



# *De novo* Transcriptome Assembly of the Clown Anemonefish (*Amphiprion percula*): A New Resource to Study the Evolution of Fish Color

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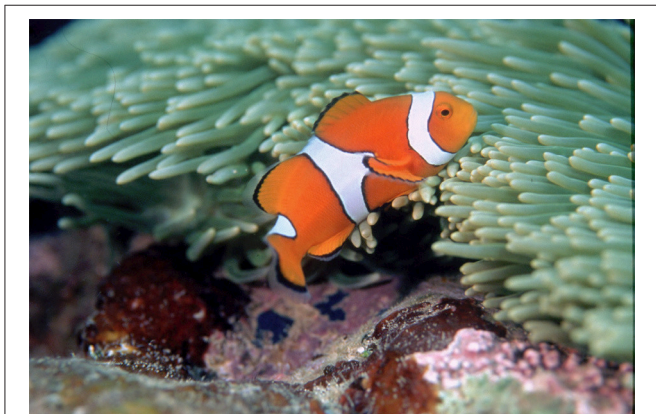
A fundamental question of evolutionary biology is, why are some animals conspicuously colored? This question may be addressed from both a proximate (genetic and ontogenetic) and ultimate (adaptive value and evolutionary origins) perspective, and integrating these perspectives can provide further insights. Over the last few decades we have made great advances in understanding the causes of conspicuous coloration in terrestrial systems, e.g., birds and butterflies, but we still know relatively little about the causes of conspicuous, “poster” coloration in coral reef fishes. Of all coral reef fishes, the clownfish *Amphiprion percula*, is perhaps the most conspicuously colored, possessing a bright orange body with three iridescent white bars bordered with pitch black. Here, we review what is known about the proximate and ultimate causes of the conspicuous coloration of clownfishes *Amphiprion* sp.: coloration has a heritable genetic basis; coloration is influenced by development and environment; coloration has multiple plausible signaling functions; there is a phylogenetic component to coloration. Subsequently, to provide new insights into the genetic mechanisms and potential functions of *A. percula* coloration we (i) generate the first *de novo* transcriptome for this species, (ii) conduct differential gene expression analyses across different colored epidermal tissues, and (iii) conduct gene ontology (GO) enrichment analyses to characterize function of these differentially expressed genes. BUSCO indicated that transcriptome assembly was successful and many genes were found to be differentially expressed between epidermal tissues of different colors. In orange tissue, relative to white and black, many GO terms associated with muscle were over-represented. In white tissue, relative to orange and black tissue, there were very few over- or under-represented GO terms. In black tissue, relative to orange and white, many GO terms related to immune function were over-represented, supporting the hypothesis that black (melanin) coloration may serve a protective function. Overall, this study presents the assembly of the *A. percula* transcriptome, and represents a first step in an integrative investigation of the proximate and ultimate causes of conspicuous coloration of this iconic coral reef fish.

**Keywords:** marine ecology, evolutionary ecology, transcriptome, genome, melanin pigment, carotenoid pigment, structural coloration, *Amphiprion percula*

## INTRODUCTION

Conspicuous coloration is widespread throughout the animal kingdom, yet represents an evolutionary paradox because high visibility should increase predation risk (Ruxton et al., 2004). Decades of research on coloration, however, has produced compelling insights into both the proximate (genetic and ontogenetic; Hoekstra et al., 2006; Rosenblum et al., 2010; Reed et al., 2011; Gallant et al., 2014; Kunte et al., 2014) and the ultimate (Guilford, 1988; Alatalo and Mappes, 1996; Joron and Mallet, 1998; Sherratt and Beatty, 2003; Dale et al., 2015; adaptive value and evolutionary origins) causes of animal coloration (Hill and McGraw, 2006a,b; Hubbard et al., 2010; Kronforst et al., 2012; Cuthill et al., 2017; Endler and Mappes, 2017). The vast majority of work on color pattern formation has been conducted in terrestrial systems, despite the fact that some marine groups, e.g., coral reef fishes (Longley, 1917; Lorenz, 1962), express a tremendous diversity of color pattern variation paralleling that of the most diverse terrestrial groups. Among the coral reef fishes, the clownfishes of the genus *Amphiprion* are perhaps the best known and most conspicuously colored (Figure 1).

Considering the proximate causes of variation in *Amphiprion* coloration, various lines of evidence indicate it has a heritable genetic basis. First, substantial interspecific variation in color pattern exists among the 29 species (Allen, 1972; Fautin and Allen, 1992; Froese and Pauly, 2018): the coloration of the body and fins ranges from yellow to orange, pink to red, brown to black; the fish have zero to three distinct white bars on the body, that can reflect UV (Buston, unpublished data). Second, hybrids, found in the wild and produced in the lab, have intermediate or unusual color patterns (Marliave, 1985;



**FIGURE 1** | Photo of the clown anemonefish *Amphiprion percula*, illustrating the conspicuous orange, white and black coloration of the genus. In fishes, chromatophores, containing either colored droplets (xanthophores and erythrophores) reflecting organelles (iridophores) or pigment granules (melanophores), are responsible for skin coloration. In *Amphiprion*, the orange color is carotenoid based, the white color (which also reflects in the UV) is likely a structural color based on intracellular stacks of guanine crystals, and the black color is melanin based. Coloration of other members of the genus can be seen on FishBase (Froese and Pauly, 2018). [<http://www.fishbase.org/identification/SpeciesList.php?genus=Amphiprion>].

