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**DOMINANCE AND EXPERIENCE:
AGGRESSION AND THE EVOLUTIONARY ORIGINS OF SOCIAL BEHAVIOR**

BY

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THESIS

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	v
CHAPTER	PAGE
GENERAL INTRODUCTION.....	1
I. CUMULATIVE EFFECTS OF BODY SIZE AND SOCIAL EXPERIENCE ON AGGRESSIVE BEHAVIOR IN A SUBSOCIAL BEE.....	18
Introduction.....	18
Methods.....	24
Results.....	28
Discussion.....	31
Tables and Figures.....	36
II. SOCIAL AGGRESSION, EXPERIENCE, AND BRAIN GENE EXPRESSION IN A SUBSOCIAL BEE.....	40
Introduction.....	41
Methods.....	44
Results.....	49
Discussion.....	52
Tables and Figures.....	60
GENERAL CONCLUSIONS.....	65
LIST OF REFERENCES.....	68

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ABSTRACT

DOMINANCE AND EXPERIENCE: AGGRESSION AND THE EVOLUTIONARY ORIGINS OF SOCIAL BEHAVIOR

by

Jacob R. Withee

University of New Hampshire, May, 2016

Sociality as a life history strategy has many overt benefits, but its origin from solitary living is not fully understood. The cooperation necessary for formation of even basic social groups can present natural selection paradoxes that many models are unable to reconcile. Conversely, aggression is a key component to the formation of dominance hierarchies, a very basic form of social group. These hierarchies can give way to reproductive hierarchies, which are in turn the basis for some of the most complex forms of social organization. The focus of this thesis is to use aggression in an incipiently social bee species to characterize behavioral and genetic patterns useful for further study of the mechanisms behind the evolution of sociality.

In agonistic encounters and contests between conspecifics, the outcome can be determined by physiological traits like size, age, or reproductive activity, by prior experience, or by a combination of these factors. Past experience can inform future efforts, resulting in repetition of the same outcome, while physiological traits can create hierarchies of size, age, etc. Repeated pairings of small carpenter bees (*Ceratina calcarata*) by circle tube forced association revealed cumulative roles for both size and

experience in determining contest outcomes. Size predicted initial dominance, while experience determined subsequent outcomes. I posit that these results may hold true for other species at simple stages of social complexity, and may be important for behavioral studies of social evolution.

The brain gene expression corresponding with these behavioral results similarly yielded targets for evolutionary study. Behavioral syndromes resulting from repeated interactions in the first experiment resulted in significant upregulation of genes of memory, learning, axonogenesis, and transcription regulation in dominant individuals. These genes and their behavioral contexts matched those of a variety of taxa, as did a number of gene ontology terms with similar functions. Enrichment of several transcription factor binding motifs also revealed potential behavioral functions for *cis*-regulatory elements that are conserved across taxa. Overall, the results suggest key roles for genes, ontology terms, and *cis*-regulatory elements in behavioral response to aggression, in both simple and complex social groups. I propose that these be used as the foci for future experiments in order to determine the relative role of each of these target factors.

Combining behavioral and genetic data with comparisons to a wide range of taxa gives a more detailed look at the factors that may have influenced the evolution of sociality. These behavioral patterns and target genes/regulatory elements may provide valuable insights to further understanding the origins of animal societies.

GENERAL INTRODUCTION

The evolution of social behavior is important to a range of taxa, and animal societies present some of the most interesting examples, from marine shrimp to humans. In particular, eusocial colonies provide some of the most intriguing questions about cooperation and the evolutionary development of such social structures. Given the necessary high level of cooperation and complexity involved, the evolutionary origins of eusocial behavior can tell volumes about the manner in which any number of social systems formed. This, in turn can help broaden our understanding of animal societies in general.

1) Social Evolution

1.1) A Natural Selection Paradox

Much of the interest in understanding social evolution comes from the inherent evolutionary problem that cooperation represents. Evolution by natural selection logically favors selfishness, yet, in what is known as the paradox of altruism, numerous species display a level of cooperation that contradicts basic fitness models (Hamilton, 1972). Cooperation is any behavior that benefits another individual and that has evolved at least in part as a result of this benefit (West *et al.*, 2007a), but it is not fully explainable by any one hypothesis. Broadly, keeping guard over offspring while others forage reduces predator and parasite threats, and any system involving cooperative care for offspring tends to lead to mutual assured fitness gains regardless of immediate relatedness (Kocher & Paxton, 2014). The paradox emerges when an individual

foregoes any amount of the direct fitness it might gain by propagating its own genes through its own offspring for the express purpose of cooperation. This is the reason for the use of the term 'altruism' in describing this phenomenon. Thus, relatedness is a major factor thought to drive cooperation. This is described by the theory of kin selection, wherein indirect fitness is gained by cooperatively raising relatives' offspring, even if the cooperating individuals themselves do not reproduce directly (Hamilton, 1964). Genetic material is still propagated to a new generation, and depending on the relatedness of the reproducing and cooperating individuals, the latter still receives some degree of the fitness gains it might achieve through direct reproduction. As long as the cost of cooperating does not exceed the relatedness to and the benefit of the reproductive individual, cooperation will result in gene proliferation in a population, and the adaptation for cooperation remains. This has long been accepted as a mathematic principle, 'Hamilton's Rule' (Hamilton 1964). Cooperation, however, is only one component of sociality.

1.2) Preconditions for Sociality

In order for any species to be social, individuals must have several important pre-adaptations: extended maternal care, individual longevity, tolerance for one another, and a predisposition for staying together at the nest (Michener, 1985; Nowak *et al.*, 2010; Wilson, 1971). Prolonged care from the mother is the most basic of all social organization, involving behaviors as simple as nest maintenance, guarding, and keeping developing offspring clean (Sakagami & Maeta, 1977). Staying and attending in this

manner marks the difference between solitary and social. Longevity, meanwhile, is necessary for any generational overlap and consequent intergenerational interaction, an important component of the most complex social systems. Generational overlap allows for certain dominance-based interactions that may drive social complexity, and longer-lived species are thus in a position to establish greater social organization, such as multi-female provisioning (Rehan & Richards, 2010). Without tolerance, no social grouping would be possible, since tolerance is the basis for cooperation. Without a group living situation, no opportunity for such cooperation can exist. For sociality to evolve in any form, individuals therefore must stay together and exhibit mutual tolerance, after which all further organization may follow, allowing for the formation of societies (Wilson, 1971).

1.3) Social Complexity and Definitions

From least to most complex, sociality can be described generally by the categories of solitary, subsocial, and eusocial (Michener, 1974), all determined by the amount and type of social behavior. In the context of social evolution, social behavior specifically refers to interactions of conspecifics in a shared nest, exclusive of mating or purely agonistic encounters (Kocher & Paxton, 2014). On this basis, species are considered solitary if their only contact with conspecifics is to mate (Michener, 1974). Free of cooperation, solitary species represent the most straightforward life history strategy: individuals using all resources strictly for their own survival and reproduction. In contrast to solitary species, whose maternal care may be brief or nonexistent,

subsocial mothers provide long-term care to offspring, sometimes until the young reach adulthood (Michener, 1974; Wilson, 1971). The fitness method employed by subsocial species is to increase offspring survival rate by prolonged maternal protection and resource provision.

Eusociality, the most complex form of social grouping, denotes reproductive division of labor, generational overlap, and cooperative care of offspring (Michener, 1974). That is, to be considered eusocial, a colony must include a dominant, reproductive caste as well as non-reproductive subordinates, multiple generations must cohabitate in the same season, and the non-reproductive caste must care for the reproductive caste's offspring. Within this classification is primitive and advanced eusociality, distinguished by the degree of morphological differences between castes (Michener, 1974). Primitive eusocial groups display cooperative brood care and functional castes for reproduction and for work. The behavioral castes are morphologically similar and capable of reproduction, despite only one caste bearing offspring. Advanced eusocial groups follow these same principles, but with far greater physiological differences between castes, including a fully sterile, morphologically distinct worker caste (Michener, 1974). The result is a highly complex and organized society consisting of one or few dominant, reproductive members, and a majority who live only to serve the dominant caste. The evolution of these advanced eusocial groups is the most extreme demonstration of the paradox of altruism, and for this reason the evolutionary mechanism of trajectory toward such complexity is an important target for understanding the transition from solitary to social.

To study social evolution requires a study system with ample diversity of social organization for comparison. The Hymenoptera (ants, wasps, hornets, and bees) possess such a social diversity, including many eusocial taxa (Wilson, 1971), making this order a highly informative target for investigation. Complex eusociality, while rarely attained, may be easily lost, (Danforth *et al.*, 2003) and Hymenoptera are a rich resource for comparative studies of such social evolution in any direction.

1.4) Transitioning from Solitary to Social

Kin selection, in considering group formation and the means by which assured fitness returns can favor cooperation in a caste system (Gadagkar, 1990; 1997), explains why complex systems like eusociality may persist. It does not, however, address how or why eusociality may originate. Cooperation can be more common than indirect fitness models dictate (Nonacs *et al.*, 2006), and even the author of the theory admits that it is not a complete solution (Hamilton, 1972). Kin selection is only a weak selective factor working either toward or against eusociality, depending on the circumstances (Wilson & Hölldobler, 2005), and it is therefore more likely a consequence of eusociality than a cause.

While various theoretical approaches consider selective pressures at various life history (e.g. environmental effects; Kocher *et al.*, 2014) and developmental stages (e.g. order of eclosion; Schwarz & Woods, 1994), the formation of more basic levels of social organization, as in the case of dominance hierarchies, plays a significant role in social evolution. Reproductive hierarchies, the basis for complex forms of sociality, are

preceded by the establishment of dominance hierarchies (West-Eberhard, 1967). Even species that may be prone to solitary living can display the underpinnings of sociality necessary to form social dominance hierarchies when forced to interact (Arneson & Wcislo, 2003), suggesting that behavior during interaction can result in strong effects on social organization. However, the tools for forming hierarchies may not be as specific as the preconditions for sociality, relying less on physiological traits and more on behavioral effects (Chase *et al.*, 2002). To better understand how interactions may shape social structure, a useful avenue of study would therefore be to explore the influences involved in dominance behavior and the formation of hierarchies.

2) Social Experience and Behavior

Studies of social experience theory consider behavior changes in terms of the binary outcomes of 'winning' and 'losing'. In interactions involving dominance behavior and aggression, a winner and a loser are normally assigned based on retaining territory vs. fleeing, aggression vs. avoidance, or by likelihood of initiating agonistic interactions (Whitehead, 2008). Typically, the outcomes of winning or losing are predictive of identical contest outcomes in the future, with each repetition of an outcome increasing the probability of future repetition of that same outcome (Hsu *et al.*, 2006). Thus, an individual's past experiences may predict its future behaviors via the phenomena known as the 'winner effect' and the 'loser effect'. This experience-based system is described by two similar hypotheses, the social cue hypothesis and the self-assessment hypothesis (Rutte *et al.*, 2006). The social cue hypothesis states that victory and defeat

leave lasting impressions, which dictate future decisions. By contrast, the self-assessment hypothesis suggests that winners and losers evaluate their own contest abilities relative to their group over time, and make decisions accordingly. These hypotheses have been tested in a broad range of taxa representative of many levels of social organization. The similarity of results across these various phylogenetic distances lends much more strength to the conclusions drawn, making them key to understanding the behavioral evolution of sociality.

Wong & Balshine (2011) found that aggression increased in the group-living cichlid *Neolamprologus pulcher* as individuals ascended in rank during social hierarchy reestablishment, satisfying the expectations of the winner effect. However, aggression was aimed primarily at same-rank individuals, suggesting that this behavior was due more to conflict of rank than the effects of past experience. Thus, in a simple linear dominance hierarchy (one individual per dominance rank), aggression is only likely to occur as a means of reestablishing rank, after which time agonistic behavior subsides. Individuals within the hierarchy assess their own ability and then maintain a status quo. This pattern of punctuated aggression is fairly common. In the highly territorial Mediterranean field cricket, *Gryllus bimaculatus*, isolated individuals behaved much more aggressively on contact with one another than did individuals who cohabitated (Stevenson & Rillich, 2013). With repeated encounters, however, aggression waned until these same isolated individuals acted as tolerantly as cohabitating individuals. The researchers concluded that, because individuals of *G. bimaculatus* are predisposed to aggressive behavior, the significant reduction of aggression observed after individuals

achieved familiarity and began cohabiting with conspecifics is a manifestation of the loser effect, and may explain the high social tolerance seen in these groups.

