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SYMPOSIUM

Social Aggression, Experience, and Brain Gene Expression in a Subsocial Bee

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Synopsis The genetic mechanisms behind aggressive behaviors are important for understanding the formation of dominance hierarchies, and thus social systems in general. Studies into the effects of social experience and agonistic contest outcomes have shown significant changes in brain gene expression resulting from repeated winning and losing, as well as changing dominance rank, primarily in obligately social species. However, our knowledge of the genetic underpinnings of behavior in subsocial organisms is relatively poor, yet understanding the behavioral genetics of this simplest form of sociality provides the basis for understanding all other forms of social living. Here, we measured the effects of aggression on brain gene expression in the subsocial bee, *Ceratina calcarata*, in individuals that had experienced repeated winning, repeated losing, or a change in rank during repeated encounters. Consistent winning accounted for the majority of variation in brain gene expression, followed by changing rank over maintaining rank. Candidate genes for aggression are identified through comparative transcriptomics across 21 invertebrate and 6 vertebrate taxa. Lastly, we identified significantly over-represented *cis*-regulatory elements, namely transcription factor binding motifs deeply conserved across a wide range of taxa and broadly implicated in differential regulation of genes related to aggressive/dominant behavior. We present evidence for both genetic and *cis*-regulatory mechanisms for aggression that may have broad importance to social evolution.

Introduction

Genomic studies of aggressive behavior are key to our understanding of social evolution (Robinson et al. 2008; Gadau et al. 2012; Rehan et al. 2014a et al). Aggression is observed across many taxa (Huntingford 1976). In a socially organized species, it may drive the establishment of dominance relationships (Syme 1974; Wong and Balshine 2011). Such dominance hierarchies precede reproductive division of labor and complex social organization (Gadagkar 1980; West-Eberhard 1967). Thus, understanding many aspects of social evolution requires first understanding the genetic basis for aggression. Genes involved in memory and learning have been implicated in aggressive behavior (Fischman et al. 2011; Nighorn et al. 1991; Woodard et al. 2011), as have those involved in axonogenesis (Edwards et al. 2006; Toth et al. 2014). These genes are both important to first form brain connections and second recognize competitors. Determining conserved genes for behavior and the context in which they are expressed are foundational to understanding the proximate mechanism of aggression, reproductive division of labor, and ultimately social evolution (Rehan and Toth 2015; Toth and Rehan 2017). Because conserved genes are similarly expressed in dominance and aggressive contexts across taxa, they may be important to both aggressive behavior and dominance hierarchy formation (Toth et al. 2014).

Agonistic encounters have lasting effects on social behavior, and prior performance tends to predict the outcome of future interactions (Hsu et al. 2006). Accordingly, in an interaction resulting in a "winner" and "loser," the individual that wins is more likely to win repeatedly in future interactions, and the losing individual is likely to lose again (Rutte et al. 2006).

Given that social groups necessarily involve repeated interactions among the same individuals, such behavioral interactions can be strongly indicative of dominance hierarchy formation and basic social organization.

Beyond focal genes of interest, transcription factors are regulatory elements shared among numerous genes and deeply conserved across a broad range of species. Underlying conservation of a number of *cis*-regulatory elements has been linked to aggressive and social behavior in birds (Clayton 2013) and honey bees (Lutz and Robinson 2013). Neuroendocrine signaling transcription factor motifs are associated with behavioral function in honey bees, mice, and sticklebacks (Rittschof et al. 2014). The goal of these studies is to link specific genes or molecular elements to behaviors of interest. For example, a transcription factor binding motif associated with *Adh Transcription Factor 1* (*Adf1*) is linked to learning and memory (Cristino et al. 2006), while *COUP Transcription Factor 1* (*NR2F1*) initiates transcription of its target genes in specific behavioral contexts (Rittschof et al. 2014). However, these studies have primarily focused on obligately social species, with far less attention given to aggressive effects on sociality in species representing earlier stages in the evolution of complex sociality.

