense and herbivory between C_3 and C_4 plants; between domesticated and wild plants; throughout ecological succession (Rasmann et al., 2011) and along environmental gradients of temperature, precipitation, soil and fire frequency; yet these predictions remain largely untested in grasses, let alone below-ground.

Given the size and diversity of the Poaceae, there are likely many undiscovered and unexplored chemical defenses and defense strategies to be found belowground. In particular, there may be much to learn from studies of allelopathic secondary metabolites produced by plant roots. Numerous examples exist of phytotoxic compounds synthesized and exuded by grass roots, which have not been tested for roles against root herbivores, or at least, such tests have not been reported. Examples include diterpene momilactones produced by the roots of rice, which are induced by jasmonic acid (Kato-Noguchi and Peters, 2013), antimicrobial triterpene saponins known as avenacins which accumulate in oat roots (Mylona et al., 2008), and sorgoleone, a hydrophobic ρ-benzoquinone exuded from Sorghum root hairs (Weston et al., 2013). In surveying the relevant literature, we noted an apparent lack of consideration given to the possibility that grass rhizosheaths may play a role in defense against herbivores. We also identified a need to characterize the contribution of root composition (in terms of cellulose, lignin, callose, suberin, silica, and calcium oxalate) to root toughness, and the significance of root toughness for defense against herbivores. More broadly, we hope that in surveying the relevant literature, we have equipped researchers with candidate grass root defenses for further hypothesis-driven research.

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BM and SJ conceived the review article. BM reviewed the literature and wrote the paper with significant input from SJ.

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Invertebrate Biosecurity Challenges in High-Productivity Grassland: The New Zealand Example

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To protect productive grasslands from pests and diseases, effective pre- and at-border planning and interventions are necessary. Biosecurity failure inevitably requires expensive and difficult eradication, or long-term and often quite ineffective management strategies. This is compared to the early intervention more likely for sectors where there is public and political interest in plants of immediate economic and/or social value, and where associated pests are typically located above-ground on host plantings of relatively limited distribution. Here, biosecurity surveillance and responses can be readily designed. In contrast, pastures comprising plants of low inherent unit value create little, if any, esthetic interest. Yet, given the vast extent of pasture in New Zealand and the value of the associated industries, these plants are of immense economic importance. Compounding this is the invasibility of New Zealand's pastoral ecosystems through a lack of biotic resistance to incursion and invasion. Further, given the sheer area of pasture, intervention options are limited because of costs per unit area and the potential for pollution if pesticides are used. Biosecurity risk for pastoral products differs from, say, that of fruit where at least part of an invasive pathway can be recognized and risks assessed. The ability to do this via pastoral sector pathways is much reduced, since risk organisms more frequently arrive via hitchhiker pathways which are diffuse and varied. Added to this pasture pests within grassland ecosystems are typically cryptic, often with subterranean larval stages. Such characteristics make detection and response particularly difficult. The consequences of this threaten to add to the already-increasing stressors of production intensification and climate change. This review explores the unique challenges faced by pasture biosecurity and what may be done to confront existing difficulties. While there is no silver bullet, and limited opportunity pre- and at-border for improving pasture biosecurity, advancement may include increased and informed vigilance by farmers, pheromone traps and resistant plants to slow invasion. Increasingly, there is also the potential for more use of improved population dispersal models and surveillance strategies including unmanned aerial vehicles, as well as emerging techniques to determine invasive pest genomes and their geographical origins.

Keywords: pastoral, invasive species, hitchhiker, quarantine, border biosecurity, biosecurity risk

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INTRODUCTION

Biosecurity, as described broadly by the FAO (2003), is a holistic process that seeks to manage biological risks associated with food and agriculture, not the least of which are invasive alien invertebrates. As in all agricultural sectors, the threat of arrival and establishment of pasture pests will only soar in the future as drivers such as trade, travel, and climate change continue to intensify and diversify. Climate change is already linked to the extending distribution of some pest species in response to warmer temperatures (FAO, 2008).