Chase *et al.* (2002) aimed to find out whether physiological traits or social interactions played a larger role in the formation of linear hierarchies. If physiological traits played a larger role, this would suggest a predetermined system of hierarchy formation, whereas if social interactions were found to be more important, this would suggest a self-organizing system. Examining female interactions in the cichlid species *Metriaclima zebra*, the researchers found that, while linear hierarchies are influenced by physiological traits, social experience is in fact necessary, and thus the primary driving force (Chase *et al.*, 2002). Seebacher and Wilson (2007) compared the effects of physical strength and previous experience in forced associations of the slender crayfish *Cherax dispar*, and found that while the winner and loser effects were observed during repeated encounters between individuals, they disappeared when a new opponent was introduced. In this species, physical strength is how initial dominance is determined when encountering an unfamiliar opponent, but experience from past encounters determines future social hierarchy with familiar opponents. This is believed to be an adaptive mechanism to minimize the number of costly fights experienced in populations, potentially related to the self-assessment hypothesis. Future research may benefit from integrating an assessment of multiple innate traits and social environmental effects to better explore the phenomenon of social hierarchy formation and the evolution thereof (Hsu *et al.* 2006).

3) Differential Expression of Genes in the Brain

3.1) Genetics and Behavior

To understand the evolution of social behavior requires an understanding of what biological mechanisms underlie these behaviors. The transition from solitary to social, and from simple to greater social complexity, involves both behavioral and genetic changes. A behavioral phenotype, like any physiological trait, is a consequence of an individual's gene expression (Robinson, 2004). Natural selection is a subtle interplay between expressed traits, such as behavior, and how well these traits support an organism's fitness given its environment. Thus, exploring the genetic mechanisms of an observable behavioral suite may be a highly informative practice in evolutionary study. This field of behavioral genetics aims to determine how gene expression and regulation affects the neurological processes that manifest as behaviors, and how selective pressures affect these relationships (Anholt & MacCay, 2010). Of particular importance in behavioral genetics is the expression of genes in the brain, linked to numerous behaviors across different life history and evolutionary timescales (Zayed & Robinson, 2012).

The study of behavioral evolution requires an integration of the concept of stratification of social complexity (i.e. solitary and subsocial through primitive and advanced eusocial life history strategies; Michener 1974) and a current understanding of the mechanisms of evolutionary transitions. Detecting genetic differences among species representative of various social paradigms may reveal genes that are essential in the evolution of social complexity (Rehan & Toth, 2015). By unraveling the

fundamental genetics underlying essential precursory behaviors for social complexity (e.g. aggression vs. tolerance) we can better visualize the evolutionary mechanisms that allow for transition between social strategies.

3.2) Genetic Toolkits

'Genetic toolkits' are highly conserved genes with specific functions, which become coopted for new functions over time (hence the notion of repurposing a set of 'tools;' Toth & Robinson, 2007; West-Eberhard, 2003). Frequently employed in the field of evolutionary developmental biology, genetic toolkits allow for a careful investigation of the evolutionary aspects of an organism's developmental pathways and thus, the dynamics of morphological evolution (Carroll, 2008). Toolkits need not be a strict set of specific genes, but may rather entail a general, functional group of similar genes (Berens *et al.*, 2015). It is popularly held that these toolkits can become repurposed: while their genetic sequence is unaltered, variations in the regulation of their expression may alter their function, leading to rapid evolutionary change (Roux *et al.*, 2015). A repurposing of a genetic toolkit could be expressed as a highly variable phenotype, often the focal target for selective factors (West-Eberhard, 2003), and thus a significant driver of evolution. Even minor repurposing of genetic toolkits involved in the development and expression of an organism's behavior could result in significant alterations in the evolutionary trajectory of its social structure (Bloch & Grozinger, 2011; Toth & Robinson, 2007; 2010). Included in this framework is the maternal heterochrony hypothesis (Linksvayer & Wade, 2005; Rehan & Toth, 2015; West-Eberhard, 2003), in

which changes in timing of gene expression can lead to significant behavioral changes, such as division of labor and cooperative care of offspring. Essential solitary behaviors (i.e. aggression, reproduction, and foraging) may have arisen from changes in the function of highly conserved genes, due to chance variations in their regulation (Daugherty *et al.*, 2011; Toth & Robinson, 2010).

Many different genes can contribute to roles of dominance in the form of aggression, with at least 57 different genes implicated in the regulation of aggressive behavior in *Drosophila* alone (Edwards *et al.*, 2009). Aggressive behavior is most commonly attributed to interactions between serotonin and norepinephrine in vertebrates (Nelson & Chiavegatto, 2001), with changes in relative hormone concentrations leading to changes in levels of aggression. In invertebrates, aggression is primarily caused by octopamine, the analog of vertebrate norepinephrine (Pflüger & Stevenson, 2005). Differences in aggression resulting from similar hormone pathways are even related to dominance hierarchy rank in vertebrates, including the African cichlid (*Astatotilapia burtoni*) (Loveland *et al.*, 2014). Given the behavioral similarities between dominance hierarchies across diverse taxa, it is likely that such genetic targets will remain useful in further studies. Aggression is a highly conserved trait in solitary individuals, and coopting this behavior for social interaction and social structure establishment fits the understanding of how toolkits may lead to increased social organization (Daugherty *et al.*, 2011; Toth & Robinson, 2010).

3.3) Possible Candidates

There are many possible avenues to better understand the evolution of social complexity given the number of potential factors leading to diversification of genes and gene functions. The first and most likely route lies with genes exclusively involved in aggression. In addition to genes coding for aggression and dominance, molecular regulation of stress response appears to be an essential driver in divergences in aggressive behavior (Alaux *et al.*, 2009). Africanized and European honey bee subspecies, each representative of significantly disparate aggression profiles, were found to differ primarily in genes coding for stress response to threats. Genetic effects of dominance and aggression have also been found in repeated interactions relying on social experience (Manfredini *et al.* 2013). Social experience is also known to affect aggression response in honey bees (Alaux *et al.*, 2009), while social responses to heat and stress are conserved gene pathways associated with behavioral differences across social organizations (Toth *et al.*, 2010). Thus, not only is it important to target genes that are differentially expressed between individuals of varied aggressive behaviors, it is also critical to consider genes that may be differentially expressed as a result of social experience (Robinson *et al.*, 2008). Aggression may be a useful precursory behavior for social evolution, but considering aggression *and* experience together will likely yield a more complete understanding.

Another direction of investigation is to target genes that are not directly involved with aggression, but that are rather coopted from functions not directly associated with sociality, such as those coding for metabolism, foraging, and nutrition: genes involved in brain metabolism may also be largely responsible for controlling aggressive/dominance

behaviors in honey bees (Li-Byarlay *et al.*, 2014), while carbohydrate metabolism, gland development, and signal transduction genes are all heavily implicated in social evolution (Woodard *et al.*, 2011). Genes for lipid metabolism are conserved, and associated with aggressive behavioral differences across multiple taxa and social organizations (Toth *et al.*, 2010); in *Polistes* wasps, nutrition-related genes also affect division of labor (Daugherty *et al.*, 2011). In this case, the same set of genes normally coopted for aggression are being used instead for further differentiation of social role. Many types of genes and functions are conserved across a broad diversity of taxa (Fischman *et al.*, 2011), and given their roles in the establishment of social organization, they are each viable targets for studies of social evolution.

4) Methodology and a Model System

4.1) Forced Associations and the Circle Tube Assay

Forced interactions in artificial arenas have proven an effective means for directly observing behaviors between individuals. The simple setup allows for direct viewing of all behaviors that occur, and the resulting action patterns are identical to those seen in the wild (Bell & Hawkins, 1974). In an assay particularly suited to social insects, two individuals are placed in a clear plastic tube whose diameter is large enough to allow them to pass by one another, but narrow enough to allow either to actively block passage of the other. The ends of the tube are then joined to form a circle leaving the two individuals forced to interact with one another (Breed *et al.*, 1978). Observing the nature of each interaction (aggressive, tolerant, avoidant, or following) based on

predetermined action patterns can then give a measurable profile for each individual's social standing.

The power of the so-called circle tube assay lies in how it enables broadly inferential conclusions from its ostensibly simple results. Packer *et al.* (2003) used circle tubes to evaluate the social organization of *Halictus (Seladonia) lanei*, a Neotropical species whose nest behavior had not yet been observed. The patterns of dominant and subordinate behaviors between pairings of different castes were consistent with those of eusocial groups, suggesting that this species was also eusocial. The dramatic head size dimorphism of the species is the most extreme of all known halictids, and potentially indicative of corresponding caste dimorphism. This, along with the social organization of its sister species, supported their assessment that *H. lanei* is eusocial. Following the success of this study, Packer (2006) later applied the same principles to 14 Chilean halictid species whose nest behavior was also unknown. Captured individuals of different castes and functional groups were paired in circle tubes, and their behaviors were compared with known patterns of different social organizations to make conclusions about each species' natural nest behavior. Based on the results of Packer (2006), Richards & Packer (2010) sought to elucidate the social organization of a solitary halictid, *Xeralictus bicuspidariae*, a member of the subfamily Rophitinae, which are ancestrally solitary (Danforth *et al.*, 2008). Utilizing circle tube assays, the researchers were able to compare the previously unobserved behaviors of *X. bicuspidariae* to those of other known solitary species, and again demonstrated the power of the circle tube assay to allow for the inference of social status. Given what can

be learned about the role of dominance in the evolution of sociality via circle tube analysis, a similar investigation of dominance and social experience could be carried out in a socially polymorphic species, while simultaneously exploring the corresponding genetics of these behavioral phenomena.

4.2) *Ceratina calcarata*: Choosing a Model Organism

Owing to the power of comparative studies, taxa that represent a wide diversity of social forms, such as the Hymenopteran clade Anthophila (Kocher & Paxton, 2014), establish bees as a uniquely informative target for explorations of social transitions. Multiple species of halictid bees are parasocial and primitively eusocial (Michener, 1990a), and phylogenies show that there have been multiple gains and even more losses of eusociality in the allodapines (Schwarz *et al.*, 2007). These evolutionary gains and losses can function as replicates due to the independent occurrences of these transitions in either direction (Kocher & Paxton, 2014). Within the subfamily Xylocopinae alone there is a wealth of social diversity, representative of a variety of transitional forms. Ancestrally this group exhibited simple sociality, but has since experienced at least four reversions to solitary living (Rehan *et al.*, 2012). The subfamily presently consists of species that are generally solitary or sub-social, with varying degrees of social plasticity. This manifests in some Xylocopine species as a primitive, pseudo-caste system with alloparental care of siblings (Michener, 1990b). This relatively unique social polymorphism allows for unprecedented study of the dynamics of social evolution within the Xylocopinae.

Ceratina are a small, stick-dwelling genus of mass provisioning carpenter bees, long considered primarily solitary (Michener, 1974). Studies on Japanese *Ceratina* species revealed behaviors associated with subsocial species (Sakagami & Maeta, 1977), including maternal nest maintenance, brood cell cleaning, nest guarding, and care of offspring up to and sometimes into adulthood. The Australian small carpenter bee (*Ceratina australensis*) is incipiently social, mostly prone to solitary living (Rehan *et al.*, 2010). The North American small carpenter bee (*Ceratina calcarata*) is subsocial instead of solitary, and occupies a very wide geographic range. Because they occur across so wide a latitudinal range, different populations of *C. calcarata* likely experience very different life cycles owing to significant phenological variation, and this variance is likely reflected in the social organization of these disparate populations (Rehan & Richards, 2010). *C. calcarata* is capable of nestmate recognition, a trait usually associated with eusocial species, and this recognition affects aggression levels (Rehan & Richards, 2013). Typically, in a eusocial colony, a queen behaves more aggressively toward workers, while workers show no aggression to one another. Similarly, in the subsocial *C. calcarata*, reproductively active mothers are most aggressive, pre-reproductive females are less so, and daughters and post-reproductive females are largely passive. Observed aggression is closely tied to the seasonal patterns of a females' reproductive state, making this behavior more ephemeral than might be seen in a fully eusocial system.

Given what is understood of the many factors and social precursors that lead to sociality, an investigation of *C. calcarata*, focusing on dominance behavior, social

experience, and brain gene expression, could be highly informative. Positioned at the cusp of complex sociality, *C. calcarata* displays a significant social plasticity which has the potential to reveal a great deal about the behavioral and genetic mechanisms of the evolution of complex sociality.