The small carpenter bee, *Ceratina calcarata*, is subsocial (Rehan and Richards 2013) and shows behavioral similarities to primitive dominance hierarchies (Sakagami and Maeta 1977; Withee and Rehan 2016). Solitary species rarely interact and do not form dominance hierarchies in nature, but still exhibit social aggression in forced association experiments (Boesi and Polidori 2011; Flores-Prado et al. 2008; Breed et al. 1978; Wcislo 1997). Here, we present brain gene expression data for *Ceratina calcarata* females as a result of repeated agonistic interactions in order to better understand the genetic basis of aggression and the corresponding effect of experience. In this study, we characterize specific expression patterns based on winning over losing and on maintaining versus switching rank. We also compare results to genes upregulated in socially dominant individuals across taxa in order to identify conservation of genes associated with aggression and social evolution. Finally, we identify transcription factor binding motifs associated with differentially expressed genes in order to assess *cis*-regulation of gene expression and to compare these elements with those across diverse taxa.

Materials and methods

Bee sampling and behavior trials

Fifty-four *Ceratina calcarata* females were collected in Strafford County, New Hampshire (43°08'N 70°55'W), between June 11 and July 10, 2014, during peak reproductive activity, when they are most aggressive (Rehan and Richards 2013). Dominant and subordinate behaviors were quantified using the circle tube method (Breed et al. 1978). Two bees are introduced into a small tube simultaneously to eliminate established territorial effects (Wcislo 1997). Behavioral trials were performed for 20 min, at the first sign of activity from either individual. All interactions were recorded as aggressive, avoidant, following, or tolerant, based on the previously established ethogram and methodology for *C. calcarata* (Rehan and Richards 2010 2013; Figure 1). Aggression and avoidance are negatively correlated (Withee and Rehan 2016), allowing us to label the more aggressive individuals "winners" and the more avoidant individuals "losers". This terminology followed standard dominance indices (Bang et al. 2010) and allowed for simplified binary assessment of dominance rank as per Manfredini and colleagues (2013).

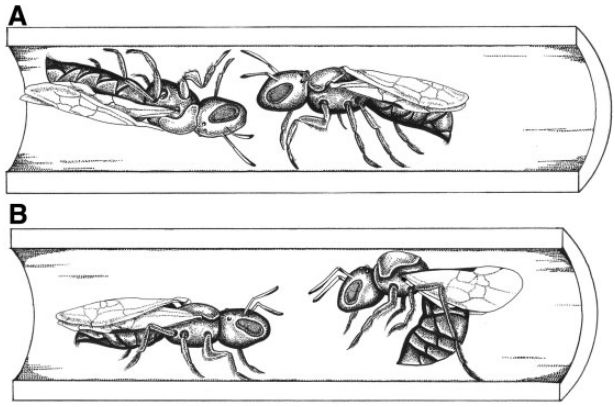

Fig. 1 Examples of (**A**) tolerance behavior and (**B**) aggressive behavior in *Ceratina calcarata* during frontal encounters, as observed in a circle tube trial. While the majority of interactions tend to be tolerant in this species, individuals often assert aggressive dichotomies similar to those of a dominance hierarchy.

After the initial behavioral trial, individuals were given a 20-min recovery period and then re-paired randomly with a second individual for another 20-min trial in a new circle tube. Individuals were randomly re-paired to achieve all possible combined outcomes after two trials. Individuals were again assigned ranks of "winner" and "loser" based on behavior differences, and they were summarized with behavioral classes based on combined outcomes of the two trials, respectively: winner–winner = WW ($n = 17$), winner–loser = WL ($n = 12$), loser–winner = LW ($n = 10$), and loser–loser = LL ($n = 15$). Immediately after completion of the second behavioral trial, bees were flash frozen in liquid nitrogen and stored at -80°C.