Litsios and Salamin, 2014; Pedersen, 2014a; Dibattista et al., 2015; Gainsford et al., 2015; Balamurugan et al., 2017). Third, there is substantial intraspecific variation in color pattern associated with genetic divergence among populations (*A. clarkii*, Moyer, 1976; Bell et al., 1982; *A. melanopus*, Drew et al., 2008). Finally, unusual color patterns arising spontaneously via mutation can be artificially selected in the lab (Pedersen, 2014b; Talbot, 2014).

Developmental color variation also occurs in *Amphiprion* (Fautin and Allen, 1992). In *A. percula*, larval settlers are pinkish in color with a single white bar, and only acquire the conspicuous orange coloration, with three white bars, bordered with black, once the fish have successfully recruited to a group (Buston, personal observation). In *A. percula* and *A. ocellaris*, the development of the white bars is influenced by diet and stocking density (Avella et al., 2007; Chambel et al., 2015); in *A. ocellaris*, dietary carotenoids influence orange hue, saturation and brightness (Tanaka et al., 1992; Yasir and Qin, 2010; Ho et al., 2014; also *A. frenatus*, Hekimoglu et al., 2017), as does the light environment in which the fish are kept (Yasir and Qin, 2009a,b); in multiple species (*A. chrysopterus*, *A. clarkii*, *A. percula*, *A. polymnus*) a varying combination of island location, depth, anemone species, anemone size, and social rank are associated with variation in melanism (Moyer, 1976; Militz et al., 2016).

The ultimate causes of coloration in *Amphiprion* are less clear because there have been no explicit tests of alternative hypotheses for the adaptive value of the color pattern traits, e.g., quality, attractiveness, strategy, genetic compatibility, kinship, individual identity or presence (Dale, 2006). It is possible, however, to gain some preliminary insights by considering signal properties (Dale, 2006). In *A. percula*, the pinkish color of settlers may be a dishonest signal of presence (i.e., camouflage) to residents (McDonald, 1993; Buston, 2003a). In *A. clarkii* and others, the differences in coloration associated with social rank may be a signal of reproductive strategy, either female vs. male or breeder vs. non-breeder (Moyer, 1976; Fautin and Allen, 1997; Militz et al., 2016). In *A. percula* and *A. ocellaris*, the highly variable orange, black and white patterning may be a signal of individual identity to other group members (Fricke, 1973; Nelson et al., 1994; Dale et al., 2001; Buston, 2003b; Tibbetts, 2004).

Finally, turning to consider the evolutionary origins of the various components of the color pattern, close inspection of the clownfish phylogeny reveals a likely phylogenetic component (Litsios et al., 2014; Rolland et al., 2018). Within the genus there are two main clades which separated 11–12 million years ago (MYA): a smaller clade that is bright orange with three white bars (*A. percula* and others); a larger clade that is dominated by species with two white bars (*A. clarkii* and others). Within the larger clade there are three other small clades of note: the first, which diverged 5–6 MYA, tends to have one or zero wide white bars (*A. melanopus* and others); the second, which also diverged around 5–6 MYA, tends to be more pinkish in color with one or zero narrow white bars and a dorsal white stripe (*A. perideraion* and others); the third, which diverged 2–3 MYA, tends to be more blotchy in its color patterns (*A. polymnus* and *A. sebae*).

In sum, the clownfishes of the genus *Amphiprion* present a wonderful opportunity to investigate the proximate and ultimate causes of coloration in coral reef fishes. This opportunity is enhanced by the recent development of transcriptomic (*Amphiprion bicinctus*, Casas et al., 2016) and genomic (*Amphiprion frenatus*, Marcionetti et al., 2018; *Amphiprion ocellaris*, Tan et al., 2018) resources for the genus. Here, we used a combination of RNA sequencing, transcriptome annotation, differential gene expression analyses and gene ontology enrichment analyses to (i) generate a *de novo* transcriptome for *A. percula*, (ii) identify over-represented genes associated with orange, white, and black epidermal tissues, and (iii) identify potential functions of those genes in *A. percula*. We found patterns consistent with over-representation of GO terms associated with muscles in orange regions, as well as over-representation of GO terms involved in immune response in black regions. While our results, by design, represent only a preliminary assessment of color-specific gene expression in the epidermis of *Amphiprion*, they represent a first step in identifying the genetic mechanisms underlying color pattern formation in this iconic group of coral reef fishes.

## MATERIALS AND METHODS

### Source Population

All work was conducted with the approval of Boston University IACUC. Research was carried out at Boston University (Boston, MA). Clownfish used in the study, *Amphiprion percula*, all originated from wild populations in Papua New Guinea and were supplied by Quality Marine and Sea Dwelling Creatures. At the time of sampling all individuals had fully developed white bars, bordered with black and all individuals were non-reproductive—*A. percula* is a sequential, protandrous hermaphrodite and all individuals start out as non-reproductive, then transition to male and female in their lifetime. Experimental fish were housed in 1 L tanks, which were part of a larger re-circulating saltwater aquarium system. Flow through each tank was approximately 9 L/h  $\pm$  0.5 L. Abiotic conditions were maintained as constant as possible: pH = 8.1  $\pm$  0.1, temperature = 26.1  $\pm$  0.6°C, salinity = 32  $\pm$  0.5 ppt with ambient light. Water samples were tested every 2 weeks for nitrate, nitrite, and ammonia (Salifert test kits, Amsterdam, The Netherlands; Red Sea test kits). These measurements were 0 ppm for the entirety of the experiment.