The international importance of biosecurity is widely recognized (e.g., Barlow and Goldson, 2002; Waage and Mumford, 2008) and prevention of pest establishment is key to effective biosecurity (Simberloff et al., 2013). To this end, the benefits of stringent risk assessment (Keller et al., 2007) and operational prevention of arrival, early detection, and eradication are feasible for many land-based industries. However, New Zealand's high-performance, improved pastures present their own unique and demanding biosecurity challenges. There the country's cost-effective pastoral farming methods are based on year-round production of high-producing grass and clover varieties. While such simple ecosystems create great value, they are also almost uniquely vulnerable to invasive pests and diseases (e.g., Goldson et al., 2014a) and, based on the current pest burden, around NZD $1 billion dollars' worth of production loss and costs are already being incurred by the sector (e.g., Goldson, 2014). This contribution reviews reasons why generally effective biosecurity strategies in other sectors are particularly challenged in pastures and comments on what may be done to deal with the threat of invasive pest species in the future.

THE EXISTING NEW ZEALAND BIOSECURITY SYSTEM

New Zealand has developed one of the most comprehensive agricultural biosecurity systems in the world (**Figure 1**). This has arisen as a consequence of the significant dependence of the country on peerless primary production exports and vulnerability to invasive species. Also the country has the advantage of comprising distant islands, and hence borders that are more defensible than those found in jurisdictions within large regions.

For New Zealand, the present biosecurity model shows activity pre-, at-, and post-border (**Figure 1**). Initial pre-border stages deal with threats at their place of origin. Thus for the importation of goods, evidence is required from the exporter demonstrating that offshore biosecurity requirements have been fulfilled, including compliance with any specifically designated product import health standards. These pre-border actions are often supplemented by pathway risk analyses in conjunction with physical interventions at the border itself, such as passenger baggage, vehicle and container inspection. This may include use of detection systems such as sniffer dogs and X-ray. The information gained based on actual interceptions is fed into ever-developing sophisticated risk models designed to assist in decision-making and determination of the biosecurity requirements associated with various types of freight (e.g., Jamieson et al., 2013). A large part of such effort assumes that the target threats and their places of origin are as reasonably well known as possible, for example, with fruit or wood product imports. The benefit here is that it provides the opportunity to monitor and manage recognized pest threat pathways known to be associated with the imported biological products. In turn this facilitates threat interception and disinfestation through treatments such as pesticides, heat-treatment or washing.

Issues around passenger arrivals have been well-managed via baggage X-rays, sniffer dogs, and passenger profiling (Ministry for Primary Industries [MPI], 2013a), but there remains the vast challenge posed by the arrival each year of c. 600,000 containers, 90,000 used vehicles and machinery and 17,000,000 tonnes of cargo (Ministry for Primary Industries [MPI], 2013b). This huge task is variously tackled by the presentation of bills of lading, pathway risk-profiling, use of transitional unloading facilities, and employment of trained accredited inspectors. Irrespective, hitchhiker pests continue to arrive via this route, including insects that are attracted to port and ship lights and/or arrive as contaminants of plant or soil material on inanimate objects (McNeill et al., 2011; Hulme, 2015). A close container inspection survey has revealed that of c.1000 containers landed at the main centers in New Zealand c.13% carried potentially threatening contaminants (Gadgil et al., 2002).

THE UNIQUE CHALLENGE OF PASTORAL BIOSECURITY

The value of the pastoral sector to New Zealand is very significant at c. NZD$24.3 billion p.a. and as such contributes >40% of the country's merchandisable exports[1]. The base for this comprises high-producing ryegrass and clover varieties that are well attuned to the country's favorable climate and these plants contribute NZD17.2 billion p.a. to the national GDP (NZIER, 2015). Improved pasture now occupies c. 10.6 m ha in New Zealand (c. 40% of the total land area)[2] and about a third of this consists of intensively managed sward of mainly ryegrass/clover. From virtually pest-free origins in the 19th century, these pastures have now acquired a significant burden of exotic pest species; Barlow and Goldson (2002) noted that 90% of the country's pasture pest species are exotic. The most damaging of these include the clover root weevil (*Sitona obsoletus* Gmelin), the Argentine stem weevil (*Listronotus bonariensis* Kuschel), the lucerne weevil (*S. discoideus* Gyllenhal) and the blue green aphid (*Acyrthosiphon kondoi* Shinji; Goldson et al., 2005). Further, African black beetle (*Heteronychus arator* Fabricius) is causing increasing damage in the North Island partly as a result of climate change. In Australia, it has been observed that with the rapid expansion in improved pastures since the 1950s there was a widespread decline in productivity of pasture legumes in the1970s and 1980s. This is considered, in part, to have been due to the occurrence of new