RNA sequencing and differential expression analyses

RNA was extracted using the Qiagen RNeasy Mini Kit from the brain tissue of nine individuals per each of the four behavioral classes (WW, WL, LW, LL). Three brains were pooled per extraction, with three biological sequencing replicates for each of the four behavioral classes, totaling 12 RNA samples. RNA libraries were prepared and sequenced at Genome Quebec using the Illumina TruSeq RNAseq sample preparation kit. RNA libraries were multiplexed with six samples per lane and sequenced for 100 bp PE reads on two lanes on a HiSeq 2500, producing 422 Mb of 100 base pair paired-end reads for all samples (Supplementary Table S1). Raw data have been submitted to the NCBI sequencing read archive (SRA) with accession number SRX1547420. RNA reads were aligned to the *C. calcarata* genome (Rehan et al. 2016) and differential gene expression analyses were conducted in R (version 3.1.3; detailed methods in Supplementary File 1). Principal components analysis (PCA) was performed in FactoMineR (version 1.25; Husson et al. 2013).

Comparative analyses

Differentially expressed genes (DEGs) and GO terms among the four behavioral categories (WW, WL, LW, and LL) in *C. calcarata* were compared to all known published findings based on aggression versus avoidance and dominance versus subordinance in both vertebrate and invertebrate studies including: ten bee species, two species of paper wasp, eight ant species, fruit fly, three-spined stickleback and African cichlid, laying hen, mouse and Wistar rat, and domestic dog (Supplementary Table S2). We identified putatively homologous sequences between *C. calcarata* and other species using tBLASTx (*E*-value $\leq$ 1*e*-4). We tested for significant overlap in differentially expressed genes between pairs of species using a two-tailed hypergeometric test.

Detection of *cis*-regulatory elements

To establish *cis*-regulatory elements, we looked for common transcription factor binding motifs in flanking regions of each *C. calcarata* DE gene set (WW, WL, LW, LL). We searched for consistent, repeated instances of motifs near each gene set to identify transcription factor regulation of that gene set, based on windows of 1 kb and 5 kb upstream (these windows yielded different results and so were not considered redundant). We used the Motif Enrichment Tool (Blatti and Sinha 2014) to test for these motifs using honey bee (*Apis mellifera*) orthologs included in the interface. The highly conserved motif scoring profiles were compiled from core FlyFactorSurvey motifs (Zhu et al. 2011), JASPAR (Portales-Casamar et al. 2010) and TRANSFAC PUBLIC (Matys et al. 2003) vertebrate motifs, and reported with a significance threshold of $P < 0.001$ correcting for multiple testing (Benjamini and Hochberg 1995).

Results

Brain gene expression

Differential gene expression in *C. calcarata* brains was treatment-specific, with notable inverse patterns of regulation between opposite behavior classes (Supplementary Figure S1). In total, there were 457 differentially expressed genes across all treatments. Individuals ranked as winners in the first trial had high similarity in expression profiles (80% bootstrap support), as did individuals ranked as losers in the first trial (87% bootstrap support; Supplementary Figure S1). WW individuals displayed significant downregulation of genes that were upregulated in LL individuals, and *vice versa*. Likewise, expression of genes in WL and LW individuals were largely inversely related (Supplementary Figure S1). These differences between conserved rank (WW and LL) and swapped rank (WL and LW) can be seen in a PCA of expression patterns (Figure 2). Winning in both trials accounted for 68% of variation in expression, after which swapping rank over maintaining rank (WL-LW > WW-LL) accounted for 19% of variation. The outcome of the final behavioral trial (second trial win over second trial loss, WW-LW > WL-LL) accounted for the remaining 13% of variation in gene expression.

