Behavioural Genetics and Social Environment in the Small Carpenter Bee, *Ceratina calcarata*

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Abstract

Most bees are solitary but the eastern small carpenter bee, *Ceratina calcarata,* are both subsocial and facultatively social. Females form associations of parents and a single generation of offspring, often including a smaller under provisioned dwarf eldest daughter (DED) who feeds her adult siblings. To study the influence of social environment on this species, firstly, observation nests were constructed, and secondly, I conducted an experiment in the field to compare gene expression profiles among ages and phenotypes of foraging females. Observation nests were treated by removing either only mothers, or both mothers and DEDs. In the absence of mothers offspring were more tolerant, and aggression was significantly greater in the absence of both mother and DED. Here I also present brain gene expression profiles of foraging mothers, DEDs in the presence and absence of mothers, and regular daughters. I found significant differences in gene expression associated with age, size and social environment.

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Chapter I: General Introduction

Eusocial organisms, exhibiting reproductive division of labour, cooperative brood care and overlap of generations are among the most intensively studied biological systems (Wilson 1971, 1975). Understanding how eusociality has evolved is one of the most compelling challenges of evolutionary biology. Eusocial organisms are not only highly cooperative, engaging in cooperative brood care, group foraging, nest construction and defense, but also display altruism, as workers forgo independent reproduction to assist in maintaining homeostasis within a social group (Wilson 1971). Eusociality thus represents a major evolutionary transition, analogous to the evolution of multicellularity (Szathmáry and Smith 1995). Despite intensive study of eusocial species, a basic understanding of the proximate mechanism underpinning the transition from solitary to eusocial organization is only beginning to emerge.

Eusociality has been a highly successful strategy, with ants (Formicidae) and termites (Isoptera) composing the majority of biomass in some tropical habitats (Hölldobler and Wilson 1990). Despite the adaptability and ecological success of sociality as a strategy it has had relatively few independent origins, suggesting that multiple derived traits are required before eusociality may emerge. While eusociality has evolved in highly divergent taxa, including mammals (Sherman et al. 1991) and crustaceans (Duffy et al. 2000), independent origins of eusociality are mostly concentrated within the Hymenoptera: ants, bees and wasps (Hines et al. 2007, Cardinal and Danforth 2011, Rehan et al. 2012, Gibbs et al. 2012).

Social organization in Hymenoptera can be broadly classified according to increasing social complexity. Solitary species lack extended intraspecific association; subsocial species engage in extended parental care; incipiently social species cooperate in brood care but lack reproductive division of labour; primitively eusocial species exhibit reproductive division of labour; advanced eusocial species exhibit reproductive division of labour with overlapping generations and morphological castes

(Rehan and Toth 2015). While advanced eusocial termite and ant species represent extremes of both social complexity and altruism, they lack the variation in social organization required for comparative study of solitary and social organization, and thus offer little opportunity for testing hypotheses regarding the proximate mechanisms of the transition from solitary to eusocial behaviour. The study of bees and wasps, within which closely related species show variation in social behaviour, and even intraspecific variation in social complexity, provide the most informative models for understanding the proximate mechanisms driving the transition to sociality (Rehan and Toth 2015, Shell and Rehan 2018b). Facultatively social species in particular, representing solitary and social phenotypes, provide ideal systems for understanding this transition (Shell and Rehan 2018b).

The small carpenter bee *Ceratina calcarata* Robertson (Hymenoptera: Apidae) offers a highly informative example of facultatively social behaviour. *C. calcarata* may be subsocial, engaging in extended maternal care of offspring into maturity, or weakly social, in producing a small worker-like daughter, also termed dwarf eldest daughter (DED), who engages in sibling care (Rehan and Richards 2010a). *C. calcarata* thus offers a model system in which to study the proximate mechanisms of sociality, through experimental manipulation of their social environment.

Parental Manipulation

Ultimate explanations for the evolution of sociality have been provided by the theory of kin selection, that cooperative behaviours evolve from benefits to altruistic traits expressed in offspring (Hamilton 1964), and multilevel selection, that selection may act on individual and group level traits (Wade 1985, Wilson and Hölldobler 2005). Not mutually exclusive with these theories is a potential role for parental manipulation (Alexander 1974, Michener and Brothers 1974, Charnov 1978, Craig 1979a). Parental manipulation theory proposes that sociality may evolve by direct selection on parental traits that induce offspring to cooperate within a social group, thereby increasing parental fitness (Alexander 1974, Michener and Brothers 1974, Charnov 1978, Craig 1979a). Such parental

manipulation may be imposed by social competition, whereby reproduction of offspring is suppressed without their loss of potential fitness, e.g. through aggressive threats or consumption of offspring eggs (Michener and Brothers 1974, West-Eberhard 1987), or via subfertility, directly reducing the reproductive potential of offspring (Craig 1979a, West-Eberhard 1987). Parental manipulation may work in tandem with kin selection, as cooperating workers may or may not gain inclusive fitness benefits from cooperation (Alexander 1974, Michener and Brothers 1974, Eberhard 1975, Crespi and Ragsdale 2000).

Models incorporating parental manipulation are highly suggestive of social evolution at its earliest stages, as they provide a mechanism for selection on traits expressed in reproductive individuals, rather than sterile workers (Alexander 1974, Linksvayer and Wade 2005). Parental manipulation thus provides a potential proximate mechanism for the differentiation of plastic social behaviours, in the form of maternal care (Alexander 1974, Linksvayer and Wade 2005)

Phenotypic plasticity, variation in traits by environment, is likely highly important in most transitions to sociality, as differences in behaviour and physiology of socially defined individuals stem from a common genotype (West-Eberhard 1989). Eusociality may be derived from plasticity arising from social interactions in subsocial ancestors (Alexander 1974, Michener and Brothers 1974, West-Eberhard 1987), after which selection may act on mutations that allow greater plastic differentiation by social environment (accommodation), leading to the defined castes found in derived social species (West-Eberhard 1987, Gadagkar et al. 1988, Linksvayer and Wade 2005).

In subsocial species, targets for maternal manipulation include social cues, such as aggressive behaviours and pheromonal signals, as well as nutrition, through differential allocation of provisions to offspring (Michener and Brothers 1974, Crespi and Ragsdale 2000, Kapheim et al. 2016). Physical aggression leading to dominant-subordinate relationships is common within incipiently and primitively eusocial Hymenoptera (West 1967, Arneson and Wcislo 2003, Jandt et al. 2014). Paradoxically, aggression may lead to the mutual tolerance required for social grouping, if residual traces or ‘memory’

of past outcomes (social-cues), or honest self-assessments of competitive ability serve to resolve conflicts after a limited number of agonistic encounters (Rutte et al. 2006).

Within the primitively eusocial *Polistes* genus of paper wasps all individuals remain reproductively competent, but reproductive and foraging roles are decided through dominance interactions (Pardi 1948, West-Eberhard 1996). Behavioural assays have found that within some primitively eusocial sweat bees (Halictidae) the presence of a reproductive dominant is enough to suppress mating in workers, who are otherwise reproductively viable (Greenberg and Buckle 1981). Within sweat bees specialization of reproductively active individuals in nudging and biting behaviours suggests that manipulation plays a greater role in social organization than worker altruism, as workers remain relatively behaviourally undifferentiated and capable of reproduction, but resorb ovaries when repeatedly aggressed (Brothers and Michener 1974, Michener and Brothers 1974).

Within advanced eusocial species chemical signals are common mediators of social interactions and function to determine caste roles (Wilson 1971). It has been hypothesized that such chemical signals are derived from ancestral social hierarchies established through physical aggression (Ratnieks et al. 2006, Kocher and Grozinger 2011). Chemical signals have also been found to play a role in caste determination within halictid species, along a social spectrum from obligately eusocial (e.g. *Lasioglossum malachurum*), to the facultatively eusocial (e.g. *Halictus rubicundus*), thus suggesting that aggressive interactions may be replaced by chemical communication during early stages of social evolution (Steitz et al. 2018).