5) Research Aims

The goal of this research is to examine the mechanisms for aggression and dominance hierarchy formation, in order to better understand this aspect of social evolution. The bee *Ceratina calcarata* is used because of its unique status as a simple, subsocial species with close relatives that exist at extremes of social complexity. Chapter I focuses on the relative effects of physiological traits and social experience on contest outcomes during repeated interactions. The goal here is to characterize what determines dominance on a very basic level in order to have a broader idea of what factors may contribute to hierarchy formation in early social evolutionary history. Chapter II then expands on these findings by identifying the gene expression behind the behaviors. Observing the effect of aggression and experience on gene expression in the brain allows for a better understanding of how these behaviors work. Comparison of these genetic findings to similar behavioral contexts in other taxa reveals conservation of genetic function and potential broader implications for the use of key genetic elements in many levels of social complexity. This chapter is followed by a brief summary of the general conclusions reached by this research, both in terms of aggression and dominance specifically, and social evolution as a whole.

CHAPTER I:
CUMULATIVE EFFECTS OF BODY SIZE AND SOCIAL EXPERIENCE
ON AGGRESSIVE BEHAVIOR IN A SUBSOCIAL BEE

ABSTRACT

Dominance hierarchies represent some of nature's most rudimentary social structures, and aggression is key to their establishment in many animal species. Understanding the behavioral origins of dominance hierarchies can therefore answer some fundamental questions about the origins of sociality. Previous studies on hierarchical structure have focused on the relative influences of prior experience and physiological traits of individuals in determining social rank through aggression. Although these studies span many taxa, they deal almost exclusively with obligately social species, allowing for minimal comparison to more incipient stages of social organization. Here we examine the behavioral potential for dominance hierarchy formation in the subsocial small carpenter bee, *Ceratina calcarata*, using circle tube forced association. This species exemplifies the simplest form of social living, yet exhibits several traits indicative of more complex sociality, making it an appropriate target for evolutionary comparison. Both physiological traits and social experience were found to play partial roles in predicting future interactive behavior in *C. calcarata*. Our results suggest that individual size is associated with dominance in initial encounters, while prior experience plays a larger role in predicting dominance in subsequent encounters. Social systems in the early stages of social evolution may well have

followed these same predictive factors and our findings provide important insights on the behavioral origins of dominance hierarchies. Similar future studies on related species at various stages of social complexity can allow for extensive comparison and broader inferences into the transitions from simple to complex societies.

INTRODUCTION

Aggression is one of the most common types of behavior between conspecifics, and is exhibited across numerous taxa (Huntingford, 1976). However, many social species rely on cooperation to function (Wilson 1971; West *et al.* 2007b), requiring a trade-off between aggression and cooperation. Aggression can often be used to establish dominance through the formation of hierarchies, and subsequent aggressive behavior maintains this order (Bang & Gadagkar, 2015; Daws *et al.*, 2002; Kim & Zuk, 2000; Wong & Balshine, 2011). Dominance manifests as aggression in many species (Syme, 1974), and aggressive behavior has been repeatedly linked to sociality (Arneson & Wcislo, 2003; Cameron & Jost, 1998; Wcislo, 1997). As the organization of a social system becomes more complex and cooperation increases, aggression within a social unit decreases (Arneson & Wcislo, 2003; Sumana & Gadagkar, 2001; West *et al.*, 2007b). Thus, varying levels of aggression in social species help indicate evolutionary transitions in social organization. Studying the influences of aggression and dominance on social behavior can further explain the evolution of complex social systems.

Solitary living is the simplest life history strategy of a species, with conspecific interactions limited to mating (Michener, 1974). Accordingly, solitary life represents an evolutionary antecedent to all forms of social organization. The simplest form of social

behavior is subsociality, in which a mother stays with and provides extended care for offspring (Michener, 1974). The most derived form of social organization, eusociality, is defined by generational overlap, a dominant reproductive caste, and non-reproductive subordinates, which care for the offspring of the dominant reproductive (Michener, 1974). One of the more significant aspects of eusociality is the reproductive hierarchy of its caste system, which is evolutionarily preceded by dominance hierarchies (Gadagkar, 1980; West-Eberhard, 1967), and which may be enforced by aggression in more primitively eusocial groups (Platt *et al.*, 2004). Even largely solitary species may possess the behavioral precursors necessary to form social dominance hierarchies when forced to interact (Arneson & Wcislo, 2003). This suggests that latent differences in behavioral tendencies among solitary individuals (Garamszegi & Herczeg, 2012; Jandt *et al.*, 2013) can provide insights into the earliest stages of social group formation. Hymenopterans (bees, wasps, and ants), in particular, exemplify a diversity of social structures, from solitary to a range of social interactions (Wilson, 1971), making these a useful target for study.

Individuals' physiological traits, such as size, are known to play a significant role in determining dominance (Rowland, 1989; Tokarz, 1985). This is typically gauged by consistency of contest outcomes in repeated encounters, and by predictive correlations between traits and winning (Brace *et al.*, 1978; Rutberg & Greenberg, 1989). Both size and age are frequent considerations in studies of repeated interaction outcomes (Heinze & Oberstadt, 1999; Higashi *et al.*, 1994; Hughes & Strassmann, 1988; Kasumavic *et al.*, 2009; Kim & Zuk, 2000). Reproductive status has also proven a major

contributor to dominance (Arneson & Wcislo, 1993; Cameron & Jost, 1998; Chandrashekara & Gadagkar, 1991; Rehan & Richards, 2013; Wcislo, 1997).

In agonistic interactions, the outcome of a contest can largely predict future behavior for each individual (Earley & Dugatkin, 2006; Hsu *et al.*, 2006; Sneddon *et al.*, 1997). More aggressive individuals, who win in contests, will be more likely to escalate future interactions, while more subordinate individuals, who lose these contests, will have decreased willingness to engage in future encounters. The lasting impressions of victory and defeat dictate future decisions, the basis of what is called the social cue hypothesis (Rutte *et al.*, 2006). The behavioral outcomes that result from repeated dominant and subordinate interactions rapidly become dominance hierarchies (Daws *et al.*, 2002; Fewell *et al.*, 2009; Hoogendorn & Velthuis, 1999; Kim & Zuk, 2000). However, previous studies in hymenopterans have not examined the effects of such social experience in naturally occurring simple societies (i.e. subsocial groups). This indicates that although there are specific physiological traits affecting dominance in many species, the effect of social experience at the most basic level of sociality remains unknown.

Cumulative effects of both social experience and physiological traits have also been observed (Earley & Dugatkin, 2006; Tanner *et al.*, 2011). Physiological traits and social environment are co-predictors of dominance in many systems, with greater effects resulting from experience (Chase *et al.*, 2002) or individual characteristics (Kasumavic *et al.*, 2009), and often with both contributing equally (Schuett, 1997; Seebacher & Wilson, 2007). In social insects, this is evident in several species whose

reproductive dominance is controlled by a combination of aggressive behavior, size, and age (reviewed in Hogendoorn & Velthuis, 1999). Winning and losing effects are only recently being explored in a broader array of social life histories (Bang & Gadagkar, 2015), but not with corresponding consideration for physiological trait effects against experience. By also comparing the physiologies of individuals, we can further assess the contributing influences of physiological traits and social experience on dominance.

Direct observation of individuals within a nest is often impractical or impossible, but the use of artificial arenas offers a means to replicate nest conditions (Bell & Hawkins, 1974; Breed *et al.*, 1978; Brothers & Michener, 1974). For hymenopterans, this type of observation can be performed via the circle tube assay, wherein two individuals are introduced into a clear plastic tube, the ends are joined, and the pair is allowed to interact (Breed *et al.*, 1978). These one-on-one interactions also mirror those expected inside the colony, and can even allow for characterisation of many species' whole social hierarchies without any need for intranidal observations (Packer, 2006). Circle tube observation has shown that solitary individuals tend to be much more aggressive than social individuals (Richards & Packer, 2010). Similarly, eusocial workers that are very aggressive toward non-nestmates are instead cooperative with nestmates (Packer *et al.*, 2003). The versatility, specificity, and comparability of circle tube assays allow researchers to identify behaviors that are indicative of evolutionary changes in social organization.

Within Hymenoptera, the bees represent a broad social diversity (Kocher & Paxton, 2014). The bee subfamily Xylocopinae (Hymenoptera: Apidae) exhibits not only

a wide range of social forms, but also contains a number of incipiently social species (Rehan *et al.*, 2012), providing meaningful comparisons across the full social spectrum including contrasts with both simple and complex societies (Rehan & Toth, 2015). In particular, the small carpenter bee *Ceratina calcarata* offers important insights. Previous forced association study on the species has found that it is subsocial, but capable of nestmate recognition (Rehan & Richards, 2013), a characteristic more typical of eusocial organisms (Boesi & Polidori, 2011; Flores-Prado *et al.*, 2008). While studies like these offer insights into certain pre-conditions for more advanced sociality, few studies have focused on behaviors in the context of social experience (Arneson & Wcislo, 2003). Likewise, observation of physiological traits, such as ovarian development, have found that reproductively active *C. calcarata* mothers are highly aggressive, pre-reproductive females are less aggressive, and daughters and post-reproductive females are largely non-aggressive (Rehan & Richards, 2013). Thus, *C. calcarata* expresses a behavioral repertoire similar to that of a primitive dominance hierarchy (Breed *et al.* 1978; Wcislo, 1997), making this subsocial species well-suited for investigation into physiological traits and experience effects as pre-conditions for more complex social organisation.

Here we examined the behavioral potential for dominance hierarchy formation in a subsocial bee using circle tube assays. The aims of this study were threefold: first, to determine whether the physiological traits of size, age or reproductive development have an effect on dominance behavior in *C. calcarata*; second, to determine whether social experience contributes to this behavior; and third, to determine if there is a

cumulative effect between social and physiological factors. If physiological traits predict dominance, individual traits should be significantly correlated with behavior outcomes regardless of prior experience. Conversely, if experience is predictive of dominance hierarchies, then behaviors of repeated interactions should match those of initial encounters regardless of physiological differences between paired individuals. If the effect is cumulative, both experience and physiological traits should measurably contribute to dominance after repeated interactions.

METHODS

Nesting Biology of *Ceratina*

Ceratina calcarata females create new nests each spring by excavating the pith of dead plant stems (Rehan & Richards, 2010). Solitary foundresses forage for pollen and nectar to create individual pollen balls upon which they lay their eggs. After mass provisioning and oviposition, females cap the brood cell and the process is repeated in a linear fashion, filling the nest. Females are considered to be in the 'active brood' stage when pollen masses, eggs or small larvae are present in the nest, indicating that clutches are incomplete (Daly, 1966; Rehan & Richards, 2010). It is during this active brood stage in early summer that females are the most aggressive (Rehan & Richards, 2013), making this the ideal time period for studies of dominance behavior. Mothers continue nest maintenance and guarding until autumn. In autumn, both male and female adult offspring eclose and remain in the nest for overwintering until dispersal and mating occurs the following spring (Rehan & Richards, 2010).

Bee Collection

Active brood nests were collected prior to 8 am between June 11 and July 10, 2014 from dead, broken stems of staghorn sumac, *Rhus typhina*. Nests were collected in Strafford County, New Hampshire (43°08'N 70°55'W), and chilled at 4°C until processing. The nests were then split longitudinally to extract adult females, which were kept on ice in microfuge tubes until initiation of the behavior trials. All behavioral trials were conducted no more than two hours after nest processing to minimize stress-induced behavioral changes in the bees (Pabalan *et al.*, 2000). Pairs were randomly chosen and individuals were uniquely color coded with a Sharpie brand paint marker on the top of the thorax in such a manner as not to affect movement (Arneson & Wcislo 2003; Rehan & Richards 2013).

Behavior Trials

Ceratina calcarata individuals were simultaneously placed in opposite ends of a clean, unused polyethylene tube with an internal diameter of 4 mm and a length of 30 cm, twice the average *C. calcarata* head width and 40 times the average body length, respectively. These dimensions allowed for the possibility of both mutual passage and forced blockage (Packer, 2005), while simultaneous introduction eliminated established territorial effects (Wcislo, 1997). The tube's ends were joined and trial timing (t = 20 minutes) began when either individual became active. Behavioral interactions were recorded every time the bees were within one body length of each other (Kukuk, 1992;

Packer, 2005). Encounter behaviors were classified as either aggressive, avoidant, following, or tolerant (Table 1). Depending on the species, following can be considered representative of any one of the other three behavioral categories, so these interactions were classified separately as per Packer (2006). Mutually performed tolerance behaviors were recorded as single events for both individuals, whereas all other behaviors were recorded as separate events per individual (Table 1). Behaviors were recorded in terms of latency to first instance and frequency of each event. To assess pair-wise differences, behaviors were also quantified as a difference in frequency between the two bees in a pair. Bees were randomly assigned as 'Bee 1' or 'Bee 2', and frequency of each behavior was subtracted (Bee 1 – Bee 2) so that relationships between behavior differences could be assessed.