Of the 172 DEGs in aggressive individuals (WW > LL), among the most highly expressed were 13 with known functions (Figure 3; Supplementary Table S3). These genes include those with known brain synaptic function (e.g., the genes *headcase*, *couch potato*, *still life*, *longitudinals lacking*, *ß Spectrin*, *ultraspiracle*, and *paralytic*), learning and memory (*dunce*, *radish*, and *Synapsin*), transport (*paralytic*), and transcription regulation (*pixie*, *Eip93F*, *Sin3A*). Synaptic action, learning and memory, and transcription regulation genes were also significantly upregulated in females that swapped rank over those that maintained rank, including *headcase*, *couch potato*, *ultraspiracle*, and *dunce* (WL-LW > WW-LL; Supplementary Table S3).

Gene ontology enrichment

A total of 109 GO terms were significantly enriched in *C. calcarata* females from behavioral comparisons: WW > LL ($n = 94$), WL-LW > WW-LL ($n = 10$), and

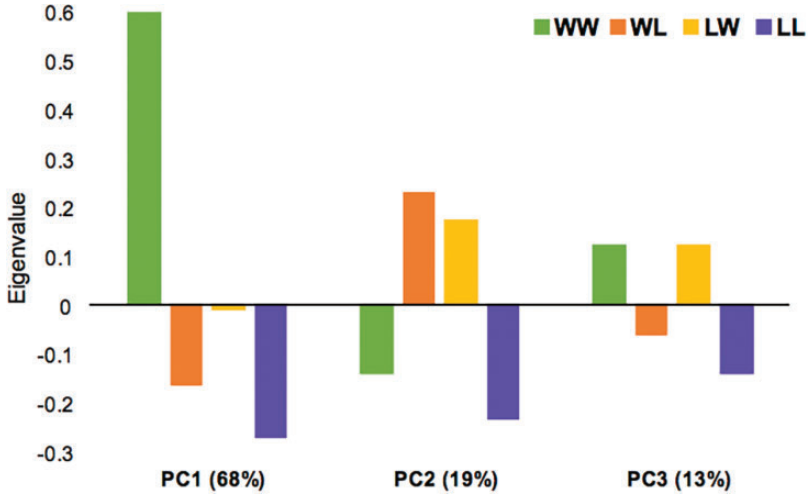

Fig. 2 Principal component analysis (PCA) of gene expression across behavioral classes. Repeated dominance/aggression (winning in both trials, WW) compared to all other classes accounted for 68% of the variation observed, while switching rank over maintaining rank (WL-LW > WW-LL) accounted for 19%, and a second trial win over a second trial loss (WW-LW > WL-LL) accounted for 13% of variation in gene expression.