Within Hymenoptera manipulation of larval nutrition is particularly important to social organization, as it may both determine adult body size, indirectly affecting dominance-subordinance roles in social hierarchies (Packer and Knerer 1985, Hunt and Nalepa 1994, Richards and Packer 1994a, Smith et al. 2009, 2019, Kapheim et al. 2011a, 2012, Brand and Chapuisat 2012), and directly affecting opportunities for independent nesting, encouraging subordinates to remain within the group (Tepedino and Torchio 1982, West-Eberhard 1987, Wade 2001). Physiological variation may be the

basis for caste-biasing in societies composed of otherwise undifferentiated and totipotent individuals (Kapheim et al. 2012, Kapheim 2017).

Within Hymenoptera the endocrine system plays a key role in status within dominance hierarchies (Kapheim 2017). Within primitively eusocial bumble bees *Bombus terrestris* and *Bombus impatiens* aggression influences vitellogenin (Vg) expression, and thus reproductive status (Amsalem et al. 2014, Padilla et al. 2016). Within colonies of the primitively eusocial paper wasp *Polistes dominula* juvenile hormone levels are also responsive to social environment, and associated with increased aggression in workers in the absence of queens (Tibbetts and Huang 2010a). It has been proposed that changes in the regulation of these systems resulting in their decoupling from reproduction may explain the diversity of social systems in insect societies (West-Eberhard 1987, 1996).

In an otherwise solitary small carpenter bee, *Ceratina japonica,* social cohabitation based in dominance hierarchies may appear when nesting substrates are limited (Sakagami and Maeta 1984). Behavioural assays of *C. calcarata* suggest that aggression may be preferentially directed towards nestmates (Rehan and Richards 2013), but that maternal care is positively correlated with reduced aggression and avoidance between offspring (Arsenault et al. 2018). Social hierarchies produced by dominance of mothers may thus lead to increased tolerance within social groups, and ritualized aggression between nestmates may substitute for costly conflicts. Within *C. calcarata* worker-like DEDs are of smaller size, the direct result of reduced nutritional quantity and quality (Lawson et al. 2017), and this likely limits the probability of independent nest founding as well as the positive outcome of agonistic conflicts (Withee and Rehan 2016). *C. calcarata* thus offers a tractable model to test the relative roles of physiological plasticity and social context in the production of social roles in the transition from solitary to social behaviour.

Ground Plans and Genetic Toolkits

Several related frameworks for understanding the evolution of eusociality propose that social castes are derived from changes in the timing and expression of genes governing modular behavioural and physiological traits found in solitary ancestors (Smith et al. 2008, Toth and Rehan 2017). The ovarian ground plan hypothesis suggests that eusociality is derived from an uncoupling of foraging and reproduction behaviours found during the life-cycle of solitary species, resulting in queen-like reproductives and worker-like foraging phenotypes (West-Eberhard 1996). At the molecular level the ovarian ground plan thus predicts that gene networks related to these specializations will be differentially regulated among castes. The maternal heterochrony hypothesis proposes that sibling care found in social species is derived from a modification in the timing of maternal care behaviours found in subsocial ancestors (Linksvayer and Wade 2005). Genes related to maternal care are thus expected to be precociously expressed, before reproduction, in workers (Linksvayer and Wade 2005).

Consideration of the modularity core to evolutionary developmental biology, along with the diversity of ancestral life histories leading to social behaviours within insects, has led to the more general genetic toolkit hypothesis (Toth and Robinson 2007a). The genetic tool hypothesis proposes that changes in the regulation of modular behavioural and physiological traits in solitary ancestors forms the basis for diverse routes to sociality (Toth and Robinson 2007a). The genetic toolkit hypothesis thus predicts that convergent social phenotypes will share similar patterns of gene expression across diverse taxa, and that these patterns will be regulated by key transcription factors and cis-regulatory elements (Toth and Rehan 2017).

Each of the above-mentioned models offer a useful framework in which to consider how behaviours in solitary ancestors of social taxa may have changed in their timing and function during the evolution of sociality. The maternal heterochrony and genetic toolkit models offer testable hypotheses for the molecular mechanisms of sociality in *C. calcarata*. For instance, within *C. calcarata* aggressive behaviours could be redirected from their function in competition (e.g. during nest usurpation) towards

offspring, or differential allocation of nutrition involved in different investment between sexes could be redirected to produce smaller subordinate female offspring. Moreover, within worker-like DEDs, sibling care behaviours could be explained by up regulation of genes involved in maternal care, in agreement with the maternal heterochrony hypothesis.

Sociogenomics

The increasing availability of next generation sequencing techniques has led to the rapid expansion of the field of sociogenomics which investigates the genetic correlates of social behaviours and their interaction with the social environment (Robinson 2002). Sociogenomic research has tended to focus on model organisms and caste determination in highly derived eusocial species (Robinson et al. 2005, 2008, Smith et al. 2008, Sumner et al. 2018). A candidate gene approach (Fitzpatrick et al. 2005) has revealed some key mechanisms in advanced eusocial *A. mellifera* that also play important roles in organisms that are not social, representing highly conserved modules that may have been coopted at the earliest stages of social evolution (Robinson et al. 2005, 2008, Toth and Robinson 2007a, Smith et al. 2008). Naturally occurring allelic variation in the *foraging* (*for*) gene, coding for a cGMP dependent protein kinase (PKG), of the fruit fly *Drosophila melanogaster* produces sedentary or actively foraging phenotypes (de Belle and Sokolowski 1987, Feil et al. 2005). The *for* gene is highly conserved, being found in diverse eukaryotes, including *Paramecium,* nematodes and humans (Hofmann 2005) and has maintained an association with feeding behaviour within diverse lineages (Kaun and Sokolowski 2008). For instance, mutations in the *for* ortholog *egl-4* in the solitary nematode worm *Caenorhabditis elegans* are associated with increased roaming in the presence of food (Fujiwara et al. 2002). Within highly eusocial Hymenoptera division of labour is typically defined by reproductive queens and sterile workers specializing in foraging. *For* is closely associated with this division of labour in several eusocial insects, through expression rather than allelic variation or mutation (Kaun and Sokolowski 2008). Expression levels of *for* orthologs within *A. mellifera* are

closely associated with age-based transition from nursing to foraging behaviours in workers (Ben-Shahar et al. 2002, Ben-Shahar 2003). Within harvester ant *Pogonomyrmex barbatus*, *for* expression is associated with foraging/nest work division of labour (Ingram et al. 2005). Along with *for* the clock gene *period* (*per*), involved in circadian rhythms (Konopka and Benzer 1971) and mating behaviour (Wheeler et al. 1991) in *D. melanogaster* is also associated with *A. mellifera* worker task specialization, in either nursing or foraging (Toma et al. 2000), though association of this relationship with the *per* product is not known (Fuchikawa et al. 2017). Also involved in *A. mellifera* foraging is the ortholog of a highly conserved gene *malvolio* (*mvl*) (Ben-Shahar et al. 2004), mediating sucrose response in *D. melanogaster* (Rodrigues et al. 1995). Highly conserved genes influential on the behaviours of solitary species continue to play a role in social behaviours in advanced eusocial species, making them interesting candidates for genes involved in the earliest transition from solitary to eusocial behaviour.

Broader studies of gene expression in brain or larval tissue of eusocial Hymenoptera have revealed extensive differences in gene expression between castes, involving numerous genes and gene networks (Robinson et al. 2005, 2008, Smith et al. 2008, Sumner et al. 2018). Studies of brain gene expression among *A. mellifera* workers of different ages, and of nurse or foraging behavioural phenotypes, have found high levels of differential gene expression (39% of ~5500 genes tested), highly predictive of behavioural types (Whitfield et al. 2003). Whole transcriptome analysis of *A. mellifera* workers and queens show widespread differences in expression (4596-4787 genes depending on larval stage) involved in diverse metabolic and cellular functions (Chen et al. 2012). While some differentially expressed genes (DEGs) are unique to *A. mellifera*, highlighting the importance of novel genes in advanced eusocial organisms, overall, differentially expressed genes are largely related to highly conserved metabolic and reproductive processes (Evans and Wheeler 1999, 2000, Wheeler et al. 2006, Corona et al. 2007, Barchuk et al. 2007). These include the insulin signaling pathway (Wheeler et al. 2006, Corona et al. 2007) and metabolic enzymes (Evans and Wheeler 2000). It has been proposed that these widespread differences in gene expression are orchestrated by endogenous JH and

ecdysteroids in response to nutritional cues (Barchuk et al. 2007, Kapheim 2017). These results tend to confirm the expected widespread differences in gene expression between physiological and behaviourally distinct castes with derived social traits related to novel genes (Berens et al. 2015, Rehan and Toth 2015, Sumner et al. 2018).