After the 20-minute trial, individuals were removed from the circle tubes and placed back on ice in microfuge tubes. Following a 20-minute recovery period, individuals were then paired with a different partner and the trial was repeated as before. Upon completion of their second trial, all bees were flash frozen in liquid nitrogen and stored at -80°C for future dissection.

Physiological Measurements

Immediately before the behavior trials, head width and wing wear of individuals were measured using a Nikon SMZ800 dissecting scope with mounted Unitron 15854 LED light. Head width, measured as the maximum distance across the compound eyes, is an accurate predictor of body mass in this species (Rehan & Richards, 2010). Wing

wear, damage on the wing margin ranked on a scale from zero to five (unmarred to heavily damaged) serves as a proxy for age and foraging activity (Mueller & Wolf-Mueller, 1993). Metasomas of frozen bees were thawed in 70% ethanol for dissection. Reproductive development was quantified as the sum of the three largest terminal oocyte lengths in each individual. The ovaries enlarge as eggs develop throughout the active brood stage, and then are resorbed after the breeding season, making ovary development a useful metric for reproductive developmental stage. These measurements allowed assessment of the effects of physiological traits on contest outcomes.

Statistical Analyses

Calculations were made in R version 3.1.1 (R Core Team, 2014). Shapiro-Wilk tests were used to test for normal distributions. Ovarian development was normally distributed, so parametric pair-wise comparisons for this variable were made with a Student's t-test, and group wise comparisons made with an ANOVA. Correlations to ovarian development were made with a Pearson product-moment correlation. Head width, wing wear and all behavior frequency and latency data were not normally distributed, so non-parametric pair-wise comparisons for these variables were made with the Mann-Whitney U test and Wilcoxon signed-rank test. Group-wise comparisons were made with a Kruskal-Wallis one-way ANOVA followed by a Tukey's honest significance test for any significant differences found. All subsequent linear correlations

were calculated using a Spearman's rank correlation. Logistic regressions were used to calculate the effect of each physiological and behavioral metric on trial outcome.

RESULTS

Behavior Trials

A total of 54 individuals were randomly paired for each of two trials (27 pairs per trial). Total frequency of interactions for combined first and second trials was 23.63 ± 18.70 encounters (mean \pm SD). Individuals' overall interaction frequency did not vary significantly from the first trial to the second (Wilcoxon signed rank test: $V = 651.5$, $p = 0.74$). Average latency to first interaction in both trials was 2.46 ± 4.96 minutes, with no significant difference between first and second trials ($V = 736$, $p = 0.96$). Separately, aggression, following, and tolerance frequencies were not significantly different between trials, while avoidance frequency was significantly lower in the second trial (4.15 ± 3.96 encounters) versus the first (5.54 ± 6.26 encounters; $V = 736$, $p = 0.03$). Latency for each of the four behavioral categories did not significantly differ between trials.

Tolerance was the most frequently observed interaction in the first trial (13.3 ± 21.2 encounters; Kruskal-Wallis: $\chi^2_3 = 30.5$, $p < 0.0001$) as well as in the second trial (15.6 ± 15.7 encounters; $\chi^2_3 = 49.9$, $p < 0.001$). Latency to avoidance was shortest of all behavior categories in both the first trial (5.22 ± 7.28 minutes; $\chi^2_3 = 30.5$, $p < 0.0001$) and second trial (5.16 ± 7.51 minutes; $\chi^2_3 = 49.9$, $p < 0.0001$). There was not a significant difference between trials for latency to aggression, following, or tolerance.

Pair-wise Behavioral Comparisons

There was a significant negative linear correlation in the differences between paired individuals for aggression and avoidance frequencies ($r_s = -0.36$, $n = 54$, $p < 0.01$; Figure 1), and a significant positive linear correlation in pair-wise behavior differences for aggression and following ($r_s = 0.57$, $n = 54$, $p < 0.0001$; Figure 1). There was a positive but non-significant correlation between the differences in frequency of aggression and tolerance ($r_s = 0.25$, $n = 54$, $p = 0.07$; Figure 1). Based on the negative correlation between aggression and avoidance in all trials, we classified the more aggressive individual in each dyad as the 'winner' and the more avoidant individual as the 'loser' as per a simplified version of accepted dominance indices and terminology from similar studies (Bang et al. 2010; Manfredini et al. 2013). By these definitions, winners in the first trial were individuals with significantly higher aggressive frequency (5.03 ± 4.76) than losers (1.55 ± 2.38 ; Mann-Whitney U test: $W = 192$, $p < 0.001$), while losers were those with significantly higher avoidance frequency (7.83 ± 7.42) than winners (3.52 ± 4.09 ; $W = 552$, $p = 0.04$; Figure 2). Likewise, second trial winners were those with significantly higher frequency of aggressive behavior (4.00 ± 3.02) than losers (1.11 ± 1.55 ; $W = 134.5$, $p < 0.0001$), and losers were those with significantly higher avoidance behavior frequency (4.96 ± 3.99) than winners (3.25 ± 3.68 ; $W = 517$, $p = 0.04$; Figure 2).

Experience effect was measured by treating first trial behavior frequency as a predictor of second trial outcome in a logistic regression. Each additional aggressive behavior in the first trial increased the odds of winning in the second trial 1.43 times

($\log(\text{odds}) = 0.359$, $p < 0.001$), and each avoidant behavior decreased the odds of winning 0.912 times ($\log(\text{odds}) = -0.0922$, $p < 0.05$). First trial following frequency also increased the odds of winning in the second trial by 1.35 times ($\log(\text{odds}) = 0.304$, $p < 0.01$).

Physiological Correlations

Average female head width was 1.95 ± 0.16 mm, and average wing wear score was 2.07 ± 1.43 . Wing wear was not significantly correlated with either head width (Spearman's rank correlation: $r_s = 0.10$, $n = 46$, $p = 0.474$) or ovarian development (Pearson's product moment correlation: $r_p = 0.02$, $n = 54$, $p = 0.43$). There was a significant positive linear correlation between head width and ovarian development ($r_p = 0.61$, $n = 49$, $p < 0.0001$), so ovarian data were subsequently normalised as the ovarian development ratio: ovarian sum (mm)/head width (mm). Average ovarian development ratio was 1.35 ± 0.28 . Aggression, avoidance and following frequencies were not significantly correlated with head width, wing wear, or ovarian development ratio in either of the two trials. The frequency of tolerance behavior also showed no significant correlation with head width or wing wear, but showed a significant positive correlation with ovarian development ratio in the second trial ($r_p = 0.35$, $n = 46$, $p = 0.02$).

Ovarian development and wing wear did not differ significantly between winning and losing females in separated first or second trials. Similarly, logistic regressions of wing wear and ovarian development ratio showed no significant predictor effect on winning in either trial. Head width was significantly greater in winning females ($2.0 \pm$

0.14 mm) than in losing females (1.88 ± 0.16 mm) in the first trial ($W = 161$, $p < 0.01$; Figure 3), but not in the second trial. Moreover, logistic regression of head width in the first trial indicated that this was a significant predictor of winning, with each 0.01 mm increase in head width raising the odds of winning 2.5 times ($p < 0.01$).

DISCUSSION

Dominance hierarchies are among the most rudimentary social structures, and aggression has been coopted as the basis of their formation (Gadagkar, 1980; West-Eberhard, 1967). Previous studies on hierarchical structure have focused on the relative influences of prior experience and physiological traits of individuals in determining social rank through aggression (Hsu *et al.*, 2006; reviewed in Syme, 1974). Although these studies span many taxa, they deal almost exclusively with obligately social species, allowing for minimal comparison to more incipient stages of social organization.

This study found that aggressive behavior between reproductively active females of the subsocial bee *C. calcarata* was higher in certain individuals than in others, resulting in an aggression/avoidance dichotomy matching the social expectations for the early stages of dominance hierarchy formation (Syme, 1974). Repeated trials demonstrated partial effects of both social experience and the physiological trait of body size on future outcomes, suggesting a cumulative role for these factors in early dominance hierarchy behavior in this species.

Physiological Trait Effects

Physiological characteristics of individuals are known predictors of social behavior (Earley & Dugatkin, 2006; Sneddon *et al.*, 1997;). When these traits serve as the principle predictors of contest outcomes, results are due to physical differences between individuals independent of experience (Kasumavic *et al.*, 2009). Given the physiological metrics quantified here for *C. calcarata*, there is evidence that physiological traits are partly predictive of contest outcome.

Winning individuals had greater head width in the first trial. Larger individuals were more likely to win and thus to be dominant (the odds of winning increased 2.5 times for every 0.01 mm larger head width). Body size has been known to contribute, in varying degrees, to behavior in other species (Daws *et al.*, 2002; Kasumavic *et al.*, 2009; Kim & Zuk, 2000). Moreover, the reproductive dominants in *Ceratina* colonies made through forced association were larger females, while non-reproductive, foraging behavior was exhibited in smaller females (Sakagami & Maeta, 1995). Although we found that body size was a very strong predictor of winning in this first trial, it was not significantly predictive in the second.

Social Experience Effects

Prior experience is another known predictor of future behaviors in many animal species (Hsu *et al.*, 2006; Jeanson & Fewell, 2008; Manfredini *et al.*, 2013; Rutte *et al.*, 2006; Seebacher & Wilson, 2007; Stevenson & Rillich, 2013; Wong & Balshine, 2011). The negative correlation between aggression and avoidance suggests two distinct behavior outcomes, whereby those two behaviors are, in their extremes, mutually

exclusive (Figure 1). These consistent binary behavior differences displayed between groups are consistent with behavioral contests involving winners and losers (Whitehead, 2008). Given the winner or loser effect that results from first trial experience (Rutte *et al.*, 2006), winning and losing as an outcome revealed patterns based on individuals with like behaviors for trial outcomes: when the behaviors of the first trial were used as predictors of second trial winning and losing, aggression, avoidance, and following behavior frequency in the first trial all significantly predicted winning and losing in the second trial.

Based on this expectation of the social cue hypothesis, behavioral responses completely attributable to social experience should manifest as constant or even increased aggression and avoidance in repeated encounters, and tolerance should decrease accordingly (Stevenson *et al.*, 2005; Stevenson & Schildberger, 2013; Rutte *et al.*, 2006;). Instead, avoidance in individuals that lost both trials and aggression in individuals that won both trials significantly decreased in the second trial, while all other behavior types were unchanged between trials. Past studies involving forced association in other *Ceratina* species have resulted in induced dominance hierarchies with the necessary mutual tolerance to achieve cooperation (Sakagami & Maeta, 1984; 1989; 1995). Pre-conditions such as mutual tolerance in forced associations facilitate more complex social evolution (Michener, 1985). The observed decrease in aggression and avoidance and the uniform tolerance between the two trials in *C. calcarata* could thus suggest similar behavioral precursors requisite for more complex sociality.

Cumulative Effects

A combined role for social experience and physiological traits is common in nature for determining dominance hierarchies (Berdoy *et al.*, 1995; Chase *et al.*, 2002; Earley & Dugatkin, 2006; Seebacher & Wilson, 2007; Tanner *et al.*, 2011), and the most likely explanation for the results of this study. Here we found that size may predict dominance in single interactions, but that prior experience predicts dominance in repeated interactions. Furthermore, this experience effect may suggest a prominent role of memory and learning in social interactions of the species, which is known to be characteristic of more complex social life histories (Dukas & Real, 1991). The short-term results of repeated interactions seen here may foreshadow those of the prolonged exposure seen in a cohabitating social group.

Future Directions

The social plasticity observed throughout the subfamily Xylocopinae offers an opportunity to compare dominance behavior across the full social spectrum of species ranging from solitary to eusocial (Rehan & Toth, 2015). An interesting next step would be to study the relative effects of physiological traits and social experience on dominance behavior in species at varying stages of social complexity. One potential candidate for this work is *C. australensis*, which is facultatively social (Rehan *et al.*, 2010; 2011; 2014a) and thus an interesting source of comparison for understanding the early stages in the evolution of sociality.

Another important consideration for future work is the role of cuticular hydrocarbons and reproductive pheromones as these are crucial chemical cues known to elicit behavioral responses associated with dominance in solitary and eusocial species (Howard, 1993). Extensive study on what factors dictate hierarchy formation can therefore provide a deeper understanding of the social environment and physiological pre-conditions required for the evolutionary transition from solitary to eusocial.