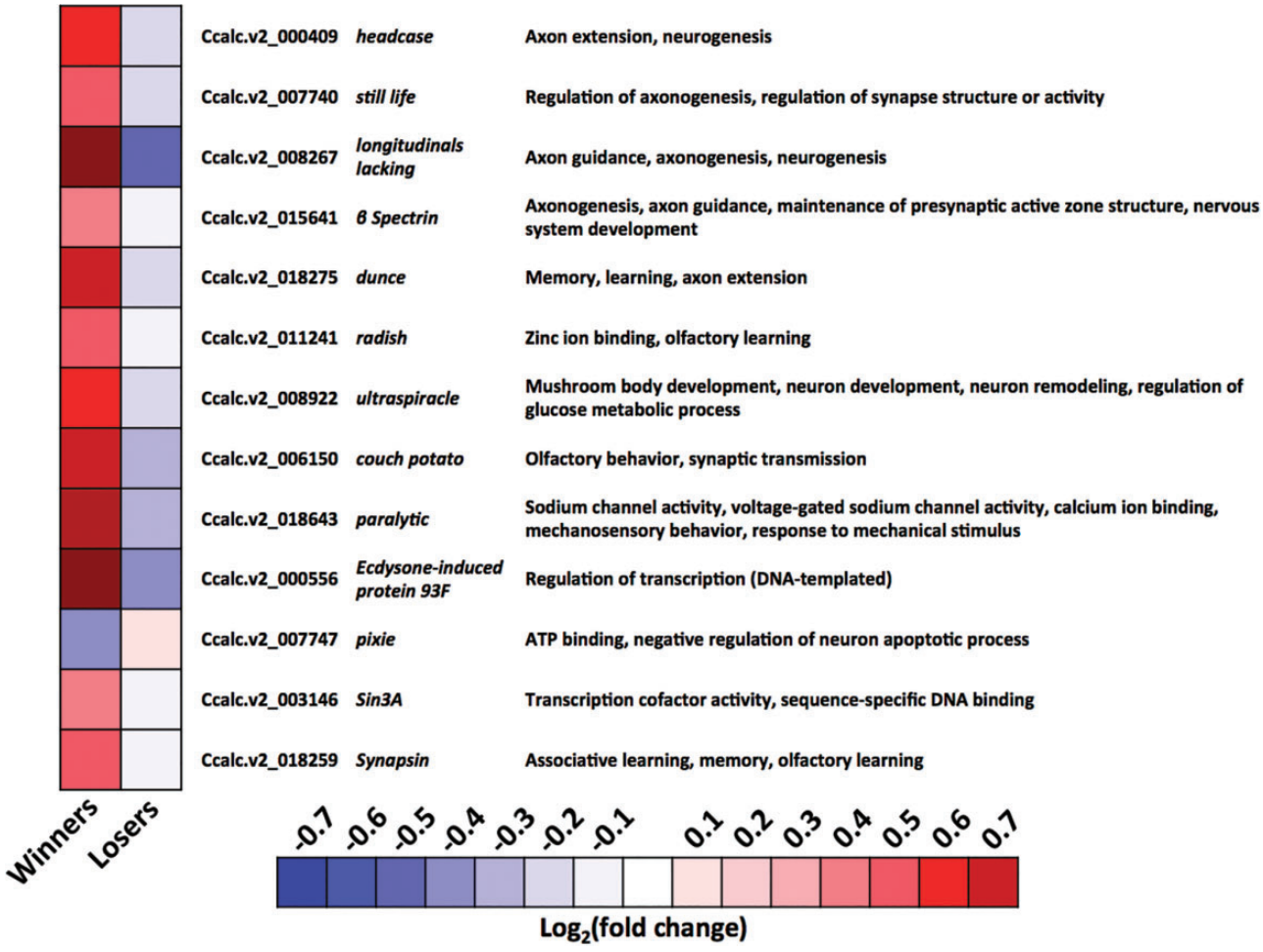

Fig. 3 Expression patterns of top 13 most highly expressed, behaviorally relevant, and significantly differentially expressed genes (FDR < 1 corrected $P < 0.05$) for WW and LL class females. Putative gene names and functions are based on *Apis mellifera* and *Drosophila melanogaster* orthologs. A total of 457 genes were significantly differentially expressed for all conditions.
Blue = downregulated, red = upregulated, white = not differentially expressed between classes, value = $\log_2$(fold change). The full list of DEGs are presented in Supplementary Table S3.

WW-LW > WL-LL ($n = 5$). Several functional terms were related to transcription regulation (Supplementary Table S4), which were overexpressed in WW > LL. Other significantly enriched terms, also overexpressed in WW > LL, involved synaptic transmission (GO:0007268), axonogenesis (GO:0007409), and olfactory learning (GO:0008355). All terms were treatment specific, with no overlap at all between conditions (Supplementary Table S5).

Comparative analysis

Gene expression related to aggression and repeated winning experience in *C. calcarata* (WW > LL) largely corresponded with expression associated with aggression and social dominance in 16 other species (Supplementary Table S6). Notable among the genes that matched aggression and dominance contexts in other studies were *dunce*, *longitudinals lacking*, and *orb2* (Figure 3). Enriched GO terms for *C. calcarata* matched to aggression, social dominance, and social organization contexts in studies on 23 species (Supplementary Table S7). These terms include: axonogenesis (GO:0007409), brain function and learning (GO:0007268 and GO:0008355), and transcription regulation (GO:0003700, GO:0006355, GO:0006357, and GO:0043565).