### Sequencing

In 2015, epidermal tissue samples were taken from nine, cold-anesthetized, *A. percula*. From each individual, nine samples were taken: three different colored epidermal tissues (orange, black, white) from three different body positions (anterior, middle, posterior; see **Figure 1**). This procedure yielded 81 samples in total (9 individuals  $\times$  3 colors  $\times$  3 positions). Samples were immediately submerged in RNAlater for preservation. Samples of the same color, from all body positions and all individuals, were later pooled, yielding three libraries for RNA-sequencing: orange, white, black. In an ideal world, we would have kept these samples separate, maximizing our sample size and enabling us to examine variation within and among individuals, but funding constraints

prevented us from adopting this strategy. Pooling tissue samples by color reduced our sample size and meant that we could not examine variation within and among individuals, but enabled us to investigate patterns of gene expression in different colored epidermal tissues against multiple backgrounds in a cost effective manner.

Following preservation, RNase-free microcentrifuge tubes with 1 ml of TRIzol Reagent (Invitrogen) were used for RNA isolation. Samples were homogenized, followed by a 5-min incubation period at room temperature. Phase separation was carried out with 0.2 ml of chloroform and centrifugation at 12,000 g for 15 min at 4°C (following Casas et al., 2016). Ethanol (200 proof) was added in equal volume to each sample, and samples were loaded onto a Purelink RNA minikit column (Life Technologies), washed three times, and eluted into 50  $\mu$ l of RNase-free water. Each total RNA sample was then treated with DNase followed by a final clean up using Qiagen's RNeasy Plus kit. An Agilent Bioanalyzer 2100 in conjunction with fluometry (Qubit) were used to assess total RNA quality and concentration. All samples scored an RNA integrity number (RIN) of at least eight and were used for library construction.

From the chosen samples, Dynabeads Oligo (dT) kit (Life Technologies) was used to isolate Poly-A mRNA from 1 to 3  $\mu$ g of total RNA. Following mRNA capture, 2  $\times$  100 bp RNAseq libraries were created using NEBNext Ultra library prep kits (New England Biolabs). 12 PCR cycles were used for library enrichment. Libraries were quality checked using KAPA's library quantification kit on the Bioanalyzer 2100 (with DNA 1000 chips). Using these results, individually-barcoded mRNA sequence libraries were multiplexed in equal proportion (10 nM) and sequenced across four lanes of Illumina's HiSeq 2000 platform on rapid run mode. All sequencing was performed at Tufts Genomics.

### Transcriptome Assembly, Assessment and Annotation

For transcriptome assembly purposes, all sequencing data were pooled, yielding over 289 million PE reads. Read quality was assessed using FastQC and raw reads were quality filtered and trimmed at the first base with mean quality score (Q-score) < 20. Sequence adaptors were removed using *trimmomatic*, leading and trailing bases with a Phred quality score below 3 were trimmed and each sequence was scanned with a 4 bp sliding window and trimmed when average Phred quality dropped below 15, resulting sequences < 36 bp were discarded and the remaining reads were then normalized *in silico* using the *normalize\_reads* function in the Trinity bioinformatics pipeline (Haas et al., 2013). *Trinity 2.2.0* (Grabherr et al., 2011) with *bowtie v1.2.0* (Langmead et al., 2009) was used to construct the *de novo* transcriptome on Boston University's Shared Computing Cluster with a 100 Gb memory node and 12 cores.

The resulting transcriptome assembly was trimmed with a custom perl script to remove contigs under 500 base pairs. Ribosomal RNA contamination was controlled by finding sequences exhibiting significant nucleotide similarity (BLASTN, *e*-value  $\leq 1 \times 10^{-8}$ ) to the SILVA LSU and SSU rRNA databases



(<http://www.arb-silva.de/>). A total of 288 contigs from the *A. percula* transcriptome, each of which matched an identifiable rRNA, were identified and removed from the transcriptome. BUSCO was then used to evaluate transcriptome completeness. BUSCO detects the presence of single-copy orthologs universal to all *Actinopterygii* (ray-finned fishes) as an overall measure of transcriptome quality. The ortholog dataset was provided by BUSCO (Simão et al., 2015) and BUSCO v3, augustus v3.2.3, hmmer v3.1b2 and blast+ v2.6.0 were used. Contigs were then annotated by BLAST sequence homology searches against UniProt and Swiss-Prot NCBI NR protein databases, using an *e*-value cutoff of  $e^{-5}$  and annotated sequences were then assigned to Gene Ontology (GO) categories (Blake et al., 2015). The annotation pipeline used here is described in detail at: <https://github.com/z0on/annotatingTranscriptomes>.