While the sociogenomic study of derived social species confirms the importance of highly conserved modules in social behaviours, these are largely correlates of specialized roles within eusocial species, and are not suggestive of the proximate causes of eusociality. Subsocial, facultatively social, and incipiently social species have received much less attention than advanced eusocial species, despite their relevance to understanding the transition from solitary to eusocial behaviours (Kronauer and Libbrecht 2018, Shell and Rehan 2018b). As the earliest transition from solitary to eusocial behaviours are expected to involve conserved genes, transcriptomic studies comparing expression profiles between different behavioural and physiological phenotypes in response to environmental cues are likely to be highly informative of the mechanisms involved in sociality at its earliest stages.

Among primitively eusocial insects that have offered informative sociogenomic subjects are *Polistes* paper wasps. Individual paper wasps are behaviourally totipotent, with reproductive and foraging roles based in dominance hierarchies established through aggressive behaviours (Pardi 1948, Keller and Reeve 1994). In *Polistes canadensis* selected DEGs between workers and queens overlap with those in three eusocial species (*A. mellifera*, bumble bee *B. terrestris*, and fire ant *Solenopsis invicta*), though no relationship with the production of ecdysteroids or JH, key regulators of caste development in advanced eusocial Hymenoptera, was found (Sumner et al. 2006). Genes related to metabolic processes were generally more highly expressed in queens than workers, as was the expression of the yolk protein *vitellogen* (Vg) (Sumner et al. 2006).

In *P. metricus* studies of DEGs of putative orthologs of genes related to worker behaviour in *A. mellifera* revealed similar expression patterns in workers and foundresses, as opposed to gynes or queens (Toth et al. 2007), as expected under the maternal heterochrony hypothesis (Linksvayer and

Wade 2005). Expression profiles of candidate genes for *P. metricus* foraging/provisioning behavioural types significantly overlap those associated with foraging in honey bees, however no significant overlap between reproductive genes exists (Toth et al. 2010). Nutrition has also been found to play a role in increased *P. metricus* foraging, and genes upregulated during starvation and in naturally foraging wasps are involved in both insulin (*ILP2*) and juvenile hormone (*USP*) signaling pathways (Daugherty et al. 2011). The overlap of genes related to foraging roles, and the interaction of nutrition with these key gene networks, mediated by the endocrine system, demonstrate similar social functions for genes in primitively eusocial wasps and an advanced eusocial bee (Toth et al. 2010, Daugherty et al. 2011), lineages that diverged as much as 150 million years ago (Branstetter et al. 2017).

Polistes metricus has also been a revealing model of the role of social environment and aggression in gene expression and the evolution of eusociality (Toth et al. 2014). Gene expression profiles between dominant and subordinate foundresses and workers show little overlap, implying discrete worker and reproductive hierarchies (Toth et al. 2014). This is in keeping with the known role of reproductive status and JH expression in the maintenance of social hierarchies in *Polistes* (Tibbetts and Sheehan 2012). Caste associated genes included many associated with metabolic processes and, potentially, lifespan, and selected DEGs in aggressive phenotypes were related to vision and eye development, suggesting a role for visual communication in establishing dominance hierarchies (Toth et al. 2014). Transcriptomic analysis of aggressive phenotypes of *P. metricus* show little overlap with genes related to pheromonal dominance in honeybees, being comparable instead to aggressive phenotypes in this species, as well as solitary *Drosophila* and mice (Toth et al. 2014). Dominance hierarchies thus appear to be the result of aggressive interactions, rather than derived pheromonal signals in this primitively eusocial species (Toth et al. 2014).

Transcriptomic studies of *C. calcarata* and the facultatively social sweat bee *Megalopta genalis* have begun to reveal the importance of genetic accommodation, social experience and dominance hierarchies in gene expression (Rehan et al. 2014, Jones et al. 2015, Withee and Rehan 2017, Arsenault

et al. 2018). Comparison of gene expression profiles from brain and abdominal tissue within different castes of *M. genalis* found significant differences in abdominal tissues, suggesting the importance of physiological and behavioural feedbacks in the evolution of sociality (Jones et al. 2017). These DEGs significantly overlapped caste-specific gene expression in *B. terrestris* and *A. mellifera.* DEGs in *M. genalis* castes were also found to correspond with genes under positive selection in multiple eusocial species (Jones et al. 2017), suggesting a common genetic toolkit is available for the elaboration of sociality from incipient to advanced eusocial organization.

Transcriptomic studies of *C. calcarata* gene expression in brain tissue have been highly informative regarding the question of the evolution of sociality, suggesting the importance of maternal care and dominance behaviour in gene regulation and correlated social behaviours (Rehan et al. 2014, Withee and Rehan 2017, Arsenault et al. 2018). Transcriptomic analysis of brain gene expression of female *C. calcarata* undergoing repeated interactions in circle tube assays revealed widespread changes in gene expression related to winning and losing, as well as changing rank, in aggressive encounters (Withee and Rehan 2017). Gene ontology enrichment analysis of up regulated genes in aggressive individuals (winning multiple trials) and individuals who changed in rank were found to be associated with axonogenesis and memory (Withee and Rehan 2017). Further circle tube assays combined with transcriptomic analyses have demonstrated increased aggression and avoidance within female *C. calcarata* from orphaned nests as opposed to those receiving maternal care, correlated with widespread differential gene expression, alternative splicing and methylation (Arsenault et al. 2018). Hierarchical clustering analysis of *de novo* transcriptomic profiles of brain gene expression among mothers, regular daughters, and DEDs during season stages (winter, spring, autumn and summer) tend to confirm the maternal heterochrony hypothesis (Rehan et al. 2014). During autumn DEDs adopt a sibling care role, and accordingly gene expression profiles of autumn DEDs and autumn mothers, both engaged in care of adult offspring/siblings, are most similar (Rehan et al. 2014). Under the alternative hypothesis, of a decoupling of reproduction and foraging behaviours in precociously foraging worker-like DEDs,

greater similarity between DEDs and foraging spring mothers is expected (Rehan et al. 2014). Taken together these results suggest a profound role for dominance interactions and maternal care in plastic social behaviours.

Further transcriptomic investigation of how increased sibling care behaviours in DEDs are affected by social cues from mothers is required. Behavioural data within a manipulated natural setting combined with such transcriptomic data will be highly revealing of the role that social interactions play in producing phenotypically plastic differences that lead to the transition from solitary to social organization.

Thesis Aims

Critical studies of the proximate mechanisms involved in the evolution of sociality at its simplest stages are still lacking. Study of advanced eusocial Hymenoptera has implicated many potential mechanisms, but highly derived and complex social traits may not reflect those specifically involved in sociality in its simplest forms. Furthermore, study of advanced eusocial species fails to account for the diversity of ancestral life histories leading to social lineages, as well as the diversity of forms sociality takes. As obligately social, advanced eusocial species also provide limited opportunity for the experimental manipulation of social environment necessary to test hypotheses regarding the key behavioural dynamics underlying the transition from solitary to social organization. In contrast, incipiently and primitively social Hymenoptera have emerged as tractable models for experimental study of social environment. In particular, study of incipiently and primitively social species has provided key insights into the role of maternal manipulation and dominance hierarchies in the formation of division of labour in simple social systems.