Transcription factor (TF) binding motif enrichment analyses revealed 250 binding motifs associated with differentially expressed *C. calcarata* genes (Supplementary Table S8). These binding motifs are associated with TFs that function in the regulation of gene expression (*USF*, *NR2F1*, and *E2F1*), memory and learning (*Adf1*, *CREB*, and *CREB1*), and neurogenesis (*POU3F2*; Supplementary Table S9). Motif matches were found in 10 species (Supplementary Table S9). Additionally, of the 95 significant DEGs with known functions found for *C. calcarata*, 12 were transcription factors associated with aggression, rank change, and second trial outcome (Supplementary Table S10).

Discussion

Here, we provide candidate genes for aggressive behavior and social experience from *C. calcarata*. Complete aggression (winning in both trials) over complete submission (losing in both trials) revealed the most dramatic changes in brain gene expression, but changing rank between trials, as well as the specific outcome of the second trial, both had significant differences. Comparisons across taxa indicate that many of the most highly differentially expressed genes associated with aggressive behaviors function in transcription regulation, axon and neuron formation, and memory/learning, suggesting that these DEGs and GO pathways are conserved across disparate taxa and ubiquitously involved in social aggression.

Winning and losing resulted in what may constitute dominant and subordinate patterns of brain gene expression, and the experience of repeated winning/losing or of reversed rank had its own effects on expression. This is confirmed by the sources of variance in gene expression (Figure 2): consistent aggression over consistent avoidance accounts for the majority of variance (68%), followed by switching of rank (19%). Consistent outcome (maintenance of rank) matches the expectations of the "winner" and "loser-effect" of social experience (Hsu et al. 2006; Rutte et al. 2006), and repeated behavioral outcomes are known to have gene regulatory and physiological consequences (Maruska 2015). Thus, the changes in brain gene expression we see in recurrent winners and losers may indicate the beginning stages of biological differences between dominant and subordinate. Conversely, the switching of rank between trials reverses any such effect, resulting in more similar gene expression patterns between individuals with reversed rank, as well as more similar gene expression between those with consistent rank, regardless of aggression and dominance context (Figure 2). Experiments in the advanced eusocial fire ant *Solenopsis invicta* found similar differences in brain gene expression due to social experience (Manfredini et al. 2013). Interestingly, those that maintained rank between repeated trials (winning or losing both times) had more similar brain gene expression than those that switched ranks (winning followed by losing or *vice versa*). Our study found that conserved genes associated with social dominance in a eusocial ant are also differentially regulated during agonistic interactions in a non-eusocial bee.

Candidate genes with known GO function in axonogenesis were significantly upregulated and overexpressed in repeatedly winning females and in those switching rank, suggesting similar genetic effects in the brain resulting from both aggression and repeated or reversed experience. One of these genes, *ß Spectrin*, is upregulated in aggressive primitively eusocial paper wasps (Toth et al. 2014), as well as in primitively eusocial and non-eusocial bee species over advanced eusocial species (Woodard et al. 2011). Moreover, in the advanced eusocial fire ant, *ß Spectrin* was significantly upregulated in winners over losers (Manfredini et al. 2013). These similar contexts and consistent upregulation in dominant individuals suggest a conserved function of *ß Spectrin* for dominance and aggression.