## Read Mapping, Differential Analysis and GO Enrichment

Across the three RNA tissue libraries, raw reads ranged from 114 to 143 million PE reads (Table 1). Fastx\_toolkit removed *Illumina TruSeq* adapters and poly(A)<sup>+</sup> tails. Sequences <20 bp in length with <90% of bases having quality cutoff scores >20 were trimmed. Resulting filtered reads (R1 and unpaired reads only) were then mapped to the *de novo* *A. percula* transcriptome described above using bowtie 2.3.2 (Langmead and Salzberg, 2012). Raw counts were then used as input into differential expression analyses (Supplemental Table 1).

Differential gene expression analyses were performed using *edgeR* v3.20.8 (Robinson et al., 2010) in R v3.4.1 (R Development Core Team., 2008). Three unique pairwise comparisons were carried out between the three tissue color libraries: orange vs. white, orange vs. black, white vs. black). Using *edgeR*, samples were used to create a *DGEList* object. Normalization factors to scale library sizes were acquired using the *calcNormFactors* function. Next, the *exactTest* function was used with a large dispersion value of 0.4, as no biological replicate libraries were available to control for variation (Chen et al., 2017). We used *EdgeR*'s suggested large dispersion values since larger dispersion values minimize the ability to detect differentially expressed genes and the typical value for the common biological coefficient of variation (BCV; square-root-dispersion) for outbred human populations has been found to be 0.4 (Robinson et al., 2010). That is, we chose a large dispersion value as a conservative measure, being aware of the limitations of our sampling strategy.

Data were then compiled into tables of normalized counts, log fold change, log counts per million, *p*-values, and false discovery rate- adjusted *p*-values for each pairwise comparison (Supplemental Tables 2–4).

Given the limitations of our sampling strategy, no specific candidate genes or metabolic pathways were explored. Rather, *p*-values resulting from the *exactTest* function were used to implement a standard “GO enrichment” analysis, based on ranking of signed log *p*-values (Dixon et al., 2015; Wright et al., 2015; [https://github.com/z0on/GO\\_MWU](https://github.com/z0on/GO_MWU)). Results were plotted in nine separate dendrograms, each representing one of three GO domains [biological processes (BP), cellular component (CC), molecular function (MF)] for one of the three pairwise comparisons. Using data acquired from GO enrichment, GO categories that were significantly enriched at a *p*-value < 0.1 were noted and recorded. Given the limitations, only GO categories that were consistently up or down regulated in both pairwise comparisons were considered significantly enriched. These categories were then explored for common trends and functions, and grouped together in “GO subdomains” using their biological relatedness as explained by the Gene Ontology Consortium’s definition for each term (Blake et al., 2015; <http://www.geneontology.org/page/ontology-documentation>). We treat these results as plausible hypotheses for future investigation.

## RESULTS

### *Amphiprion percula* Transcriptome

To compare gene expression between tissue colors, a *de novo* transcriptome was constructed. Trimmomatic quality trimmed the reads before assembly and 97.13% of both read pairs survived, in 0.89% of instances only forward reads survived and in 0.00% of instances did only reverse reads survive. In all, 1.99% of read pairs were dropped. Sequencing produced a total of 389 million paired reads after adapter trimming and quality filtering. The preliminary transcriptome held a total of 217,325 transcripts which were manually trimmed to remove contigs under 500 base pairs. The resulting transcriptome had a mean GC content of 45.91% and produced 88,074 contigs with an average length of 1,517.56 bp (N50 = 1,969) and a median contig length of 1,119. These contigs corresponded to 18,618 unique isogroups, of which 12,062 had gene annotations when searched against NCBI’s nr database. Furthermore, 12,153 of these isogroups had GO annotations. Of the 4,584 Benchmarking

**TABLE 1** | Summary of RNA libraries, including the tissue color, raw 100 bp paired-end reads (“PE Reads”), trimmed reads, proportion of reads surviving quality control, mapped reads and the proportion of reads that mapped to the *Amphiprion percula* transcriptome.

Tissue color	Reads				
	Raw PE reads (10 <sup>6</sup> )	Trimmed reads (10 <sup>6</sup> )	Proportion surviving	Mapped (10 <sup>6</sup> )	Proportion mapped
Black	114.9	96.1	0.84	83.0	0.86
Orange	130.7	114.2	0.87	94.8	0.832
White	143.8	126.7	0.88	108.3	0.86
Total	389.4	337.0		286.1	

Universal Single-Copy Orthologs (BUSCOs) for Actinopterygii, 70% were complete, and a further 15.4% were fragmented, suggesting that the *de novo* assembly was relatively successful (Supplemental Figure 1). All raw reads are archived in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) under accession number PRJNA471968, with transcriptome assembly and annotation files available at <http://sites.bu.edu/davieslab/data-code/>.

## Preliminary Investigation of Differential Gene Expression and GO Enrichment

For individual tissue RNA libraries, raw reads ranged from 114 to 143 million reads (Table 1). After quality control with *fastx\_toolkit* and *fastq\_quality\_filter* this number was reduced to 96–126 million reads. Reads that successfully mapped to the *de novo* transcriptome ranged from 83 to 108 million for each library (Table 1). Out of 18,618 isogroups, edgeR's analysis of pairwise comparisons found 327 (1.76%) differentially expressed genes (DEGs;  $p = 0.1$ ) in the orange vs. white tissue comparison, 374 DEGs (2.01%) in the black vs. white tissue comparison, and 320 DEGs (1.72%) in the orange vs. black tissue comparison. EdgeR results can be found in Supplemental Tables 2–4.