The small carpenter bee, *C. calcarata,* is an ideal model organism for understanding the earliest stages of social evolution (Shell and Rehan 2018b). This species is typically subsocial with mothers engaging in solitary care of offspring. *C. calcarata* mothers also frequently produce a single worker-

like individual (dwarf eldest daughter, DED), who participates in caring for and feeding siblings, increasingly so in orphaned nests (Johnson 1988, Rehan and Richards 2010a, 2010b, Rehan et al. 2014, Mikát et al. 2017). *C. calcarata* is thus on the brink of eusociality, and experimental manipulation of the social environment of colonies may reveal how worker-like cooperative behaviour is produced (Rehan et al. 2014).

In Chapter II, I analyze foraging and intranidal behaviours of *C. calcarata* under multiple experimental treatments. To understand the role of maternal and DED presence on nesting and foraging behaviour in *C. calcarata,* observation nests were created and subjected to three social environment treatments, i) an unmanipulated control, ii) mothers removed, and iii) mothers and DEDs removed. Behaviours within the nests, as well as foraging behaviours were recorded. This experiment assesses the hypotheses that DEDs engage in sibling care through foraging, and that mothers act social dominants, reducing aggression within nests. Colonies lacking DEDs and mothers are expected to have reduced foraging behaviours and increased aggression as compared to controls.

In Chapter III, I examine differences in gene expression among behavioural types of *C. calcarata* females under experimental treatments applied to nests in the field. Through removal of mothers and DEDs, and both, in artificially established nests, differentially expressed genes among DEDs, regular daughters and mothers, and among these groups under mother and DED presence/absence treatments are analyzed. This chapter assesses the genetic toolkit hypothesis of social evolution.

In Chapter IV, I provide a general discussion of the results of my thesis. I summarize my experiments and the insights they provide into the influence of social environment on behaviour and associated gene expression in *C. calcarata*.

Chapter II: The Influence of Social Environment on Cooperation and Conflict in an Incipiently Social Bee, *Ceratina calcarata*

Jesse L. Huisken, Wyatt A. Shell, Hannah K. Pare and Sandra M. Rehan

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Abstract

How mutual tolerance is produced, and the role of social environment in inducing cooperation in social groups, remains unstudied in many simple societies. In particular maternal and sibling care and conflict are challenging to manipulate experimentally for many species. Most bees are solitary, but mothers of the eastern small carpenter bee, *Ceratina calcarata,* engage in prolonged care of offspring, and are therefore subsocial. Females form social associations of parents and a single generation of offspring, including a smaller dwarf eldest daughter (DED) who forages and feeds her adult siblings. Adult assemblages of *C. calcarata* present a unique opportunity to study the effect of social environment on cooperation and sibling care in an otherwise subsocial bee. To study how social environment influences foraging and intranidal behaviors, observation nests were constructed, and unaltered as a control, or treated by removing either only mothers, or both mothers and DEDs. Nests were video recorded for 464 hours during summer (July-August) parent-adult offspring cohabitation. Individual and interactive behaviors were scored. In the absence of mothers offspring were more tolerant, suggesting that a hierarchy between mother and offspring produces less tolerance between offspring. Aggression was only significantly greater in the absence of both mother and DED. We found that foraging was lowest in the absence of mothers, and persisted in the absence of both mother and DED. This study provides the first detailed account of the intranidal behaviors of this species and experimentally reveals how social environment influences cooperative behavior.

Significance Statement

Understanding how particular life histories, such as extended parental care, may set the stage for more complex social behaviors, such as sibling cooperation, is critical to understanding how alloparental care evolves in group living organisms. Most species exhibiting parental care and sibling cooperation are difficult to manipulate experimentally. Though relatively uncommon in invertebrates, extended parental care is frequently found in small carpenter bees that can be carefully observed within their nests and foraging. Here we examine how the absence of mothers and worker-like daughters influences the social behavior of related adults living in close group association of such bees. This experiment presents an intranidal study of a subsocial bee's behavior, and our results suggest that mothers play a dramatic role regulating social behavior. Further, our results show that siblings are more tolerant in the absence of mothers, suggesting that mothers may maintain social hierarchies among offspring. Siblings may interact more aggressively and more frequently as they negotiate intranidal and foraging tasks in the absence of maternal care.

Keywords: aggression; tolerance; carpenter bee; Hymenoptera; maternal care; phenotypic plasticity

Introduction

Maternal care forms a key component of social environment, profoundly influencing behavior in both vertebrates (Caldji et al. 1998, Fish et al. 2006) and invertebrates (Costa 2006, Kramer et al. 2015). In its simplest form, social behavior in invertebrates consists of extended parental care of offspring, most frequently mothers, termed subsocial behavior (West-Eberhard 1969, Wilson 1971, Michener 1974). In such cases mothers may associate with offspring after they are no longer dependent on them for survival, even into fully adult stages of development (Tallamy and Wood 1986; Costa 2006). Subsocial behavior is frequently exhibited within the Hymenoptera, where mothers and, rarely,

fathers, may engage in offspring provisioning or nest guarding (West-Eberhard 1969; Wilson 1971; Michener 1974; Rehan and Richards 2010a; Mikát et al. 2019).

Within many species extended parental care may result in more complex social interactions, including cooperative behaviors among offspring resulting from dominance hierarchies between parents and offspring. Adoption of such cooperative roles within a social group is thus sensitive to changes in social environment. Alloparental care of siblings, though relatively uncommon, occurs in some species of cooperatively breeding birds, several mammal and fish species, and many insects (Wilson 1971, Michener 1974, Emlen 1984, Taborsky 1984, Stacey and Koenig 1990, Costa 2006, Brooks et al. 2017). In cotton-top tamarins, daughters that care for younger siblings are reproductively repressed in the presence of their family (Widowski et al. 1990). In many cases, aggression is the primary mechanism differentiating cooperative helpers from their siblings. In the Serengeti dwarf mongoose, subordinates acting as alloparents are the recipients of increased aggression during mating periods (Creel et al. 1992). Within the primitively eusocial sweat bee, *Lasioglossum zephyrum,* foraging and nest constructing bees are the recipients of frequent nudges from dominant reproductives (Michener and Brothers 1974). Aggressive interactions are known to play a role in determining social structure in a diversity of group living organisms, including social Hymenoptera (Gadagkar 1980, Reyer et al. 1986, Creel et al. 1992, Mulder and Langmore 1993, Keller and Reeve 1994, Cameron and Jost 1998, Arneson and Wcislo 2003, Jandt et al. 2014).

Cooperative behaviors in the socially diverse Hymenoptera are frequently determined through social interactions and environment (Wilson 1971; Michener 1974). The subsocial small carpenter bee, *Ceratina calcarata,* has a simple social system that is ideal for testing the role of the social environment in group tolerance and conflict (Shell and Rehan 2018b). *Ceratina calcarata* offspring are not only provided with provisions prior to development, but require additional feeding as adults to survive overwintering, as is found in several other temperate *Ceratina* species (Sakagami 1977; Mikát et al. 2016). The colony cycle of *C. calcarata* begins when dispersing males and females mate in early

spring (Rehan and Richards 2013). Females establish nests by excavating a tunnel in the central pith of a dead plant stem, and then begin a period of mass provisioning in early June (Rehan and Richards 2013). After provisioning, brood mothers continue to associate with their nest and care for their developing offspring by cleaning and inspecting them (Rehan and Richards 2013). Mothers engage in a second period of foraging and provisioning of eclosed adults from late July into mid-September (Rehan and Richards 2013; Mikát et al. 2017). During this period offspring typically remain in the nest, with the exception of a smaller, under provisioned daughter (also referred to as a or dwarf eldest daughter (DED)) who forages to feed her siblings often alongside her mother (Johnson 1988; Mikát et al. 2017). This second period of foraging ensures adult offspring survival during overwintering, but the DEDs, which exhibit worker-like altruism in caring for their siblings, do not survive overwintering (Rehan and Richards 2010a, Rehan et al. 2014).