This conclusion is further supported by gene ontology enrichment for axonogenesis (GO:0007409) in aggressive *C. calcarata* (WW > LL) and in paper wasps regardless of dominance rank (Berens et al. 2014). Axonogenesis also appears to be importantly linked to social over solitary behavior, as this term was only upregulated in group-housed stickleback and not separately-housed individuals (Greenwood and Peichel 2015). Furthermore, the axonogenesis gene *longitudinals lacking* was upregulated in *C. calcarata* second trial winners over second trial losers, and this gene has been implicated to function in aggression (Edwards et al. 2006; Toth et al. 2014). However, in advanced eusocial species, *longitudinals lacking* is upregulated in losers over winners (Helmkampf et al. 2016; Manfredini et al. 2013). Given the importance of aggressive behavior to many forms of sociality (Syme 1974; Wong and Balshine 2011), and the contexts of expression for axonogenesis genes specific to different levels of social complexity, axonogenesis genes upregulated during aggressive behavior may provide an underlying mechanism for the formation and maintenance of dominance hierarchies. Moreover, the process of axonogenesis could be responsible for the formation of new neuronal networks in the brain following the outcomes of behavioral contexts.

Several genes were implicated in memory and learning function, behaviors that have separately been strongly linked to aggression (Edwards et al. 2006). The gene *dunce* was upregulated in aggressive females and in rank-changing females in this study, matching expression of winners over losers in fire ant (Manfredini et al. 2013), old foragers over young nurses in honey bee (Alaux et al. 2009), and group-housed over separate individual stickleback fish (Greenwood and Peichel 2015). *Dunce* has been repeatedly linked to aggressive behavior (Fischman et al. 2011; Nighorn et al. 1991; Woodard et al. 2011), suggesting conservation of this function in *C. calcarata* as well. Upregulation associated with aggression was similarly found for the olfactory learning gene *radish* and the long-term memory gene *orb2*. A single upregulated GO term for olfactory learning, GO:0008355, was associated with more aggressive African honey bees over European honey bees (Alaux et al. 2009), as well as in genes associated with honey bee caste differences (Grozinger et al. 2007), suggesting dominance and social structure relevance. It is likely that memory and learning are important for both the winner-effect and loser-effect of social experience, potentially explaining their relationship with aggressive behavioral function. These behaviors are key characteristics of species with more complex social life histories (Dukas and Real 1991), and have been observed in *C. calcarata* previously (Withee and Rehan 2016). These memory/aggression genes may be part of the genetic mechanism for the winner- and loser-effects.

Lastly, transcription regulation genes were also substantially upregulated in this study. Orthologs of both *Eip93F* and *pixie* were upregulated in all three behavioral contexts for *C. calcarata* (WW > LL, WL-LW > WW-LL, and WW-LW > WL-LL), matching upregulation of workers over queens (Grozinger et al. 2007) and nurses over foragers (Whitfield et al. 2003) in honey bee, as well as primitively eusocial bee species (Woodard et al. 2011). A variety of transcription regulation GO terms were also enriched in WW > LL individuals. DNA-binding (GO:0003700) is enriched in aggressive *Drosophila melanogaster* (Edwards et al. 2006), in paper wasp queens over workers (Ferreira et al. 2013), and in both zebra fish (Lopes et al. 2015) and mice (Rittschof et al. 2014) responding to territorial intrusion. This provides not only additional evidence that regulation in particular was associated with aggressive behaviors, but that this function is conserved across diverse taxa. The significantly enriched DNA-binding and transcription regulation terms GO:0006355, GO:0006357, and GO:0043565 also matched these same contexts and species. The recurrence of genes and ontology terms involved in regulation, as well as the breadth of taxonomic coverage, suggest conserved regulatory mechanisms for the observed behaviors. Deeply conserved genes and regulatory pathways may have been coopted during social evolution and the context in which they are expressed are foundational to understanding genetic underpinnings of animal behavior, and how behavior evolves on a genetic level (Rehan and Toth 2015; Toth and Rehan 2017).