In orange epidermal tissue, Gene Ontology (GO) enrichment analysis revealed, an over-representation of GO terms associated with muscle in all three GO domains: “biological process” (BP), “Molecular Function” (MF), and “cellular component” (CC) (Supplemental Figures 2–7). This enrichment was most abundant in CC (Supplemental Figures 3, 6). The terms included *striated muscle contraction* (GO:0006941), *structural constituent of muscle* (GO:0008307), and *myosin filament* (GO:0032982) (Figure 3).

In white tissue, there were few enriched GO categories, but all fell under the “cellular component” domain (Supplemental Figures 3, 9). Several dealt with the extracellular matrix: *proteinaceous extracellular matrix* (GO:0005578) and *extracellular matrix* (GO:0031012) and all of these categories dealing with extracellular matrix were underrepresented (Figure 3).

In black epidermal tissue, many GO terms dealing with immunity (immune function, response to wounding/stress), were over-represented in BP (Supplemental Figures 5, 8; Figure 2). These included GO terms associated with generalized immune function such as *positive regulation of immune response* (GO:0050778), and *response to wounding* (GO:0009611), and terms associated with leukocyte movement such as *positive regulation of leukocyte migration* (GO:0002687), *regulation of leukocyte chemotaxis* (GO:0002688), and *lymphocyte migration* (GO:0072676) (Figure 2). Inflammatory response genes, including *positive regulation of inflammatory response* (GO:0050729), *regulation of inflammatory response to antigenic stimulus* (GO:0002861), as well as genes related to wounding, *response to wounding* (GO:0009611), were also over-represented in BP (Figure 2). A small number of terms dealing with immunity were also over-represented in MF in black tissue (Supplemental Figures 7, 10) and the majority of these dealt with cytokine function, including *cytokine receptor activity* (GO:0004896), and *chemokine receptor binding* (GO:0042379)

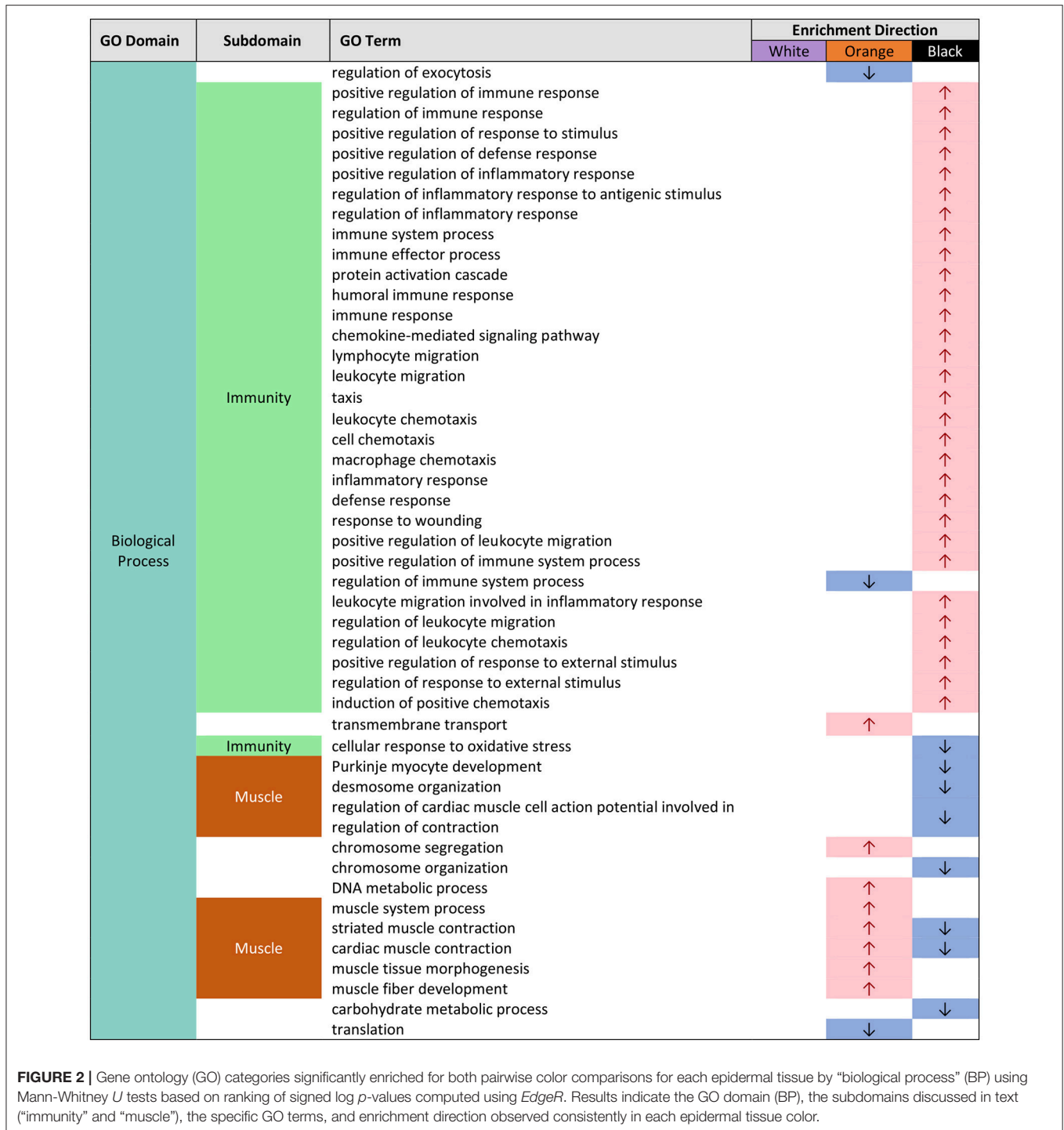
(Figure 4). No genes were enriched in CC in black tissue (Supplemental Figures 6, 9).