The maintenance of adult assemblages from late summer through early spring within *C. calcarata* nests presents a unique opportunity in which to characterize social behavior and study the role of social environment in colony cooperation. Experiments studying individual encounters in forced association circle tube assays use a single length of plastic tube connected at either end such that bees are forced to interact (Breed et al. 1978, Packer 2006). Such studies suggest that bees from orphaned nests are more avoidant and aggressive, and that mothers preferentially direct aggression towards their daughters rather than non-nest mates (Rehan and Richards 2013; Arsenault et al. 2018). It is thus likely that maternal aggression has a significant effect on offspring behavior, though the social environment of a nest differs significantly from that of temporary forced association. Intranidal behavior has not been observed in this species, and it is not known if aggressive interactions are frequent under the conditions of extended cohabitation of multiple interacting related individuals. Experience is known to play a role in aggression in *C. calcarata*, and it is thus likely that the repeated interactions found within nests lead to different social dynamics than found in temporary encounters between females (Withee and Rehan 2016).

In order to study the influence of social environment on intranidal and foraging behavior in *C. calcarata,* two experimental removal treatments were compared to control observation nests. The two treatments were: i) mother removal and ii) mother and DED removal. We predict that aggressive interactions will be frequent within nests (Withee and Rehan 2016). Given the formerly observed division of foraging labor between mothers and DEDs versus regular adult offspring, we further predict that foraging will be reduced when mothers are removed from nests, and more so when only regular offspring are present (Mikát et al. 2017). Finally, given the previous evidence for the role of maternal care in social behavior in *C. calcarata,* we hypothesize that aggression and avoidance will increase in the absence of mothers (Rehan and Richards 2013; Arsenault et al. 2018). The aims of this study are threefold: first, to document foraging rates and all observed behaviors within nests; second, to characterize the response of colony foraging behavior to mother and DED removal; third, to examine interactive behaviors across different social environments. Here we present the first intranidal observations and removal experiments to determine the role of social environment on cooperation and conflict in *C. calcarata*.

Methods

Observation nests

Nests were established by distributing a common *C. calcarata* nest substrate (cut raspberry branches, *Rubus idaeus*) mounted to bamboo stakes with zip-ties in the wild around Strafford County, NH, USA (43°08′N, 70°55′W). Established nests were identified by the presence of an entrance holes excavated in the broken ends of stems. Control nests (C) were left undisturbed and mother (M) and mother and DED removals (MD) were made when these bees first left the nest to forage. Stems were mounted vertically and enclosed within boxes to reduce ambient light within nests, and eight stems were mounted per box (Fig. 1A). A transparent plastic cup with a domed top was secured to the top of each stem to create an enclosed foraging arena, and bees were provided with flowers and feeding

stations containing water and/or sugar (Fig. 1B). Observation was facilitated by longitudinally opening branches and applying a plastic window to exposed nests (Fig. 1C). Foraging arenas and nest interiors were video monitored (Sony HDR-CX240/L for exterior, Sony HDR SR-11 Hybrid Nightshot for interior) with multiple observation periods of 50 mins each between 25 July and 28 August 2018. Interiors of nests were monitored using infra-red capable cameras (Fig. 1D). Videos were subsequently reviewed, with observers blinded to treatment, and novel behaviors were noted and recorded along with known behaviors identified in pre-existing ethograms (Rehan and Richards, 2013; Withee and Rehan, 2016). Duration of behaviors and number of bees present during an observation were also recorded. A total of 35 nests were included in the study, 13 control (C), 13 mother removal (M) and 9 mother and DED removal (MD) nests. A total of 557, 50 minute recording periods were made, over 34 days: 273 in C, 152 in M and 132 in MD ($N = 557$; total observation time = 464 hours).

Nineteen distinct behaviors were identified from video recordings (Table 1). Behaviors were considered as events and counted for each observation period, and durations were recorded. Individual behaviors (Table 1) were novel to the ethogram for this bee and defined in this study. We defined two behavioral categories for this study: i) individual behaviors, which involved one focal bee; and ii) interactive behaviors, which involved two bees within two body lengths of each other. Interactive behaviors were considered as a single event and as durations (Table 1). Interactive behaviors were classified as aggressive, avoidant, or tolerant (Rehan and Richards 2013). Because the social role of following behavior varies between species of bee it was considered as a separate behavioral classification (Withee and Rehan 2016). After behavioral assay, all bees were killed by freezing at -20°C.

Statistical methods

First, to understand how social behaviours changed overall within nests a PCA including interactive behaviors by type was created using the prcomp function in base R 3.6.1 (2019-07-05).

Behaviors were categorized as aggressive, avoidant, tolerant, averaged for each nest prior to normalization for PCA plotting using the ggbiplot function in the dplyr package (Wickham et al. 2020). To test for changes in overall activity, we built two general linear mixed models (GLMMs) with binomial distributions, with total behaviors per 50 minute observation period as response variable and treatment as predictor, one with and one without the number of bees as an offset on the binomial natural log scale to account for variation in number of bees in an observation (Zuur 2009). Removal treatments were modeled as fixed effects, and nests as a random factor. To test the effect of removal treatments on the response variables of 10 interactive behaviors, and four interactive behavioral categories (aggressive, avoidant, tolerant and follow), we built two GLMMs for the frequency of each behavior or category. Treatment was modeled as a fixed effect, and nests as a random factor, to account for varying number of nests subjected to each treatment, and differing numbers of repeated observations between nests. In analyses of frequency of behaviors, frequencies of each behavior were determined as the number of a behavior in an observation. Distributions were modeled as Poisson, or, to account for overdispersion, negative binomial (linear or quadratic). As number of bees varied among observations, frequencies were modeled with the natural logarithm of number of bees in an observation as an offset, to maintain the log scale of the Poisson or negative binomial distributions. Due to the fact that all behaviors were not observed in each period, data for some behaviors were zero-inflated (Zuur 2009; Zuur and Ieno 2016). Models for frequencies and duration of foraging behavior were fit, considering frequency and durations per bee, or duration of activity in an observation, using log offsets. Distributions and choice of inclusion of a zero-inflation parameter were selected to minimize BIC values, and models were validated by visually inspecting residual plots using the package DHARMa (Zuur 2009; Hilbe 2011; Hartig 2020). All models showed good overall fit, passed Kolmogorov-Smirnov tests, and tests for overdispersion (Hartig 2020).

All statistical tests were conducted in R version 3.6.1 (2019-07-05). Models were produced with the glmmTMB package (Brooks et al. 2017). Type II Wald chi-square tests of the effect of treatment

were conducted for each model. As behaviors were observed from the same nests, a Holm-Bonferroni correction was applied to an $\alpha = 0.05$ for behavioral categories ($N = 4$), as well as interactive behaviors considered as frequencies and durations ($N = 10$). Adjusted p-values are presented throughout. Marginal means of each frequency per bee were estimated from models, and post-hoc Tukey pairwise comparisons between treatments were conducted using the emmeans package in R (Lenth 2020).

Data Availability

The datasets generated during and/or analyzed during the current study are available in the Dryad repository: https://doi.org/10.5061/dryad.np5hqbzs6

Results

Intranidal and foraging behaviors

Across treatments bees were most frequently engaged in individual and aggressive behaviors, followed by tolerant, and then avoidant behaviors (Fig. 2). Individual behaviors were the most frequent per observation (10, 7-14; mean, 95% CI), followed by aggressive behaviors (8, 5-13). Tolerant (1.33, 0.85-2.07) and avoidant (0.58, 0.33-1.02) behaviors were much less frequent per 50 min observation period. Following was least frequent (0.069, 0.03-0.14). Frequency of overall behaviors was similarly greater than control in both removal treatments, both as raw frequencies ($\chi^2_2 = 34.145$, $N = 557$, $P < 0.0001$; Table 2) and as frequencies per bee ($\chi^2_2 = 34.495$, $N = 557$, $P < 0.0001$; Table 2).