[1]http://www.stats.govt.nz/browse_for_stats/industry_sectors/imports_and_exports/overseas-merchandise-trade-info-releases.aspx

[2]http://www.stats.govt.nz/browse_for_stats/environment/environmental-reporting-series/environmental-indicators/home/land/land-cover.aspx

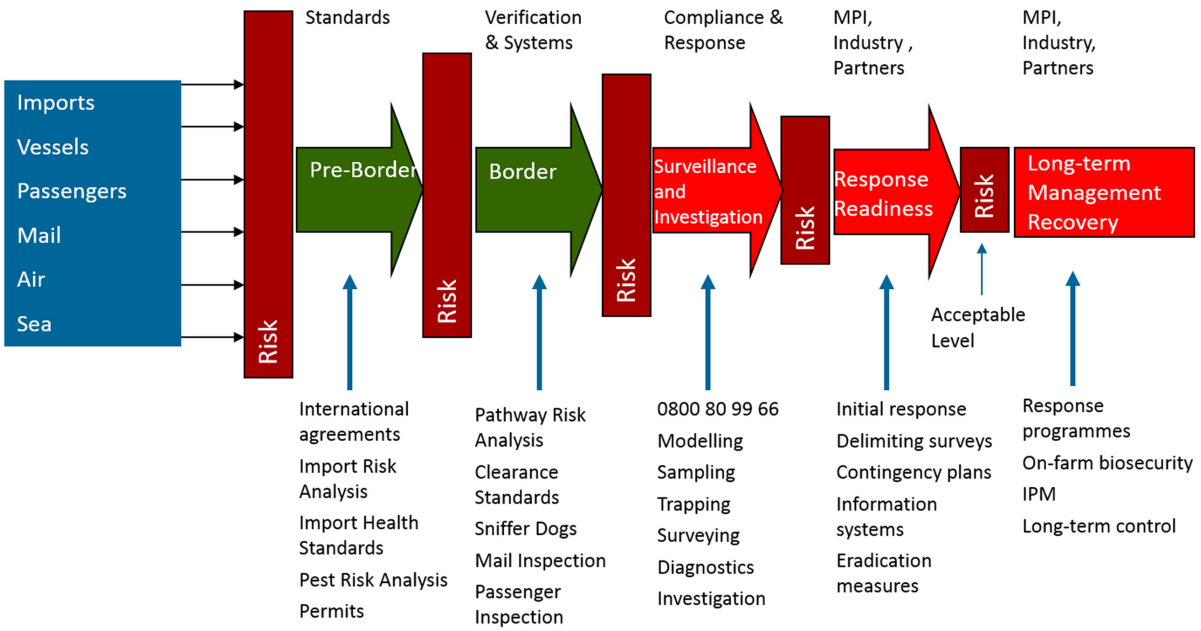


FIGURE 1 | The structure of New Zealand biosecurity depicted by MPI as a series of pre-, at-, and post-border systems and activities that are designed to successively reduce the risk from incoming sources of exotic pests, and ultimately minimize the need for long-term management.

insect pests (Wolfe, 2009). Undoubtedly, the clover root weevil has had a similar effect in New Zealand (e.g., Gerard et al., 2007).