## DISCUSSION

While typically attributed to aposematism (i.e., warning coloration), the evolution of bold, striking colors can arise via many other mechanisms, including those related to signaling (e.g., individual identity, social status, behavioral strategy, etc; Tibbetts et al., 2017). Differentiating among these competing hypotheses is complex, however, and requires detailed knowledge of the underlying genetic mechanisms responsible for color-pattern variation at multiple levels of organizations (e.g., individual, population, species). Here we assembled and annotated the first *de novo* reference transcriptome for the clownfish *Amphiprion percula*, characterized patterns of differential gene expression across colored epidermal tissue, and conducted GO enrichment analysis to characterize evidence of functional differences among differentially colored epidermal tissues as a first step toward elucidating the molecular basis of color pattern formation in *Amphiprion*.

Several lines of evidence indicate that our *de novo* transcriptome is robust. First, although our Trinity assembly produced a large number of contigs initially, filtering for contigs longer than 500 bp dramatically reduced this number and resulted in a N50 contig length approaching 2 kb, typical of full length ESTs in vertebrates (Fong et al., 2013). Additionally, these filtered contigs represent ~18 K unique isogroups corresponding closely to typical estimates of gene number in vertebrates (Prachumwat and Li, 2008; Howe et al., 2013). Benchmarking against single-copy vertebrate genes found evidence for 85% of known orthologs, which is typical of other successful transcriptome assemblies (Simão et al., 2015; Theissinger et al., 2016). For comparison, the recently completed *Amphiprion bicinctus* transcriptome (Casas et al., 2016), which was derived from brain and gonadal tissues, found evidence for completeness of 95.6% of core eukaryotic genes but looked at a smaller gene set (CEGs,  $n = 248$ ), had a median contig length of 603 bp, and an N50 value approximately 25% smaller (N 50–1,563 bp) than our assembly. Taken together, these statistics strongly suggest that the transcriptome produced here was robust.

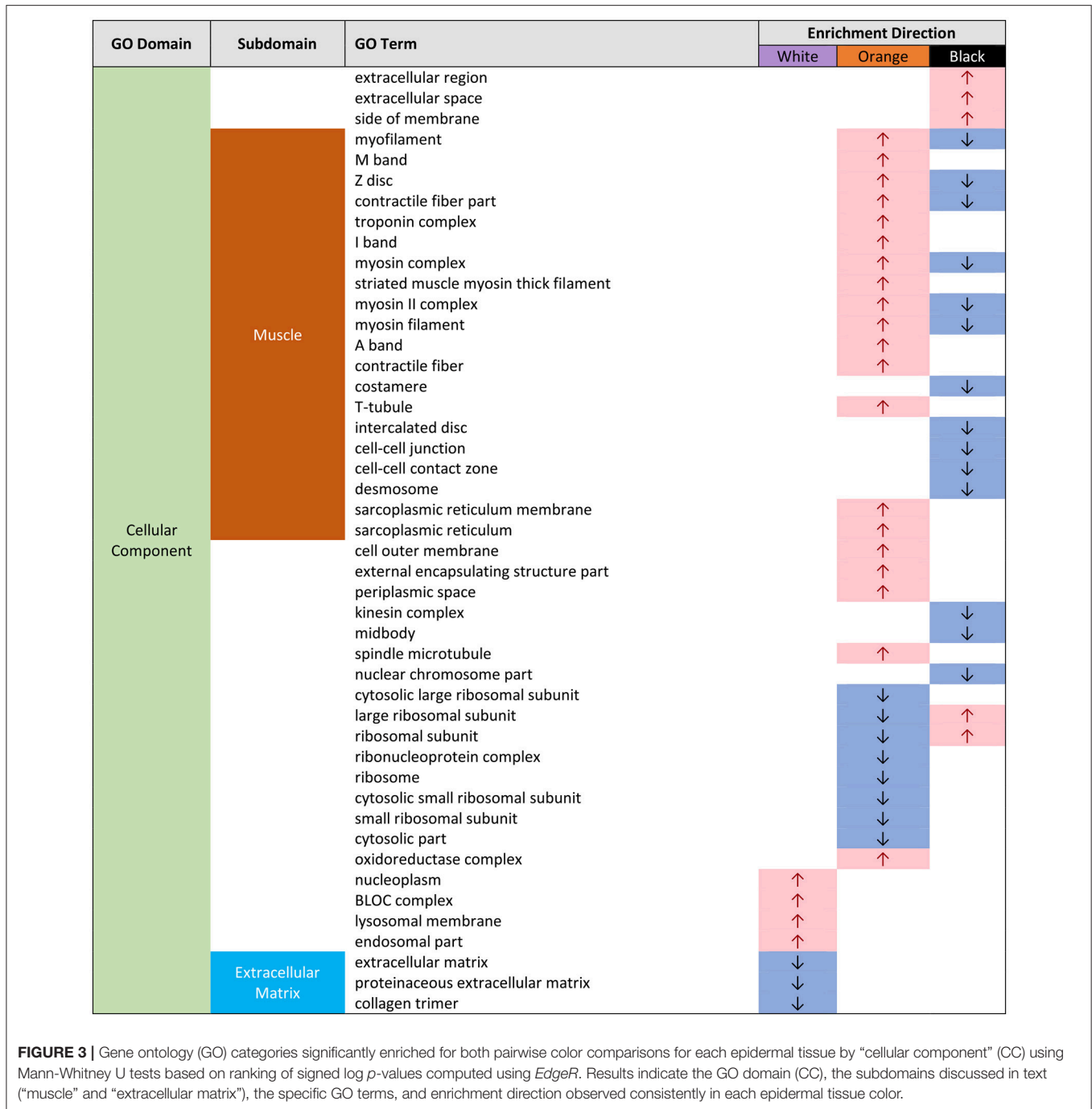
Our gene expression analyses, using the reference transcriptome, revealed striking differences among colored epidermal tissues. However, because we pooled samples from multiple individuals, our sample size is limited and these results are best viewed as preliminary. Despite this caveat, the strong differences in observed levels of expression across tissue samples is interesting for two reasons. First, pooling tissue samples from different body regions and different individuals by epidermal tissue color is expected to increase the variance in background gene expression within each pool, making it less likely to detect differences between color types. This suggests that differences between color types may have biological significance, enabling us to generate some plausible hypotheses regarding their function that will require more rigorous testing in the future. Second, variation in patterns of gene expression among epidermal tissue