CHAPTER I TABLES AND FIGURES

Table 1. Ethogram of *C. calcarata* circle tube behaviours, from Rehan & Richards (2013).

Category	Behaviour	Description
Aggression	Biting	Mandibles of one bee grab hold of body part of another
	C-posture	Curling body into a C-shape with mandibles and stinger pointed at other bee
	Nudging	One bee applying force to other with its head (Arneson & Wcislo, 2003); also called 'pushing' (Boesi & Polidori, 2011) and 'lunging'/'headbutting' (Packer <i>et al.</i> , 2003)
Avoidance	Back	Backing away from other bee without turning
	Reverse	Making a 180° turn and moving away from other bee
Following	Follow	Moving toward other bee while it moves away
Tolerance	Pass	Both bees arrange themselves to fit past one another in tube
	Antennate	In frontal encounter, stopping and touching one another with antennae
	Head-head touch	In frontal encounter, stopping with faces in contact with one another

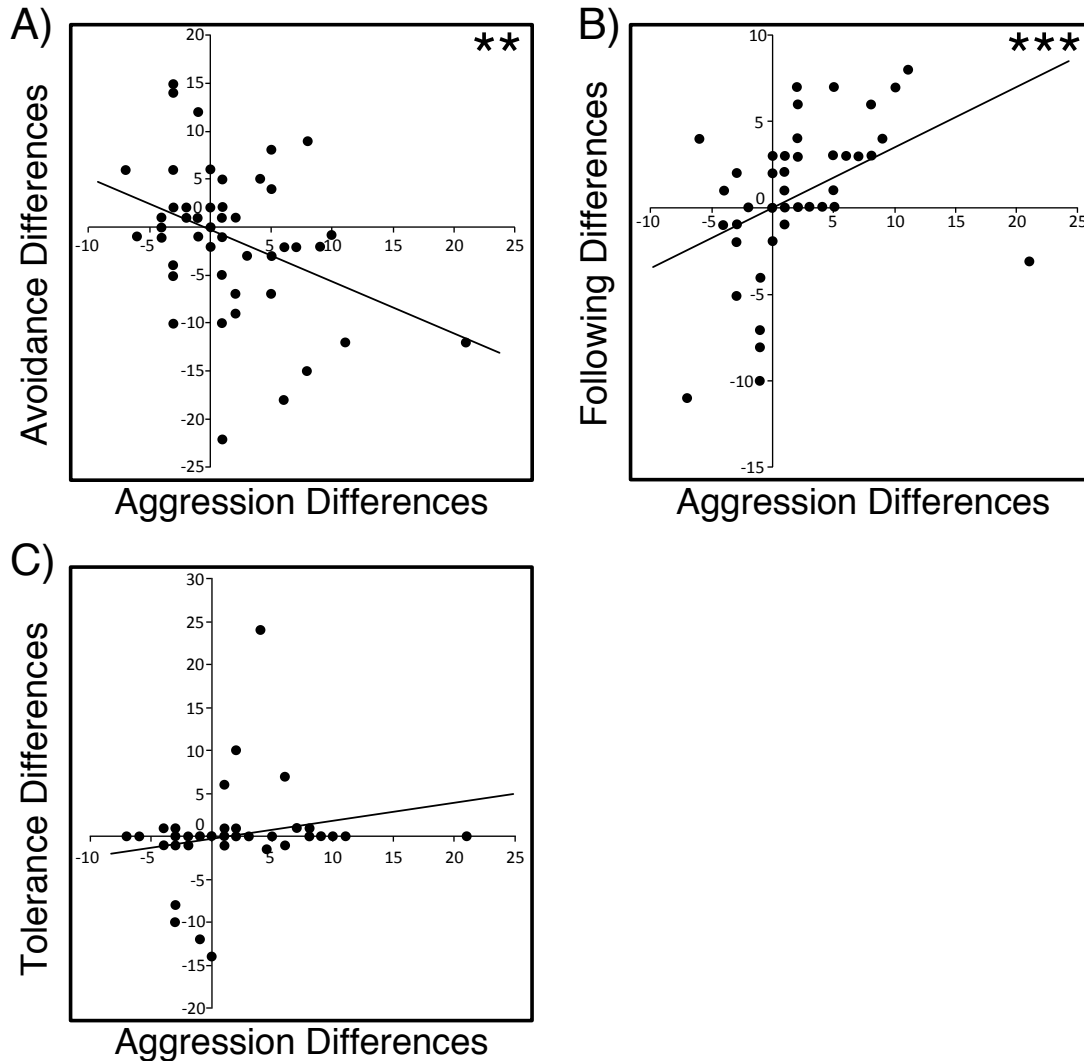


Figure 1. Pair-wise differences in individual behaviour frequency for combined trials (randomly assigned, Bee 1 – Bee 2) plotted by behaviour type and correlated by (A) aggression vs. avoidance, (B) aggression vs. following, and (C) aggression vs. tolerance. Spearman's rank coefficient for significant correlations: (A) $r_s = -0.36$, (B) $r_s = 0.57$, and (C) $r_s = 0.25$. Two asterisks denotes significance at $p < 0.01$, and three asterisks denotes significance at $p < 0.001$. The negative correlation between aggression and avoidance serves as a proof-of-concept for 'winning' and 'losing' terminology.

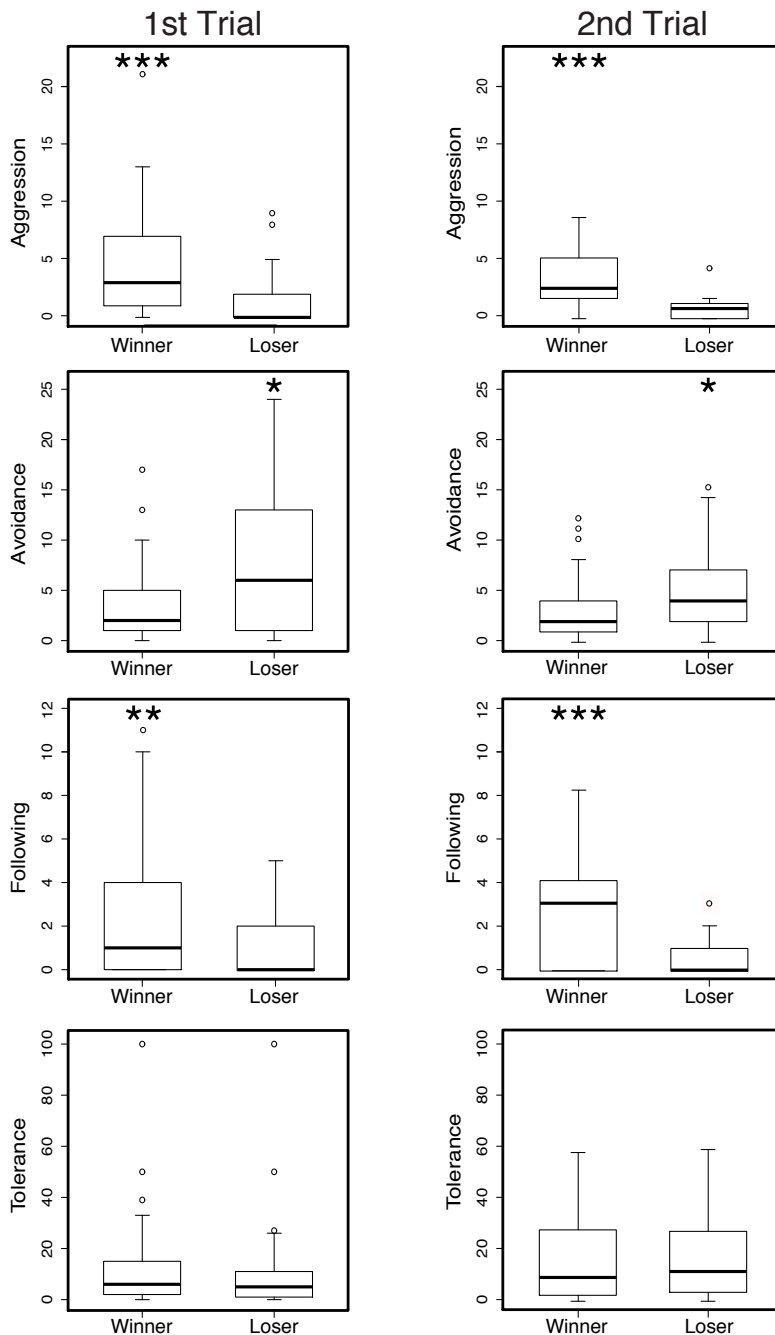


Figure 2. Behavioural comparisons demonstrating differences between designation of 'winners' and 'losers' in first and second trials for the frequency of aggression, avoidance, following, and tolerance encounters. Boxes represent medians and upper/lower quartiles, and whiskers represent maxima and minima. One asterisk denotes significance at $p < 0.05$, two asterisks $p < 0.01$, and three asterisks $p < 0.001$.

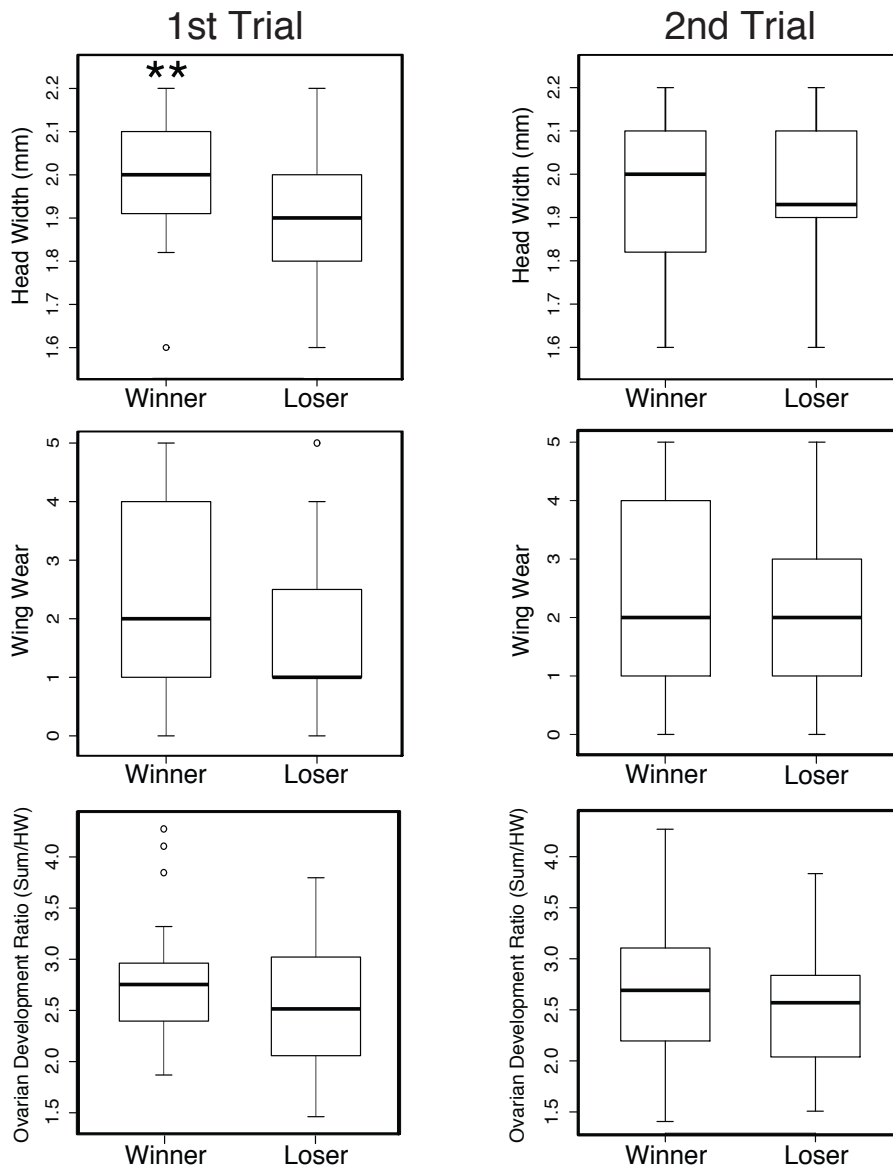


Figure 3. A physiological comparison of winners and losers in first and second trials for head width, wing wear, and ovarian development normalised for body size as the ovarian development ratio: ovarian sum (mm)/head width (mm). Boxes represent medians and upper/lower quartiles, and whiskers represent maxima and minima. Two asterisks denote significance at $p < 0.01$. Note: average head width of all individuals was significantly greater in winners, but members of each pair did not significantly differ in size for either trial.