The reasons for the severity of impact of such invasions are undoubtedly varied. For many years the New Zealand pastoral sector has been confronted with the need to pursue efficiency largely through intensification. This has resulted in elite pasture grasses and clovers being bred for traits that offer enhanced agronomic performance, but which have tended to make them more susceptible to pest damage. Significantly, however, Goldson et al. (2014a,b) have contended that New Zealand's ryegrass/clover-dominant pastoral ecosystems are notable for their lack of invertebrate biodiversity. Irrespective of whether this is strictly correct (and work is now in progress to examine this), New Zealand pastures, that comprise partial transplants of complex systems found elsewhere, are unlikely to include the same diversity of key exotic pest-suppressing species such as parasitoids, generalized predators and predatory spiders, as occur in the invasive pests' native ranges. This is in spite of many of New Zealand's pastures superficially resembling the large grassland areas found elsewhere (e.g., forb-rich European meadows). More generally, it is thought that it is this lack or difference of complexity that renders New Zealand's improved grasslands extremely susceptible to invasive exotic species (Goldson et al., 2005) and, as such, is typical of island ecosystems generally (e.g., Reaser et al., 2007). When invaders enter New Zealand pastures they encounter abundant food supply, unfilled niches, and a lack of the biotic resistance often found elsewhere in the form of interacting guilds of natural enemies and diseases (Tylianakis and Romo, 2010). This similarly applies to the functional diversity in the hedgerows and headlands of New Zealand's farmed ecosystems; again there is less exotic pest suppression capability than found in the equivalent ecosystems in the native range. It is this ecological setting that has led to the spectacularly high and damaging populations of invasive pest species that stabilize at far greater densities than those found in the native ranges (Goldson et al., 2014b). The impact of this scenario is certainly exacerbated by how very easily overlooked the damage is, for example by Argentine stem weevil, with its negative effects being attributed to poor seed germination and drought, as well as the impact of other plant stresses. Overlooking these negative factors more than anything else, has also made attainment of sustainable funding difficult for research projects to address the problem.

The incontrovertible importance of pastoral production to New Zealand and the continuing accumulation of destructive pests have resulted in an urgent need for excellent, effective, and robust pasture biosecurity measures. Unfortunately, actual biosecurity threats to pastures tend to get relatively sparse mention compared to the impacts of existing pests, e.g., in Australia (Wolfe, 2009) and in the UK (Clements, 1980; Hopkins, 2008). Often references to modified grasslands are focused more on amenity turf rather than grazed rangeland and meadow systems. For example, in the United States Vittum et al. (1999) published a comprehensive compendium of turfgrass insects of the United States and Canada. Potter and Held (2002) subsequently noted that the Japanese beetle, *Popillia japonica* Newman, an introduced scarab, had become the most widespread and destructive insect pest of turf, landscapes and nursery crops in the eastern United States. Indeed, until recently, even in New Zealand there has been a preoccupation with managing

the current pest loading rather than tackling pre-establishment biosecurity *per se* (Moot et al., 2009).

The reasons for this comparative low level of focus on pasture biosecurity are undoubtedly varied. In some ways, and despite reality, pasture is not viewed by the public as a particularly valuable 'crop'. Compared to say kiwifruit or apples, pasture plants are seen to be of low inherent unit value with not much esthetic appeal (Goldson et al., 2005). Related to this, cosmetic pest damage to forage is absolutely unimportant since forage itself is not exported. Further, many pasture pests are well-camouflaged against pasture soil and surrounding litter. Paradoxically, the light colored root-feeding subterranean early stages in the soil are sometimes easier to detect visually than the adults, although this does require turning the soil which is a further hindrance. However, even the advantage of exposure is offset by their typically indistinct morphology which limits taxonomic resolution to only a few species (AgPest[3]). Another consideration is that although these exotic pest species frequently cause severe pasture plant damage, it may only become visually apparent at certain times of the year, typically during peak spring and autumnal growth. This frequently leads to misidentification of the problem (e.g., poor seed quality, drought, etc.).

The subterranean habit of larval stages of many pasture pest species makes eradication of new invasive species nearly impossible once populations have established beyond a few hectares, e.g., clover root weevil, black beetle, and tropical grass webworm (Barker et al., 1996). Added to this, is the extensive distribution of pasture that determines low rates of economic return per hectare and precludes many surveillance and intervention options.