colors suggests that coloration is actively maintained in adults and not simply the result of differences in gene regulation during development.

In orange epidermal tissue, we observed many over-represented GO terms associated with muscle (Figures 2–4). Previous research has shown that smooth muscle proteins are a cellular component of the chromatophores that are

responsible for fish skin coloration and suggested that they may control the movement of intracellular bodies (melanophores, xanthophores, and erythrophores) within the chromatophore (Meyer-Rochow et al., 2001). The over-representation of muscle related GO terms in orange tissue might suggest greater motility control of the intracellular bodies in this skin type (Li et al., 2015), which might facilitate changes



**FIGURE 3 |** Gene ontology (GO) categories significantly enriched for both pairwise color comparisons for each epidermal tissue by “cellular component” (CC) using Mann-Whitney U tests based on ranking of signed log *p*-values computed using *EdgeR*. Results indicate the GO domain (CC), the subdomains discussed in text (“muscle” and “extracellular matrix”), the specific GO terms, and enrichment direction observed consistently in each epidermal tissue color.

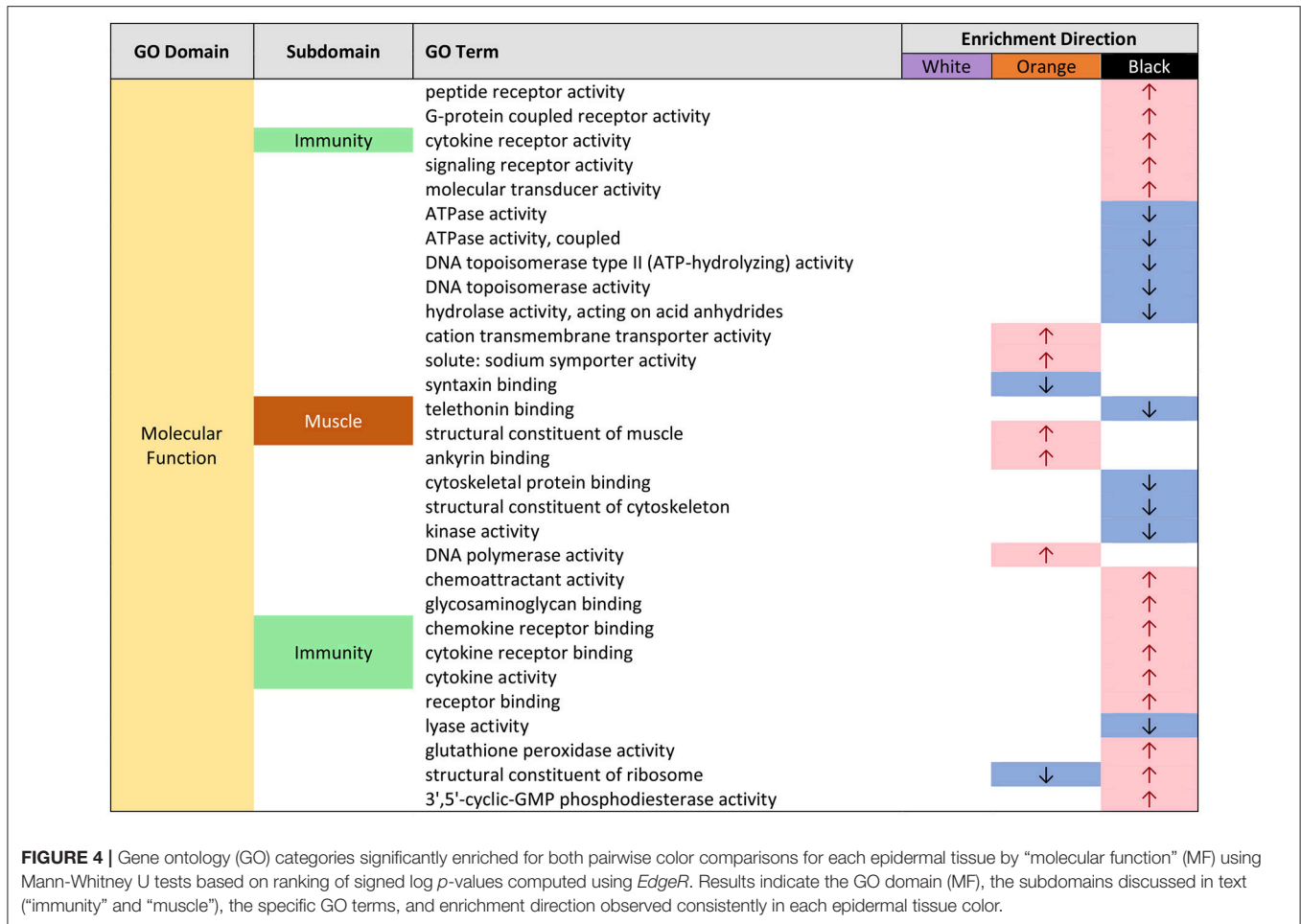
in orange coloration. This may explain how *A. percula* are able to rapidly adjust their orange coloration in response to light intensity and background color (Yasir and Qin, 2009a,b).