Foraging trips were observed 0.28 times (0.14-0.56) per observation period. Frequency of foraging per bee differed significantly among treatments ($\chi^2_2 = 6.710$, $N = 557$, $P = 0.03$). Foraging trips were more frequent in control (C) treatments as compared to mother removal treatments (M) (C > M; Tukey-test: $t_{551} = 2.456$, $P = 0.0381$) and were intermediate between treatments in mother and DED removal treatments (MD). Durations of foraging also differed between treatments ($\chi^2_2 = 6.846$, $N =$

557, $P = 0.03$). Bees spent less time foraging in M treatments compared to C (Tukey test: M < C, $t_{552} = 2.484$, $P = 0.03$).

Social environment

The first and second PCs of our PCA accounted for 65.5% and 17.6% of variation respectively (Fig. 3). PC1 was positively correlated with all behavioral types (eigenvectors: aggressive = 0.40; tolerant = 0.52; avoidant = 0.55). PC2 was positively correlated with aggression (eigenvector = 0.89) and negatively with other types (eigenvectors: tolerant = -0.05; avoidant = -0.25; follow = -0.38). Control nests were primarily negatively associated with aggressive behaviors, whereas M and MD treatments primarily positively associated with these behaviors (Fig. 3). Control nests were also negatively associated with tolerant, avoidant and following behaviors, whereas M and D treatment nests tended to be average or uncorrelated with these behaviors, with the exception of two M treatments nests which were strongly positively associated with them (Fig. 3).

Frequency of aggressive behaviors differed significantly among treatments ($\chi^2_2 = 8.3973$, $N = 557$, $P = 0.045$), was slightly but not significantly more frequent in M treatments than in control (Tukey test: C < M, $t_{551} = -2.318$, $P = 0.0541$), and was significantly more frequent in MD treatments (MD > C, $t_{541} = -2.686$, p = 0.02; Fig. 4). C-posture was more frequent in M treatment (M > C, $t_{552} = -3.168$, $P = 0.0046$; Table 3, Fig. 4).

Frequencies of tolerant behaviors also differed significantly among treatments ($\chi^2_2 = 14.69$, $N = 557$, $P = 0.002$), being significantly more frequent in M and MD treatments (M > C, $t_{552} = -3.378$, $P = 0.0029$; MD > C, $t_{552} = -3.419$, $P = 0.002$; Fig. 4). Allogrooming and dual pass were significantly more frequent in both removal treatments (allogrooming: M > C, $t_{552} = -4.332$, $P = 0.0001$; MD > C, $t_{552} = -4.843$, p < 0.0001; dual pass: M > C, $t_{552} = -3.188$, $P = 0.0043$; MD > C, $t_{552} = -3.462$, $P = 0.0017$; Table 3, Fig. 4).

Discussion

Social environment influences intranidal behavior and foraging in adult assemblages of *C. calcarata.* Our multivariate analysis found that behavior in nests with mothers and both mothers and DED removed were associated with higher aggression. We found that overall activity within nests increased significantly in the absence of mothers. Maternal presence lowered overall tolerance within nests. As aggression was only greater in the absence of both mothers and DEDs, the presence of DEDs also plays a significant role in social behavior. Both maternal and DED presence thus appear to play a significant role in maintaining social organization within nests. Foraging behavior was also lowest in nests lacking mothers but where DEDs were still present. This is likely explained by a lack of stimulus to forage directed from mothers to DEDs along with the quiescence of regular daughters waiting to receive provisions. In the absence of both mothers and DEDs, foraging behavior was observed in regular daughters indicating that they are capable of foraging, but this is not observed in the presence of mothers and DEDs (Rehan et al. 2014; Mikát et al. 2017).

Mothers primary foragers and regulators of nesting activity

Our results indicate that aggressive interactions are frequent within *C. calcarata* during the social nesting period (44% of observed behaviors). Aggressive behaviors were more frequent than the individual behaviors, such as cleaning and grooming, that comprise the next most frequent activity within nests (43% of observed behaviors). Aggression thus appears to be a regular part of social nesting behavior, confirming previous observations that aggression is directed towards nest-mates (Rehan and Richards 2013). This could support the existence of a dominance hierarchy based in aggression within *C. calcarata.* Such dominance hierarchies are typical of species exhibiting simple cooperative behaviors. Foraging helpers of cooperatively breeding pied kingfishers and superb fairy-wrens are frequently the recipients of aggressive behavior from their breeding parents (Reyer et al. 1986; Mulder

and Langmore 1993). Within Hymenoptera alloparental care, reproductive roles and dominance hierarchies are frequently determined through aggressive interactions (West 1967; West-Eberhard 1969; Michener and Brothers 1974; Gadagkar 1980; Chandrashekara and Gadagkar 1991; Wcislo 1997; Arneson and Wcislo 2003; Jandt et al. 2014). Dominance hierarchies in the polistine paper wasps, established through repeated aggressive interactions, determine reproductive or foraging and nest maintenance roles (Pardi 1948; West-Eberhard 1969; Chandrashekara and Gadagkar 1991), and aggression is similarly correlated with division of labor in some primitively eusocial halictine sweat bees (Michener 1990). Within these species foraging and nest building females are frequently smaller, and the recipients of aggressive nudging and backing from their mothers, the primary egg-layers and reproductive dominant in nests (Michener and Brothers 1974; Greenberg and Buckle 1981; Buckle 1982; Michener 1990; Boomsma and Eickwort 1993; Richards and Packer 1994). Within the *Polistes* paper wasps subordinate co-foundresses are the primary foragers, and dominant individuals are more likely to conduct low risk tasks, such as collecting nest materials (West-Eberhard 1969; Gamboa et al. 1978; Reeve and Gamboa 1987; Pratte 1989). Given that unmarked colony members were observed in this study, further research tracking individuals is a necessary next step. Such observations would further test previous finding from circle tube assays that aggression is typically directed from mothers to daughters (Rehan and Richards 2013).

The overall increase in activity under both removal treatments suggests that behavior within nests undergoes reorganization in the absence of mothers, not significantly mitigated by the presence of DEDs. Given this dramatic change, it is unlikely that DEDs substitute for mothers in the overall stability of colony functioning. Within *Ropalidia* paper wasps, removal of dominant individuals results in temporary loss of distinguishable behavioral roles, including reproductive and foraging distinctions (Chandrashekara and Gadagkar 1991). In the absence of both mothers and DEDs foraging by regular daughters for supplemental provisions represents a significant risk, as well as an energetic cost for orphaned *C. calcarata* females. The dramatic change in overall colony behavior in the absence of

mothers, and the increase in aggression in the absence of mothers and DEDs, may be the result of conflict over foraging roles among size matched siblings (Smith and Parker 1976). While previous studies suggest that DEDs are both the primary forager in the absence of mothers, and that they forage as frequently as mothers, these results suggest that ordinary siblings may contribute to intranidal activities and the overall social environment that may also be important to fitness in the absence of mothers (Mikát et al. 2017).

Mothers reduce intranidal aggression and mutual tolerance

Higher aggression in absence of mothers and DEDs suggests both maternal and alloparental care from DEDs reduce aggression between offspring. This supports prior studies that found daughters from nests retaining mothers show negligible aggressive interactions (Rehan and Richards 2013) and increased aggression between unfamiliar females from orphaned nests (Arsenault et al. 2018). This pattern of aggression from mothers resulting in reduced aggression from offspring suggests that there is a rudimentary dominance heirarchy between mothers and offspring in *C. calcarata* (Breed et al. 1978; Wcislo 1997). Dominance hierarchies result when repeated aggessive encounters establish dominant winners and subordinant losers (Smith and Parker 1976, Rutte et al. 2006). Subordinant individuals are less likely to escalate conflicts, and thus an overall decrease in aggression may be observed when a hierarchy exists (Hemelrijk 2000). Within primitively eusocial sweat bees, aggression of reproductively dominant individuals, frequently foundresses, towards nestmates is thought to orchestrate intranidal tasks, such as nest construction (Brothers and Michener 1974; Michener and Brothers 1974; Michener 1990). In other closely related species of *Ceratina, C. japonica* and *C. flavipes,* smaller individuals engage in foraging, while larger individuals are primarily occupied with nest guarding and reproduction (Sakagami and Maeta 1989, Sakagami et al. 1993). Maternal aggression in *C. calcarata* is thus most likely a mechanism by which DEDs are ejected from nests to conduct foraging (Rehan et al. 2014).