CHAPTER II:

SOCIAL AGGRESSION, EXPERIENCE, AND BRAIN GENE EXPRESSION IN A SUBSOCIAL BEE

ABSTRACT

Understanding the genetic mechanisms behind aggressive behaviors can yield insight into the formation of dominance hierarchies, and thus social systems in general. Research into the effects of social experience and agonistic contest outcomes has shown significant changes in brain gene expression resulting from repeated winning and losing, as well as changing dominance rank. Studies in a range of taxa and levels of social complexity have identified numerous target genes for aggression, primarily in obligately social species. Here we measured the effects of aggression and social experience on gene expression in the brain of the subsocial bee *Ceratina calcarata*. Using RNA seq, we compared expression profiles of individuals that had experienced repeated winning, repeated losing, or a change in rank. Out of 457 significantly differentially expressed genes, consistent winning accounted for the majority of variance in expression, followed by changing rank over maintaining rank. We then compared the resulting differentially expressed genes and corresponding gene ontologies to those of a variety of invertebrate and vertebrate taxa to determine conservation of aggressive gene function. Lastly, we identified 245 significantly over-represented *cis*-regulatory elements potentially responsible for differential regulation of genes related to aggressive/dominant behavior, and compared these to additional taxa. We present evidence for both genetic

and *cis*-regulatory mechanisms for aggression that may have broader importance to social evolution.

INTRODUCTION

Genomic studies of social behavior across numerous taxa have yielded important insights in the past decade (Gadau *et al.*, 2012; Rehan *et al.*, 2014b; Robinson *et al.*, 2005). Not only have we learned a great deal about behavior-related gene function in the brain (Zayed & Robinson, 2012), but we have also found that transcription regulation of genes in the brain is a major factor in determining behavior (Ament *et al.*, 2012; Sinha *et al.*, 2006). Of particular interest is the genetic basis for aggressive behavior.

Aggression in social species is observed across many taxa (Huntingford, 1976). In a social setting, it may result in dominance relationships (Syme, 1974; Wong & Balshine, 2011), and dominance hierarchies precede reproductive division of labor and other tenets of more complex social organization (Gadagkar, 1980; West-Eberhard, 1967).

Thus, understanding many aspects of social evolution requires first understanding the genetic basis for aggression. Numerous studies have examined this genetic basis

(Alaux *et al.*, 2009; Buitenhuis *et al.*, 2009; Edwards *et al.*, 2003; Hollis *et al.*, 2015; Renn *et al.*, 2008; Rittschof *et al.*, 2013; 2014; Sanogo *et al.*, 2012; Toth *et al.*, 2014).

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). Because a number of these 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).

However, genes alone are not solely responsible for observed changes in behavioral phenotypes. Variations in expression can have large effects – even changed timing of gene expression has been linked to social behaviors such as division of labor and cooperative care of offspring (Linksvayer & Wade, 2005; Rehan *et al.*, 2014; Rehan & Toth, 2015; West-Eberhard, 2003). Increases or decreases in gene expression can be the result of promoter activity, acting alongside the gene without necessarily coding for proteins. These *cis*-regulatory elements use some of the vast regions of genomic DNA that are non-coding (Clark & Pazdernik, 2013). A number of *cis*-regulatory elements have been linked to aggressive and social behavior in birds (Clayton, 2013) and honey bees (Lutz & Robinson, 2013), and numerous neuroendocrine signaling transcription factor motifs are associated with behavioral function in honey bees, mice, and sticklebacks (Rittschof *et al.*, 2014). Studies of differential gene expression and of *cis*-regulatory elements have revealed important details about the evolution of gene function and of sociality. For example, a transcription factor binding motif associated with the gene *Adf1* is linked to learning and memory (Cristino *et al.*, 2006), while *NR2F1* initiates transcription of its associated gene 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 incipiently social species.

Hymenopterans exemplify the complete range of social life histories (Wilson, 1971), with bees in particular showing broad social diversity (Kocher & Paxton, 2014). The bee subfamily Xylocopinae includes not only a number of different social forms, but also many incipiently social species (Rehan *et al.*, 2012). Comparisons within this group and with others can therefore allow important comparisons to all levels of sociality (Rehan & Toth, 2015). A member of this subfamily, the small carpenter bee *Ceratina calcarata*, is considered subsocial, living in small nests that consist only of a mother and a small number of offspring (Michener, 1974; Rehan & Richards, 2013). *Ceratina calcarata* shows behavioral similarities both to more complex social forms (Boesi & Polidori, 2011; Flores-Prado *et al.*, 2008; Rehan & Richards, 2013) and to primitive dominance hierarchies (Breed *et al.*, 1978; Wcislo, 1997). Given these unique social characteristics, studies of *C. calcarata* behavior and corresponding brain gene expression can inform us on the evolution from solitary to social in comparison with studies on strictly complex social species.

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. Agonistic encounters have lasting effects, and prior performance tends to predict the outcome of future interactions (Hsu *et al.*, 2006). Accordingly, in a fight 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 syndromes can be

strongly indicative of dominance hierarchy formation and basic social organization. Experiments in the advanced eusocial fire ant, *Solenopsis invicta* found measurable differences in gene expression in the brains of individuals 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). We aimed to replicate these experimental conditions to find whether genes associated with social dominance in a eusocial ant are also differentially regulated during agonistic interactions in a subsocial bee.

This study aimed to determine differential gene expression resulting from aggression and social experience, targeting specific expression patterns based on winning over losing and on maintaining vs. switching rank. We also compared 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 looked for 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 & Richards, 2013). Dominant and subordinate behaviors were quantified using the circle tube method (Breed *et al.*, 1978) and previously established behaviors and methodology for *C. calcarata* (Rehan & Richards, 2010; 2013). Individuals were placed into each end of an unused polyethylene tube, the ends of the tube were joined, and a 20-minute trial began at the first sign of activity from either individual. All interactions were recorded as aggressive, avoidant, following, or tolerant. Aggression and avoidance were negatively correlated (Chapter I), 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 *et al.* (2013).

Individuals were given a 20-minute recovery period on ice and then re-paired with a second individual for another 20-minute trial in a new circle tube. Individuals were again assigned ranks of 'winner' and 'loser' based on the behavior differences, and they were summarized with behavioral classes based on combined outcomes: 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 for brain dissection and RNA extraction.

RNA sequencing

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). Brains were used due to the relevance of brain tissue to behavior and for direct comparison against other studies on brain gene expression. Three brains were pooled per extraction, with three biological sequencing replicates for each of the four behavioral classes, totaling 12 RNA samples sent to Genome Quebec for Illumina TrueSeq RNAseq sample preparation kit, which included Poly(A) RNA purification, fragmentation using sonification, cDNA synthesis from 200bp size selected fragments, and barcoding. RNA libraries were multiplexed with six samples per lane and sequenced for 100bp PE reads on two lanes on a HiSeq 2500 rapid mode producing 422 Mb 100 base pair paired-end reads for all samples (Supplementary file 1, Table S1). Raw data have been submitted to the NCBI sequencing read archive (SRA) with accession number SRX1547420.

Data pre-processing

Adapter sequences were removed using `fastx_clipper` from the FASTX-Toolkit (Version 0.013) (Gordon & Hannon, 2010). Raw reads from each library were visualized using FastQC; overall the quality of the data is very high. Reads were quality filtered (threshold greater than or equal to 20 with a length threshold of 50 bases) using the Trim perl script (Nikhil Joshi, unpublished; full script available from <http://wiki.bioinformatics.ucdavis.edu/index.php/Trim.pl>). Approximately 4% of the reads were removed from the libraries after adapter removal and quality filtering (% surviving reads; Table S1).

Read mapping, abundance estimation, and differential expression

After quality filtering paired-end reads were aligned to the *C. calcarata* genome (Rehan *et al.*, 2016) using TopHat2 (Kim *et al.*, 2013). Read counts for each of the *C. calcarata* behavior classes were determined by quantifying transcript abundance in each library with HTSeq (Anders *et al.*, 2014). Differential expression among *C. calcarata* behavior classes was determined using the R statistical package DESeq (Anders & Huber, 2010). Heatmaps of scaled read counts were constructed with the R package heatmap.2 in gplot (Version 2.12.1) (Warnes *et al.*, 2010). Principal components analysis (PCA) was performed in the R package FactoMineR (Version 1.25) (Husson *et al.*, 2013).

Comparative analyses

Differentially expressed genes (DEGs) among the four behavioral categories (WW, WL, LW, and LL) were compared to published findings based on aggression and avoidance, dominance and subordination, and queen and worker behavior in other social colony, insect and animal gene expression studies (Supplementary File 2, Table S5). First, we identified putatively homologous sequences between *C. calcarata* and other species using tBLASTx (E-value $\leq 1e-4$). With these putatively homologous sequences, we tested for significant overlap in differentially expressed genes between pairs of species using a two-tailed hypergeometric test. Comparisons were made to microarray and RNAseq datasets for maximum social and taxonomic diversity: ten bee

species, two species of paper wasp, seven ant species, fruit fly, three-spined stickleback and African cichlid, laying hen, mouse and Wistar rat, and domestic dog (see Supplementary File 2, Table S5 for complete list of comparison species and references).

Gene ontology (GO) analysis

Functional annotation of genes was performed with Blast2GO (Conesa *et al.*, 2005), using default settings for GO assignment based on a querying of the NR database (01/05/2015) using BLASTP (E-value < 10^{-5}). Differentially expressed genes were compared to the complete *Ceratina calcarata* genome and pairwise hypergeometric tests were performed on DE gene lists from WW, WL, LW, and LL females to determine whether there were statistically significant GO enriched terms. Resulting *p*-values were then adjusted for multiple testing using the method of Benjamini & Hochberg (1995) for calculating false discovery rate.

Detection of *cis*-regulatory elements

To establish *cis*-regulatory elements, we looked for common transcription factor binding motifs in flanking regions of each *Ceratina 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 1kb and 5kb upstream (these windows yielded different results and so were not considered redundant). We used the Motif Enrichment Tool (Blatti & 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), vertebrate motifs from JASPAR (Portales-Casamar *et al.*, 2010), and vertebrate motifs from TRANSFAC PUBLIC (Matys *et al.*, 2003) and reported with a significance threshold of $p < 0.001$. Multiple hypothesis testing was corrected for via the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995).

RESULTS

Gene Expression Patterns

Differential patterns of gene expression in *Ceratina calcarata* brains were treatment-specific, with notable inverse patterns of regulation between opposite behavior classes (Figure 1). In total there were 457 differentially expressed genes across all treatments. 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 (Figure 1). Furthermore, genes that were significantly differentially expressed in WW and LL individuals tended not to be differentially expressed in WL and LW, while genes significantly differentially expressed in WL and LW individuals were largely not differentially expressed in WW and LL. 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). True dominance/aggression (winning in both trials, WW, over losing) accounted for 68% of variation in expression, after which swapping rank over maintaining rank (WL-LW > WW-LL) accounted for 19%

of variation. Lastly, outcome of the second behavioral trial (second trial win over second trial loss, WW-LW > WL-LL) accounted for 13% of the variation.

Based on these differences in patterns of expression, we looked at the identities of the top differentially expressed genes for aggression/dominance: WW > LL (n = 172). A subset of the 13 most highly expressed of these genes with known functions is shown in Figure 3 (complete list in Supplementary File 1, Table S2). Putative functions of these genes, inferred from *Apis mellifera* and *Drosophila melanogaster* orthologs, include brain and synaptic function (e.g. the genes *headcase*, *couch potato*, *still life*, *longitudinals lacking*, *β Spectrin*, *ultraspiracle*, and *paralytic*), learning and memory (*dunce*, *radish*, and *Synapsin*), transport (*paralytic*, *pixie*), and transcription regulation (*Eip93F*, *Sin3A*). Genes with these functions were significantly upregulated in WW females and downregulated in LL females (Figure 3). Forty-three of these same genes and genes with similar functions 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; Table S2).

GO Term Results

A total of 109 terms were significantly enriched in *Ceratina calcarata* females from comparisons outlined by the PCA: WW > LL (n = 94), WL-LW > WW-LL (n = 10), and WW-LW > WL-LL (n = 5). These included terms with ontologies in biological process, cellular component, and molecular function (Table 1). Several functional terms were related to transcription regulation, including GO:0003700, GO:0006355,

GO:0006357, and GO:0043565, 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 (for a complete list, see Supplementary File 1, Table S3).