Significantly, pasture biosecurity also presents less opportunity for pathway-based biosecurity intervention (**Figure 1**) such as can be implemented in high value imports and export chains. Rather, a recent assessment of exotic pests that could be hazardous to New Zealand pasture in the future pointed to the primary importance of difficult-to-manage hitchhiker pathways including containers, used agricultural machinery and passengers (Toy, 2013). In general, adventive hitch-hiker species are most likely to be recognized and dealt with as part of the ongoing risk profiling and disinfestation processes in other pathways and for other agricultural sectors.

All of these factors make the New Zealand biosecurity situation for pastures both different and difficult.

PROSPECTS FOR IMPROVED PASTURE BIOSECURITY

Given the critical importance and the vulnerability of the New Zealand pastoral sector to biosecurity failure, it is necessary to consider how a biosecurity system may be developed further to suitably accommodate the unique needs and challenges outlined above. Certainly, part of the existing suite of techniques and technologies already being applied in general to New Zealand border biosecurity will benefit pasture biosecurity. However, the question is whether and how this can be more specifically extended and augmented. There are a number of areas that merit consideration.

Pest Proofing of Pastures

Waage and Mumford (2008) have suggested that there is value in creating resilience to invasion into agroecosystems rather than 'building walls'. This is particularly applicable to pasture because of its sheer invasibility. Thus, part of any evolving biosecurity strategy for pasture could include improving resilience to pest establishment and dispersal through pasture diversification (Sanderson et al., 2013) or plant resistance. In New Zealand an enormous advance occurred in pest-proofing pastures with the discovery and adoption of naturally occurring obligate biotrophic endophytic fungi (*Epichloë* spp.). This severely suppresses or controls pests of ryegrass and tall fescue (Johnson et al., 2013) and would be anticipated to be very useful in imparting resistance to any new exotic pests. Consequently, as pasture in New Zealand still essentially comprises ryegrass and white clover, continued advancement of this endophyte technology would provide an extremely useful barrier to establishment of new pests should they arrive, and thus contribute conspicuously to biosecurity for the sector as a whole. There has also been success in breeding lucerne (*Medicago sativa* L.) for resistance to aphids (Barlow and Goldson, 2002). Similarly, there is the ability to introduce new plant material (Wolfe, 2009) that may enhance existing generic biocontrols (Vattala et al., 2006). Likewise approaches could be taken to pest-proof pasture soil by manipulating the microbial ecology such that it is less acceptable to the soil-dwelling stages of some invasive species (Ferguson et al., 2012). Finally, while currently not permissible in New Zealand for societal and export reasons, the creation of new forms of resistance is possible through host plant genetic manipulation.

Industry and Farmer Awareness

Within the pastoral sector the ongoing invasion throughout New Zealand by the clover root weevil, *S. obsoletus*, has certainly raised awareness of the need for biosecurity (Basse et al., 2015). Linked to this there is real opportunity for pastoral biosecurity to advance on the basis of a strong social component (citizen science) whereby farmers in particular maintain a high level of biosecurity vigilance. This requires ready access to relevant information and data sources such as AgPest[1], as well as to the appropriate government agency (in New Zealand, MPI). Important to this also are the industry organizations groups such as Dairy New Zealand[4], Beef + Lamb New Zealand[5] and the Foundation for Arable Research[6]. These organizations play an essential role in raising suitable awareness and provide ready access to industry networks. In this respect the New Zealand Government Industry Agreement[7] (GIA) process is likely to be valuable. Such opportunities in the near-term are highly likely to involve

[3]http://agpest.co.nz/

[4]http://www.dairynz.co.nz/

[5]http://www.beeflambnz.com/

[6]https://www.far.org.nz/

[7]http://www.gia.org.nz/

increasingly sophisticated and widespread rapid access to information via hand-held electronic devices, most likely smart phones.