Comparable levels of aggression between nests with mothers and those with only DEDs may not be explained by DEDs assuming a dominant role, as they are the smallest individual in the nest (Hogendoorn and Velthuis 1999; Smith et al. 2009; Rehan and Richards 2013). Production of smaller, more easily dominated offspring through manipulation of pollen mass provisions and developmental nutrition is thought to be a method of ensuring subordinance in many incipiently and primitively eusocial Hymenoptera (Hogendoorn and Velthuis 1999; Smith et al. 2009; Kapheim et al. 2011; Rehan and Richards 2013). Examples of such size based divisions of labor are not restricted to invertebrates (Reeve 1992). Dominants within naked mole-rat colonies subject larger subordinates to more frequent shoving, eliciting increased foraging (Reeve 1992). When dominant individuals are removed from nests of naked mole-rats, smaller individuals typically perform most of the work within nests, foraging and expanding nests (Reeve 1992). Such size related dominance interactions are particularly common to social Hymenoptera. Within primitively eusocial sweat bees, body size is frequently predictive of dominance and division of labor (Packer 1986, Kukuk and May 1991, Boomsma and Eickwort 1993). Similarly, within eusocial paper wasps, size frequently predicts competitive ability and worker versus reproductive roles (Dropkin and Gamboa 1981, Sullivan and Strassmann 1984, Tibbetts and Sheehan 2012). Higher aggression in the absence of mothers and DEDs may thus result from escalated conflict among more evenly size matched individuals (Smith and Parker 1976). Such intensified conflicts in size symmetrical individuals are found in both vertebrates in invertebrates (Enquist et al. 1987, Smith et al. 1994, Pratt et al. 2003).

Size, however, is not always associated with dominance in contests between female *C. calcarata,* with experience being the decisive factor after repeated encounters (Withee and Rehan 2016). During extended cohabitation experience is thus likely to influence the outcome of conflicts between similarly sized individuals (Withee and Rehan 2016). DEDs are not only the smallest daughter, they are also the eldest daughter. Within the related allodapine bee, *Exoneura robusta,* size is only weakly associated with dominance, with the eldest individuals typically assuming this role

(Schwarz and Woods 1994). Aside from the influence of size and age, individual variation in sensitivity, e.g. to deprivation of supplementary provisions, may results in differential levels of foraging and potential food sharing among regular daughters (Calabi 1988, Page and Robinson 1991). Such variation, differences in response thresholds, may be influenced by social environment within solitary sweat bees, resulting in a rudimentary division of labor among otherwise physiologically similar individuals (Jeanson et al. 2008). While increased aggression in the absence of mothers and DEDs implies conflict over division of labor, the degree to which regular daughters share food is not clear. In the absence of mothers and DEDs, age, experience and individual response thresholds likely influence foraging and food sharing among regular siblings. Examples of food sharing between siblings within diverse taxa are common. For example, in the absence of maternal provisioning, food sharing between siblings increases in earwigs (Kramer et al. 2015). It is thus possible that regular, non-dwarf, siblings may be able to maintain their own inclusive fitness through food sharing while contributing to other tasks within the nest, such as nest guarding and cleaning.

Reduced mutual tolerance between nestmates in the presence of mothers may also result from an established social hierarchy. Within primitively eusocial sweat bees, subordinate individuals are more avoidant of dominant individuals, responding to forced encounters with characteristic reversal or withdrawal behaviors (Buckle 1982, 1985, Wcislo 1997). After repeated encounters between females, avoidant behavior decreases in *C. calcarata* (Withee and Rehan 2016), thus decreased avoidance in removal treatments could be the result of increased overall activity, and resultant repeated interactions (Withee and Rehan, 2016). Greater tolerance within removal treatments is consistent with an overall difference in social structure within nests lacking mothers. It is also possible that in the absence of mothers, who typically prevent intruders and nest usurpation by guarding nests, nestmate recognition through chemical cues may facilitate increased tolerance (Rehan and Richards 2010).

Together our results suggest that aggressive behaviors are frequent within nests, and function to maintain division of labor among age and size classes, much as in group living vertebrates, in particular

birds and mammals (Reyer et al. 1986, Creel et al. 1992, Reeve 1992, Mulder and Langmore 1993). The role of aggression in adult nests of *C. calcarata* is also similar to that commonly found in primitively eusocial paper wasps and sweat bees (West-Eberhard 1969, Brothers and Michener 1974, Michener and Brothers 1974, Dropkin and Gamboa 1981, Buckle 1982, 1985, Reeve and Gamboa 1987, Pratte 1989, Michener 1990). Behavior within *C. calcarata* is highly plastic, and through removal experiments, we have shown group dynamics in this species is sensitive to changes in social environment. How foraging and pre-overwintering feeding is negotiated among regular daughters in the absence of provisioning from both mothers and DEDs remains to be investigated. In particular, further study could focus on how individual variation in response thresholds, age, and experience may result in differential foraging and, potentially, sibling food sharing (i.e. trophallaxis) (Calabi 1988, Breed and Page 1989, Page and Robinson 1991, Fewell and Page 2000). Such threshold responses may explain allocation of reproductive division of labor found among adult *Ceratina flavipes* during forced cohabitation (Sakagami and Maeta 1987). Understanding how changes in social environment interact with individual differences to affect cooperation and conflict, and how group cohesion is maintained and modulated is critical to understanding why some individuals cooperate, and others do not.

Tables and Figures Chapter II

Table 1. Ethogram of behaviors. Behaviours identified in study, with category of behavior and counts of behaviors observed.

Category	Behavior	Description	Count
Individual	Bump	One individual bumps into another with its abdomen	376
	Cleaning	Individual collects pith, old pollen or feces to carry outside the nest	532
	Cluster	Two or more individuals huddle together for more than a few seconds	2383
	Foraging	Active time outside of nest	261
	Grooming	Individual cleans self, may articulate abdomen	2759
	Guarding	Individual's abdomen is visible in entrance of nest, or individual pokes head/upper body outside of nest	753
	Pulsing	One individual's abdomen contracts and extends rapidly	48
	Walking	Individual moves forward away from another	193
Aggressive	Bite	On individual bites another	127
	C-posture	Aggressive posture where abdomen is bent towards second individual	39
	Nudge	One individual headbutts another individual's abdomen	7097
Avoidant	Back	Individual backs away from another	290
	Reverse	Individual reorients him/herself by turning around from another bee	399
Follow	Follow	One individual pursues another	79
Tolerant	Allogrooming	Individual(s) cleans another	361
	Antennation	In a frontal encounter, individuals stop and touch one another with antennae	71
	Pass	One individual passes by another	279
	Dual pass	Two individuals pass each other	159
	Head to head	Two individuals sit with heads touching	181

Table 2. Total behaviors. Behaviours per observation period and total behaviors per bee. Contrasts are post hoc Tukey tests of treatments effects estimated from GLMM. Df = 552. Bold demarcates statistically significant results.

	Contrast	Estimate	SE	T ratio	P-value
Total behaviors	**C - M**	**-2.011**	**0.4578**	**-4.392**	**< 0.0001**
	C - MD	**-2.678**	**0.4961**	**-5.398**	**< 0.0001**
	M - MD	-0.6673	0.5050	-1.294	0.3839
Total behaviors per bee	**C - M**	**-1.5343**	**0.3358**	**-4.570**	**< 0.0001**
	C - MD	**-1.9353**	**0.3627**	**-5.336**	**< 0.0001**
	M - MD	-0.4010	0.3688	-1.087	0.5224

Table 3. Results from GLMMs. Wald type II chi-square tests of frequencies of behaviors per individual bee. Bold demarcates statistically significant results. Df = 2.