Comparative Analysis

Comparison of *Ceratina calcarata* DEGs to other behavioral studies produced matches in 15 species, with many similarities in behavioral/aggressive context to dominance between *C. calcarata* and these comparison species (Supplementary File 2, Table S6). Aggression and repeated winning in *C. calcarata* largely corresponded with aggression and social dominance in other study species. Notable among the genes that matched aggression and dominance contexts in other studies were *dunce*, *longitudinals lacking*, *orb2*, and several of the genes listed in Figure 1. Similarly, GO terms for *C. calcarata* matched those of 23 species, almost exclusively in the context of *C. calcarata* winning over losing (Supplementary File 2, Table S7). These terms matched to aggression/social dominance and to social organization contexts in many studies. Many of these terms involved axonogenesis (GO:0007409), brain function and learning (GO:0007268 and GO:0008355), and transcription regulation (GO:0003700, GO:0006355, GO:0006357, and GO:0043565).

Significant matches to all three transcription factor (TF) motif databases showed numerous potential binding motifs associated with differentially expressed *C. calcarata*

genes (Supplementary File 1, Table S4). Putative functions based on *Drosophila* orthologs indicate that the majority of the associated TFs function in the regulation of gene expression (e.g. *USF*, *NR2F1*, and *E2F1*)(Table 2). However, a few also function in memory and learning (*Adf1*, *CREB*, and *CREB1*), and neurogenesis (*POU3F2*). Motif matches were found in 10 species (Supplementary File 2, Table S8), No single TF binding motif was associated exclusively with either consistent winning or losing behavior states in *C. calcarata* or any of the comparison species, with many motifs significantly matching to all *C. calcarata* behavior contexts (Table S8).

DISCUSSION

Here we show differences in *C. calcarata* brain gene expression resulting from aggressive behavior and social experience, as well as the putative function and ontology of these genes across taxa. Specific, significant patterns of gene expression among behavior classes indicated that both aggression and experience affected expression for individuals. Complete aggression (winning in both trials) over complete submission (losing in both trials) were primarily responsible for these affects, but changing rank between trials, as well as the specific outcome of the second trial, both played small roles in determining expression patterns also. Comparisons to other taxa and gene ontology indicate that many of the most significantly differentially expressed genes function in transcription regulation, axon and neuron formation, and memory/learning, suggesting that social aggression plays an important role in the brains of simple social animals.

Gene Expression Patterns and Ontology

The significant inverse patterns of expression per behavior class suggest a substantial behavioral effect on individual gene expression resulting not only from agonistic interactions, but from experience (Figure 1). Aggression and avoidance resulted in what may constitute dominant and subordinate patterns of brain gene expression, and consistency or reversal of this experience had its own effects on expression. This is confirmed by the PCA (Figure 2): consistent aggression over consistent avoidance accounts for the majority of variance, followed by switching of rank. 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 these behavioral syndromes are known to have regulatory consequences (Maruska, 2015). Thus, the expression we see in winners of both trials and losers of both trials may indicate the beginning of this behavioral syndrome. 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/dominance context (Figure 2, PC2). This same pattern has been observed in the fire ant *Solenopsis invicta* (Manfredini *et al.*, 2013). Similarities in behavioral genetic effects between a simple social bee and an advanced eusocial ant reinforce the potential importance of these behavioral patterns across levels of social organization.

The putative functions of the differentially expressed *Ceratina calcarata* genes (inferred from orthologs) featured several interesting patterns. Axonogenesis, neurogenesis, and axon extension genes and GO terms 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 experience. One of these genes, *β Spectrin*, is upregulated in primitively eusocial paper wasps (Toth *et al.*, 2014), as well as in primitively eusocial and non-eusocial bee species (Woodard *et al.*, 2011). In the advanced eusocial fire ant, it was significantly upregulated in winners over losers (Manfredini *et al.*, 2013). Another of these genes, *headcase*, is upregulated in honey bee workers over queens (Chen *et al.*, 2012; Grozinger *et al.*, 2007). Similarly, the gene *still life* is not only upregulated in workers (Grozinger *et al.*, 2007), but also upregulated in non-eusocial over eusocial bee species (Woodard *et al.*, 2011). Both of these patterns suggest that increased expression of these genes may be more associated with simpler forms of sociality. This is further supported by gene ontology: the enriched axonogenesis term GO:0007409 was also enriched in paper wasp queens and workers alike (Berens *et al.*, 2014), and only significantly enriched in primitively eusocial bee species (Woodard *et al.*, 2011). Gene ontology enrichment in workers and in simple societies with opposite underrepresentation in an advanced eusocial species may indicate an important role in establishment of simple hierarchies, but not full caste systems. Axonogenesis also appears to be importantly linked to social behavior over solitary behavior, as it was only upregulated in group housed stickleback and not individually housed (Greenwood &

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). Given the importance of aggressive behavior to many forms of sociality (Syme, 1974; Wong & Balshine, 2011), aggressive roles for axonogenesis genes may mean that these are important to the formation and maintenance of dominance hierarchies.

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 WW over LL females and in rank-changing females, 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 stickleback (Greenwood & Peichel, 2015). This gene has been repeatedly implicated to function in aggressive behavior (Fischman *et al.* 2011; Nighorn *et al.* 2001; Woodard *et al.* 2011), suggesting conservation of this function in *C. calcarata* as well. Similar upregulation was found for the olfactory learning gene *radish* and in the long-term memory gene *orb2*. Interestingly, patterns of expression for these two genes were opposite one another in honey bees, with *radish* upregulated in workers over queens, and *orb2* upregulated in queens over workers (Grozinger *et al.*, 2007). 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). It is likely that memory and learning are important for both the winner effect and loser effect of social experience, as

these behaviors are key characteristics of species with more complex social life histories (Dukas & Real, 1991), and have been observed in *C. calcarata* previously (Chapter I). This social importance could account for the opposing social contexts of these findings. It could also mean that these genes, like *dunce*, are associated with aggressive and social behaviors.

A final focal gene set that was substantially upregulated in this experiment consisted of transcription regulation genes. Orthologs of both *Eip93F* and *pixie* were upregulated in all three PCA contexts for *C. calcarata*, 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 GO terms also showed transcription regulation activity for WW > LL individuals. The DNA-binding gene ontology term GO:0003700 is enriched in aggressive *Drosophila melanogaster* (Edwards *et al.*, 2006), in paper wasp queens (Ferreira *et al.*, 2011), and in both zebra fish (Lopes *et al.*, 2015) and mice (Rittschof *et al.*, 2014) responding to territorial intrusion. Several other significantly enriched gene ontology terms also matched these same contexts and species, including GO:0006355, GO:0006357, and GO:0043565, all of which function in DNA-binding and transcription regulation. The diversity of genes and ontology terms involved in regulation, as well as the breadth of taxonomic coverage, suggest important regulatory mechanisms for the observed behaviors.

Conserved *cis*-Regulatory Elements

Significant database hits for transcription factor binding motifs matched several species with behavioral contexts similar to those of *Ceratina calcarata* (Table 2; Supplementary File 2, Table S8). For example, the motif associated with *Adf1* was significantly enriched in all three *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 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, it may also be possible that these *cis*-regulatory elements have similar importance for such aggression/dominance contexts. Conversely, a binding motif for a regulator of neurogenesis, *POU3F2*, was enriched in dominant *C. calcarata* females as well, but underrepresented in aggressive stickleback (Sanogo *et al.*, 2012), suggesting a more general function in both aggressive and subordinate interactions overall. The lack of specificity for *C. calcarata* behavioral context of other motif hits supports such a general functionality. Most of the other motif hits are associated with TFs whose only known function is, in general, regulation of their downstream genes, with matches to honey bee (Rittschof *et al.*, 2014), five ant species (Simola *et al.*, 2013), fruit fly (Rhee *et al.*, 2014), zebra fish (Lopes *et al.*, 2015; Oliveira *et al.* 2016), and stickleback (Rittschof *et al.*, 2014; Sanogo *et al.*, 2012). Although these particular motifs

are not strictly associated with TFs that function in behaviors, their co-occurrence across multiple taxa may suggest conservation of regulatory mechanisms.

The relative lack of conserved DEGs and GO terms across taxa compared with the diversity of taxa across which TF binding motifs were conserved suggests that a great deal of the variation observed in *C. calcarata* may be due to regulatory changes, and that these *cis*-regulatory elements are important for similar mechanisms of gene regulation in other taxa. Whether these elements are associated specifically with aggressive behavior is uncertain, but the use of regulatory elements to repurpose genes for behavioral functions can have large effects on overall social organization (Bloch & Grozinger, 2011; Toth & 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 could explore and compare similar brain gene expression in incipiently social taxa, including the facultatively social species *Ceratina australensis* (Rehan *et al.*, 2010; 2011; 2014a). Additional, closely related species are important to our understanding of the conserved mechanisms controlling aggression in an earlier stage of social evolution. 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 individuals that changed rank were more similar in gene expression to one another than to those with consistent rank. 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.

CHAPTER II TABLES AND FIGURES

Table 1. A selection of 20 most significantly enriched (FDR $p < 0.05$) GO terms putatively associated with behaviour, aggression, and transcription regulation. A total of 109 terms were significantly overexpressed across the three conditions determined by PCA: WW > LL (n = 94), WL-LW > WW-LL (n = 10), and WW-LW > WL-LL (n = 5). A full list of terms may be found in Supplementary File 1, Table S2.

Ontology	<i>C. calcarata</i> GO ID	GO Term	Condition
Biological process	GO:0006355	Regulation of transcription, DNA-templated	WW > LL
	GO:0006418	tRNA aminoacylation for protein translation	WL-LW > WW-LL
	GO:0015876	acetyl-CoA transport	WW-LW > WL-LL
	GO:0007409	Axonogenesis	WW > LL
	GO:0007268	Synaptic transmission	WW > LL
	GO:0035556	Intracellular signal transduction	WW > LL
	GO:0006357	Regulation of transcription from RNA polymerase II promoter	WW > LL
	GO:0008355	Olfactory learning	WW > LL
	GO:0000381	Regulation of alternative mRNA splicing, via spliceosome	WW > LL
Cellular component	GO:0005667	Transcription factor complex	WW > LL
	GO:0009343	biotin carboxylase complex	WL-LW > WW-LL
	GO:0016021	integral component of membrane	WW-LW > WL-LL
Molecular function	GO:0003700	Sequence-specific DNA binding transcription factor activity	WW > LL
	GO:0004812	aminoacyl-tRNA ligase activity	WL-LW > WW-LL
	GO:0016740	transferase activity	WW-LW > WL-LL
	GO:0004871	Signal transducer activity	WW > LL
	GO:0043565	Sequence-specific DNA binding	WW > LL
	GO:0003705	RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity	WW > LL
	GO:0005509	calcium ion binding	WW > LL
	GO:0046872	metal ion binding	WW > LL

Table 2. A selection of matches to 12 transcription factor binding motifs associated with significantly upregulated (FDR $p < 0.05$) DEGs. A full list of motifs, matches, and references may be found in Supplementary File 2, Table S8.