In white epidermal tissue, we detected few over- or under-represented GO terms, consistent with the relative paucity of knowledge regarding the genetic basis of structural coloration in vertebrates (Hubbard et al., 2010). The few GO terms that were consistently enriched were associated with cellular components

and within this GO ontology several GO terms associated with extracellular matrix were consistently underrepresented in white tissue (Figure 3). It seems plausible that these over- and under-represented categories might be related to the formation of the white/UV structural color, which is formed by stacks of intracellular guanine crystals in other fishes (Levy-Lior et al., 2010; Zhao et al., 2012; Gur et al., 2015).

In black epidermal tissue, we observed an over-representation of many GO terms associated with immune function:





**FIGURE 4 |** Gene ontology (GO) categories significantly enriched for both pairwise color comparisons for each epidermal tissue by “molecular function” (MF) using Mann-Whitney U tests based on ranking of signed log *p*-values computed using *EdgeR*. Results indicate the GO domain (MF), the subdomains discussed in text (“immunity” and “muscle”), the specific GO terms, and enrichment direction observed consistently in each epidermal tissue color.

leukocyte/lymphocyte movement, cytokines, inflammation, immune response, and wounding response (Figures 2, 4). Upregulation of immune defense in melanic tissue is consistent with previous research: in invertebrates, melanin has been shown to act in defense reactions and wound healing (Sugumaran, 2002; McGraw, 2005); in vertebrates, melanocytes and melanosomes are known to inhibit skin infection by microorganisms, and melanin may play a role in the regulation of immunological cytokines (Mackintosh, 2001). This raises the question of why *A. percula* exhibit black, melanin-based pigments and its associated up-regulation of immune system function? Some insights may be gained by examining the distribution of melanistic *A. percula* in nature. Militz et al. (2016) found that melanistic *A. percula* are much more frequent in the gigantic sea anemone *Stichodactyla gigantea* when compared to the magnificent sea anemone *Heteractis magnifica*. *S. gigantea* is a more aggressive anemone that can sting badly and remove skin from fingers (Buston personal observation). It seems plausible that melanin pigments and associated up-regulation of immune defense may facilitate wound-healing and allow *A. percula* to tolerate these aggressive host anemones.

In conclusion, here we have reviewed the proximate and ultimate causes of conspicuous coloration in clownfishes of the

genus *Amphiprion*. The conspicuous coloration of this genus (i) has a heritable genetic basis, (ii) exhibits developmental and environmental plasticity, (iii) has multiple plausible signaling functions, and (iv) exhibits a strong phylogenetic component. By generating a *de novo* transcriptome for *A. percula*, conducting differential gene expression analyses and GO enrichment analyses, we have revealed an over-representation of transcripts associated with immune function associated with melanin pigments, a pattern previously observed in many other vertebrates and invertebrates. Relating this to what is known about the distribution of melanistic morphs in the wild, suggests that melanism may serve a protective function when these fishes are associated with more noxious anemones. This study represents a first step in an integrative investigation of the proximate and ultimate causes of conspicuous coloration of these iconic coral reef fishes.

### DATA AVAILABILITY STATEMENT

All raw reads are archived in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) under accession number PRJNA471968, with transcriptome



assembly and annotation files available at <http://sites.bu.edu/davieslab/data-code/>. Additional count data and EdgeR output files can be found in **Supplementary Materials**.

## AUTHOR CONTRIBUTIONS

SD, SM, and PB designed the project; AM, GS and SM acquired the data; AM, SD, and SM analyzed the data; AM, SD, SM, and PB wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00284/full#supplementary-material>

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

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