Conserved *cis*-regulatory elements

Significant enrichment for transcription factor binding motifs matched honey bee, *Drosophila*, five ant species, and two fish species with behavioral contexts similar to those of *C. calcarata* (Table 1), suggesting conservation of behavioral function for these regulatory factors. For example, the motif associated with *Adh Transcription Factor 1* (*Adf1*) was significantly enriched in winners over losers in *C. calcarata* behavioral contexts (WW > LL, WL-LW > WW-LL, and WW-LW > WL-LL), and matched enrichment in honey bee workers over queens (Cristino et

Table 1 A selection of matches to 12 transcription factor binding motifs associated with significantly upregulated (FDR $P < 0.05$) DEGs

Motif	Known function of associated transcription factor	Species
Adf1	Learning/memory, long-term memory, olfactory learning	*Apis mellifera*
NRF2	Regulates the expression of antioxidant proteins	*Apis mellifera*
USF	Activates transcription	*Apis mellifera*
NR2F1	Stimulates transcription initiation	*Apis mellifera, Gasterosteus aculeatus*
ZNF354C	Nucleic acid binding; sequence-specific DNA binding	*Apis mellifera, Gasterosteus aculeatus*
CREB1	Long-term memory	*Danio rerio*
E2F1	Transcription factor activity, sequence-specific DNA binding, transcription factor binding	*Danio rerio*
REST	Represses neuronal genes in non-neuronal tissues; negative regulator of neurogenesis	*Danio rerio*
CTCF	Regulation of RNA splicing, insulation	*Drosophila melanogaster*
CREB	Long-term memory	Five ant species
POU3F2	Regulation of neurogenesis	*Gasterosteus aculeatus*
PPARG	Regulates glucose metabolism	*Gasterosteus aculeatus*

A full list of motifs, matches, and references may be found in Supplementary Table S9.

al. 2006). The known function of *Adf1* is learning and memory. Two additional, related motifs associated with memory were enriched in *C. calcarata* with significant co-occurrence in other species: the motif for *CREB*, which was found conserved across otherwise highly diversified ant genomes (Simola et al. 2013), and the motif for *CREB1*, which was enriched in zebra fish winners and losers (Oliveira et al. 2016). Based on the observed relationship of memory and learning genes with aggressive behavior, these *cis*-regulatory elements likely have conserved evolutionary importance for the regulation of such aggression/dominance social behaviors. The use of regulatory elements to repurpose genes for behavioral functions can have large effects on overall social organization (Bloch and Grozinger 2011; Toth and Robinson 2007, 2010). That the same regulatory tools here may be used in response to aggression across multiple taxa and social contexts could have implications for the mechanistic origins of simple dominance hierarchies and other forms of sociality.

Future research focusing on aggression and brain gene expression in incipiently social taxa is needed (Rehan et al. 2010; 2011; 2014b). Additional, closely related species are important to our understanding of the conserved mechanisms controlling aggression in an earlier stage of social evolution (Rehan and Toth 2015). Additionally, the differential gene expression and *cis*-regulatory elements identified in this study may serve as prime candidates for further investigation of RNAi gene silencing and specific causal expression effects into the genetic mechanisms that shape aggressive behavior.

Conclusions

Aggression and social experience significantly affected brain gene expression in *C. calcarata* females after repeated agonistic encounters. This resulted in very specific patterns of gene expression whereby repeated winning and losing had inverse gene expression effects, and initial rank was linked to greatest similarities in gene expression. The differentially expressed genes putatively function in axonogenesis, learning/memory, and transcription regulation. More broadly, these genetic functions may be associated with aggression in general, and with the formation of simple dominance hierarchies. A variety of *cis*-regulatory elements show similar and consistent patterns of enrichment across multiple taxa, suggesting regulatory mechanisms may play a substantial role in shaping aggression and social behavior. Specific similarities in gene expression, ontology, and *cis*-regulatory elements found here may indicate potential conservation of function across taxa. The notable differences, meanwhile, may be a function of differences in social complexity. Our findings provide targets for further study of the specific genetic mechanisms for aggressive behavior, as well as their associated implications for social species. If the expression patterns found across species are indeed dependent on level of social organization, then the genes identified here, as well as the *cis*-regulatory elements that regulate them, may be useful for continued study into the evolutionary origins of aggressive and social behaviors.

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Supplementary data

Supplementary Data available at *ICB* online.

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