The Use of Lists and Data Bases

There are a number of important reference sites for New Zealand biosecurity. These include, the International Plant Sentinel Network, IPSN[8] which has a focus on linking botanic gardens, National Plant Protection Organizations (NPPOs) and the work of various plant health scientists' associations. All of these can provide early warning systems of new and emerging pest and pathogen risks, including pasture and turf plant species. Further, initiatives such as The Biological Heritage National Science Challenge[9] in New Zealand, which seek to develop in depth knowledge of what species are already in the country, will provide a more solid foundation from which to recognize new species incursions. Also the Global Eradication and Response Database (GERDA) (Kean et al., 2016) aims to summarize all incursion response and eradication programs from around the world to share experience and enhance opportunities for future biosecurity responses.

In general terms, an immediate component of heightened pasture biosecurity is the identification of species with potential high impact and likelihood of arrival and establishment in New Zealand. Traditionally, this response has been to compile lists from evidence offshore of those insect species known to be damaging. Such lists are both logical and useful although they can be of mixed value if adhered to too rigidly to the species level. Irrespective, ranking of which exotic species could be a threat to New Zealand pasture can give very important broad indications of what to look out for. For example, based on the combined potential to establish and have an adverse impact, a recent report named 151 potential hazards (Toy, 2013). With reference to the ability to establish, 24 species were highly rated. Of these, 22 were insects, nine of which were Coleoptera; of the others, Diptera, Hemiptera, and Lepidoptera were represented. However, only seven of the 22 were rated high in both their establishment potential and probable impact potential. Four of these were Coleoptera, viz. *Agriotes sputator* (Linnaeus), *Hypera postica* (Gyllenhall), *S. hispidulus* (Fabricius), and *Sphenophorus venatus* (Say). Thus root-feeding beetles could be considered the most obvious group to look out for. Certainly this aligns well with New Zealand experience to date. Five of the nine severe pasture pest arrivals that have occurred since trade intensity increased in the 1920s have been Coleoptera and all are likely to have arrived as hitchhikers. It is salutary to note that there are c.100 *Sitona* spp. in the Palaearctic region (Velazquez de Castro et al., 2007), most of which have the ability to damage forage legumes and all of which are very difficult to recognize in the field as separate species (Phillips and Barratt, 2004). Interpreting lists as above also permits the cataloging of those traits and life histories that are indicative of 'types' of species that need to be prioritized as potential biosecurity threats, such as root feeding Scarabidae, rhizobial nodule feeding Curculonidae, phloem-feeding Aphidae, and vascular feeding Pentotomidae.

Obviously, the ability to classify hitchhiker pathways known to be associated with greater risk need to be advanced in terms of the taxa, goods and seasonality correlates. For example, southern hemisphere countries are sources of Coleoptera in the same lifecycle phase which makes establishment more likely. Evidence shows that Australia is a particular risk for New Zealand partly because of its geographic proximity, the survivability of the insects during brief transport and synchrony of seasons.

Emerging Technologies

Technology, albeit slowly, is increasing its capacity to assist pastoral biosecurity. It follows that improved surveillance would most usefully be focused in the vicinity of seaports, airports, rail routes, large transitional facilities, and tourist centers. There are a number of existing and new opportunities to enhance the chances of detection of exotic threats to pastures. None of these singularly suggest a breakthrough, but collectively these technologies may be brought to bear along with enhanced passive surveillance by the New Zealand community, particularly pastoral farmers.

More specifically, there is the possibility of enhanced use of 'sentinel or trap plants' that can be examined regularly to more clearly indicate the presence of a new species. Such an approach has been successfully used as a method for identifying potential pests found in off-shore pasture ecosystems or in plant collections such as botanic gardens (Roques et al., 2015). China is New Zealand's largest trading partner where there are extensive areas of pasture in similar climatic zones to those in New Zealand. Examination of pests and diseases attacking pasture plants in that country could point to potential pests that could arrive with large volumes of trade. Direct trapping can also offer enhanced detection; 'delta' sticky traps baited with *S. lineatus* synthetic aggregation pheromone have been shown to catch high numbers of various *Sitona* spp. in the vicinity of lucerne crops (Toshova et al., 2009). Similarly, smart traps for lepidopteran and dipteran pests have been shown to be effective (Liu et al., 2009). For example pheromonal lures have been useful in dealing with an outbreak of an Australian pasture tunnel moth (*Philobota* sp.) in northern New Zealand pasture (Dymock et al., 2009).