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

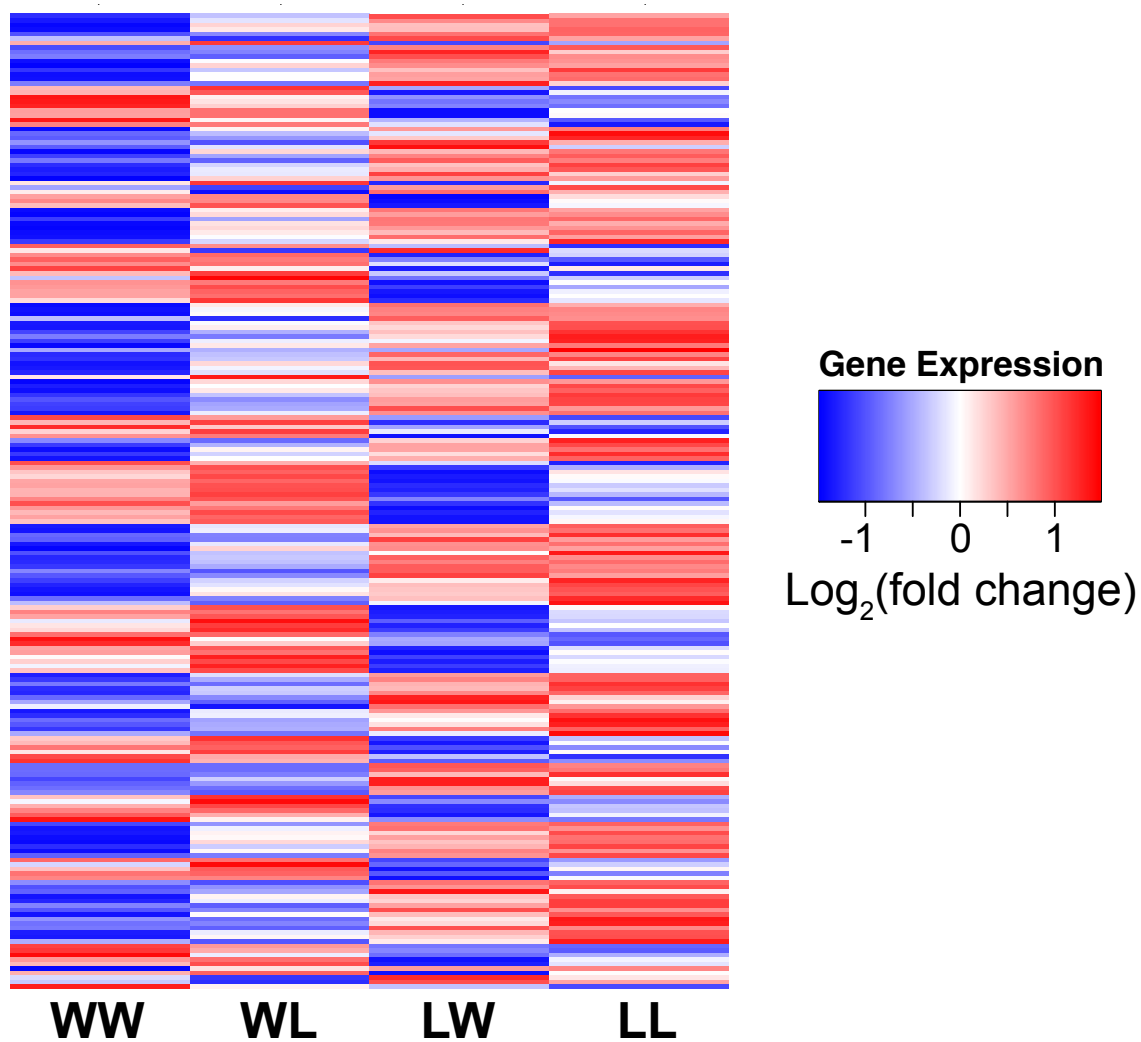


Figure 1. Heatmap of all significantly differentially expressed genes (FDR < 1 corrected p -values < 0.05; $n = 457$) by behaviour class. WW = win-win, WL = win-lose, LW = lose-win, and LL = lose-lose. Calculated clustering by class is shown. Blue = downregulated, red = upregulated, value = $\log_2(\text{fold change})$.

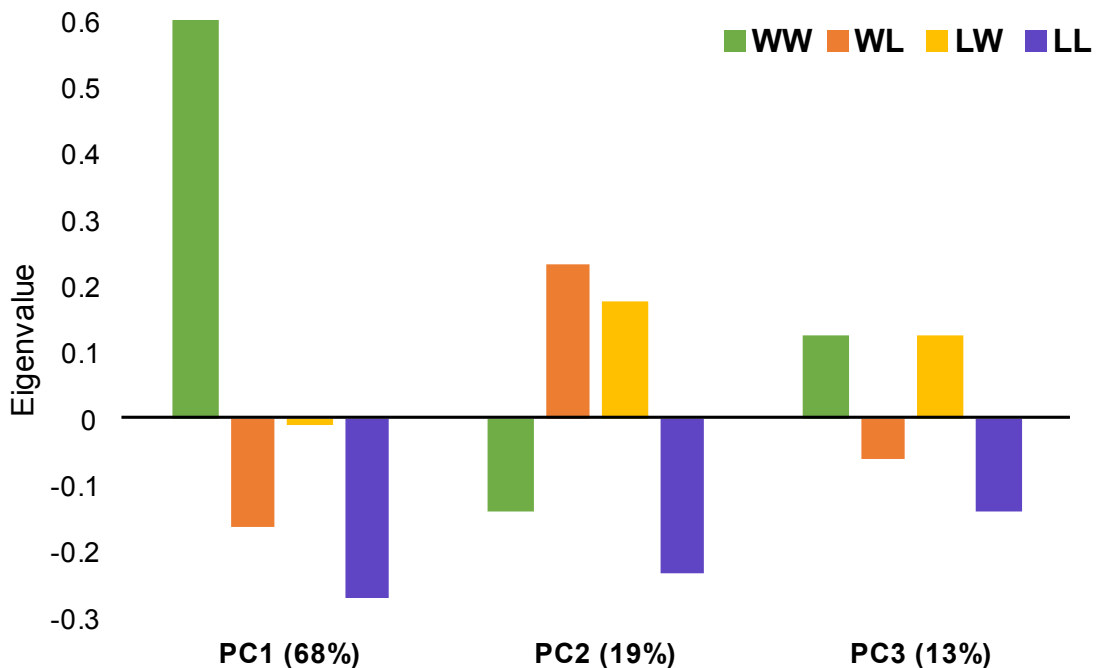


Figure 2. PCA of gene expression across behaviour classes. Repeated dominance/aggression (winning in both trials, WW) compared to all other classes accounted for 68% of the variation observed, while switching rank over maintaining rank (WL-LW > WW-LL) accounted for 19% of variation, and a second trial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

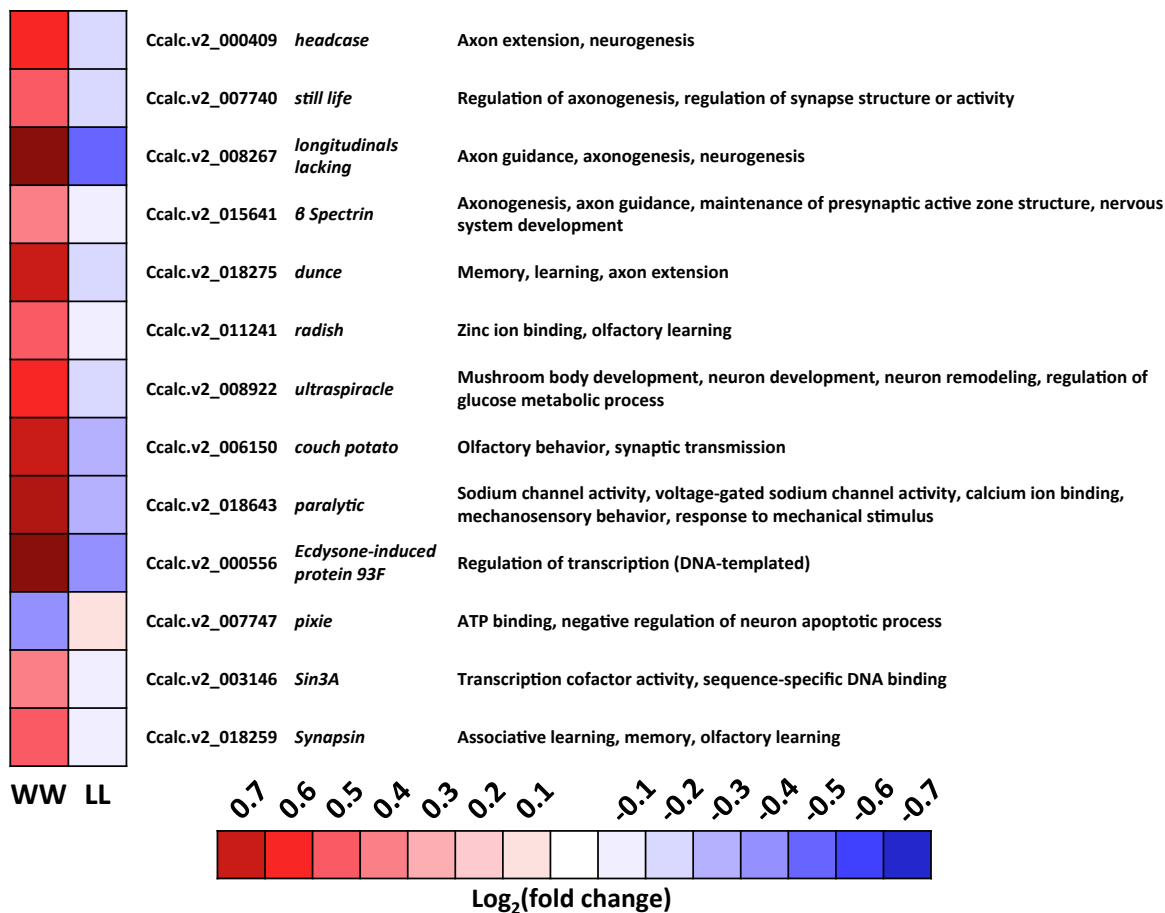


Figure 3. Expression patterns of top 13 most highly expressed and behaviourally relevant significantly differentially expressed genes (FDR < 1 corrected p -values < 0.05) for WW and LL class females. Putative gene names and functions are based on *Apis mellifera* and *Drosophila melanogaster* orthologs. A total of 457 genes were significantly differentially expressed for all conditions. Blue = downregulated, red = upregulated, value = \log_2 (fold change).

GENERAL CONCLUSIONS

The origin and elaboration of sociality is one of the major evolutionary transitions of life (Smith & Szathamary, 1995), but the underlying proximate mechanisms for this transition remains unknown. Tolerance and cooperation are key components required for the formation of social groups (Michener, 1985; Nowak *et al.*, 2010; Wilson, 1971), but aggression plays a large role in both the establishment and maintenance of simple social organization including dominance hierarchies (Bang & Gadagkar, 2015; Daws *et al.*, 2002; Kim & Zuk, 2000; Wong & Balshine, 2011). Dominance hierarchies are antecedent to reproductive hierarchies, and reproductive hierarchies are the basis for all of the most complex levels of social organization (West-Eberhard, 1967). Thus, aggression is an important behavioral focus when investigating social evolution.

Among the factors that can affect aggression and dominance behavior are individuals' physiological traits (Rowland, 1989; Tokarz, 1985) and previous experience involving these behaviors (Earley & Dugatkin, 2006; Hsu *et al.*, 2006). These factors may also work together cumulatively (Schuett, 1997; Seebacher & Wilson, 2007). Until now, however, no study of these factors had focused explicitly on a simple social species. With the research presented here, we can now conclude that for the subsocial bee species *Ceratina calcarata*, the physiological trait of size acts cumulatively with prior experience to predict aggressive behavior in subsequent interactions. Given the basic nature of this species' sociality, as well as the ability of *Ceratina* to form simple social groups under artificial conditions (Sakagami & Maeta, 1977; 1984; 1995), these findings may be applicable to future studies examining primitive dominance hierarchy formation.

In such simple societies, factors such as size or experience determining dominance status may reflect the factors that initially led to the evolution of social groups.

While answering evolutionary questions about social evolution requires explanations of ultimate causation, genetic mechanisms at the proximate level may still reveal important social evolutionary clues. Studies of gene expression in animal brains have uncovered the genetic framework for a variety of behaviors (Ament *et al.*, 2012; Sinha *et al.*, 2006; Zayed & Robinson, 2012). Among these behavioral genetic studies, aggression has been linked to genes for memory and learning (Fischman *et al.*, 2011; Nighorn *et al.*, 1991; Woodard *et al.*, 2011) and axonogenesis (Edwards *et al.*, 2006; Toth *et al.*, 2014). Similarly, *cis*-regulatory elements have been implicated in similar functions (Clayton, 2013; Cristino *et al.*, 2006; Rittschof *et al.*, 2014). Based on the aggressive behaviors and social predictors for dominance, this research has demonstrated similar patterns of expression and specific genetic mechanisms for aggressive behavior in *C. calcarata* to that of the literature. Consistent aggression and experience causes the most significant changes in brain gene expression, and the key genes involved in this aggressive behavior tend to function in learning, memory, and axonogenesis. These findings are consistent with those of a variety of taxa at various levels of sociality. The conservation of these genes across taxa suggests an underlying functional significance for aggressive behavior in multiple species regardless of social complexity.

The predictive nature of body size and prior experience on future dominance capabilities in *C. calcarata* compliment the behavioral genetic finding that experience

largely contributes to differences in brain gene expression. A consistently aggressive individual is likely to achieve higher levels of dominance through repeated interactions, and this behavioral syndrome significantly affects brain gene expression. Prolonged instances of these patterns taken to extreme degrees could eventually lead to fixed genetic and regulatory differences in behavior, like the reproductive hierarchies we see in more advanced forms of sociality. That similar genes, *cis*-regulatory elements, and gene ontology functions were consistent between dominant *C. calcarata* individuals and dominant castes and individuals from more social species suggests a recurrent role for these mechanisms in determining dominance and aggression. These results serve as useful candidates that may be targeted as a basis for further study into the factors – both genetic and behavioral – that may have led to the evolution of social behavior.

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