There are some novel approaches emerging that will be useful for all sectors. Very preliminary work has investigated the potential to detect hitch-hiker pests in confined spaces such as shipping containers, based on the detection of organic volatile compounds known to be associated with pest threats (More et al., 2007). Rapid field-based diagnostic technologies based on very fast DNA analysis, such as LAMP (Loop Mediated Isothermal Amplification) (e.g., Niu et al., 2011), are emerging that would be vitally effective in identifying new pests, thereby facilitating swift critical decision-making around containment and eradication. Work has now also advanced in the use of multiple stable isotopes to assess the natal origin of single insects as another decision-making tool. Unlike any other method, this can help determine whether the discovery of a threat

[8]http://www.plantsentinel.org/

[9]http://www.biologicalheritage.nz/home

species is part of an established population, including possible re-emergence of what was presumed to be an already 'eradicated' population, or that the discovery is in fact a new incursion (Holder et al., 2014).

Likewise, the ongoing use of metapopulation modeling using improved knowledge of pest population biology, dynamics and dispersal data will permit more targeted pasture surveillance systems to continue to be developed. Such work helps to resolve uncertainty about those ecosystem processes between introduction, full invasion and establishment (Born et al., 2004). In part, these advantages will be based on rapidly increasing computational power data-handling capability, including data-warehousing (Worner et al., 2014).

As mentioned earlier, eradication is particularly difficult with soil-dwelling species and is something that is often overlooked both socially and politically. Indeed, options for even acceptable local-site eradications are declining because of the abolition of use of various classes of pesticide, particularly those that persist in the ecosystem (Goldson et al., 2015).

Should eradication be deemed impossible, then expensive long-term control measures are required to be developed and implemented. However, before anything can be done, there is a need to understand the pest population dynamics which are often found to be markedly different from what is known of the species' native range; this means dependence on overseas literature has limitations. Significantly, strategies for dealing with a new pest species, even in the pastoral environment, can mean resorting to the use of pesticides (e.g., seed treatments) that can completely disrupt finely balanced biological control systems (Goldson et al., 2015).

Looking further into the future and with suitable social consent, eradication based on techniques such as gene-editing e.g., CRISPR–Cas9 (Webber et al., 2016) and 'Trojan gene' techniques (Gemmell et al., 2013) have the potential to cause huge reductions in populations of pest species. For example, through meiotic-drive interventions, control along the lines of the sterile insect technique (SIT) have showed promise for managing mosquito vectors of disease (Burt, 2014). Ultimately, this type of technology could be coupled with uses of unmanned aerial vehicles (UAVs) for either surveillance or delivery of control technologies, such as is starting to be used for weed control (Torres-Sánchez et al., 2013). UAVs are already capable of scouting large swaths of land and could include the use of sequential pictures with a computer algorithm to automatically screen for the effects of unexpected invasive pest species.

CONCLUSION

The confounding challenges of pasture biosecurity, as outlined in this contribution, has meant that the sector is less able to focus on this issue than the crop and horticultural sectors. In part this is because forage is neither consumed by humans nor exported.

A major constraint for pasture biosecurity is the lack of well-defined risk-species pathways, which are particularly afflicted by difficult-to-detect and difficult-to-identify hitchhiker species. This severely limits pre- and at-border opportunities for disinfestation measures. Moreover, eradication is often effectively impossible when commonly soil-dwelling life stages are involved.

While there is no silver bullet, opportunities for improving pasture biosecurity may include increased vigilance by farmers (e.g., via the pending GIA), plant resistance, more use of advanced population dispersal models and surveillance strategies, pheromone traps, and emerging genetic and isotope techniques to identify pests and their origins.

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KA and SG conceived the topic and wrote substantial parts of the text. BB revised the text critically for accuracy and intellectual content. SG, KA, and BB all approved the version to be published and agreed to be accountable for all aspects of the work.

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