Category	Behavior	χ^2	p-value
Aggressive	Bite	6.560	0.1505
	C-posture	**10.41**	**0.04482**
	Nudge	8.793	0.08182
Avoidant	Backing	8.898	0.08182
	Reverse	0.7991	0.6706
Tolerant	Allogrooming	**25.00**	**< 0.0001**
	Antennation	3.344	0.3757
	Dual pass	**13.49**	**0.01061**
	Head to head	5.526	0.1893
	Passing	7.559	0.1122
Follow	Follow	2.5484	0.5594

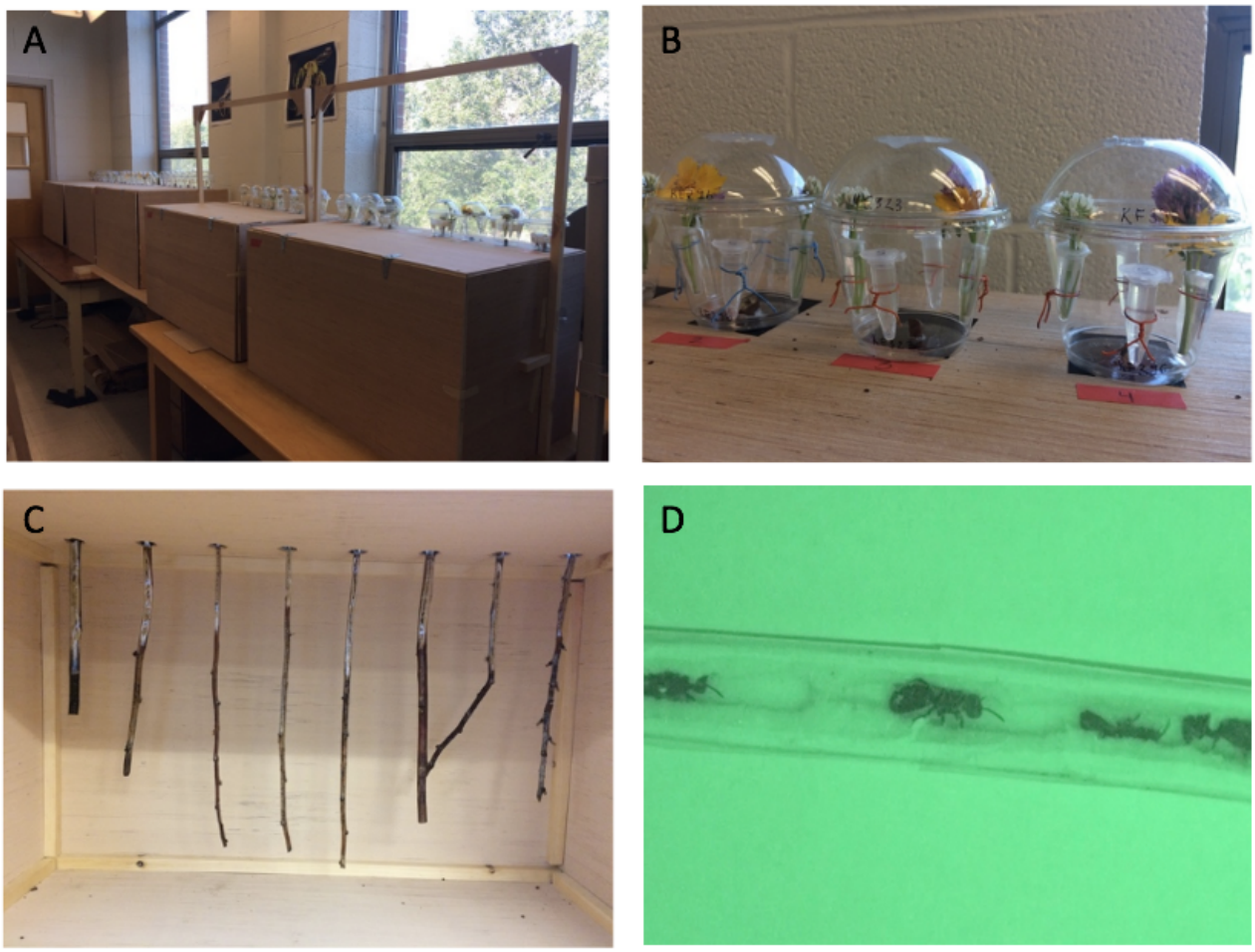

Figure 1. Observation nests. (A) experimental setup with boxes in which observation nests were housed and foraging arenas. (B) foraging arenas with flowers, water and sugar water. (C) interior view of boxes with eight observation nests. (D) still from infrared video showing interior of observation nest.

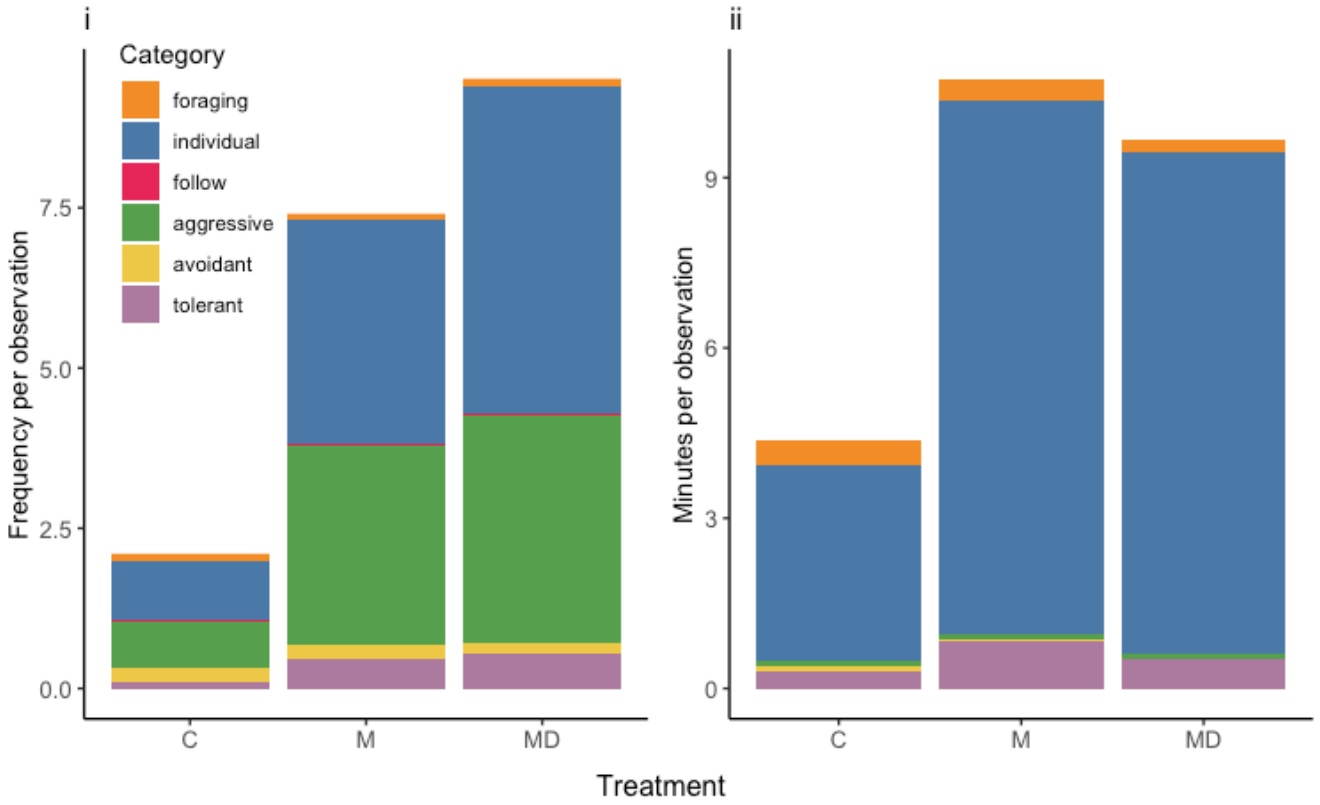

Figure 2. Multiple bar plot of behaviors by category (i) mean frequency of behavior per observation per individual bee. (ii) mean durations of behaviors per bee. C = control, M = mother removed, MD = mother and DED removed.

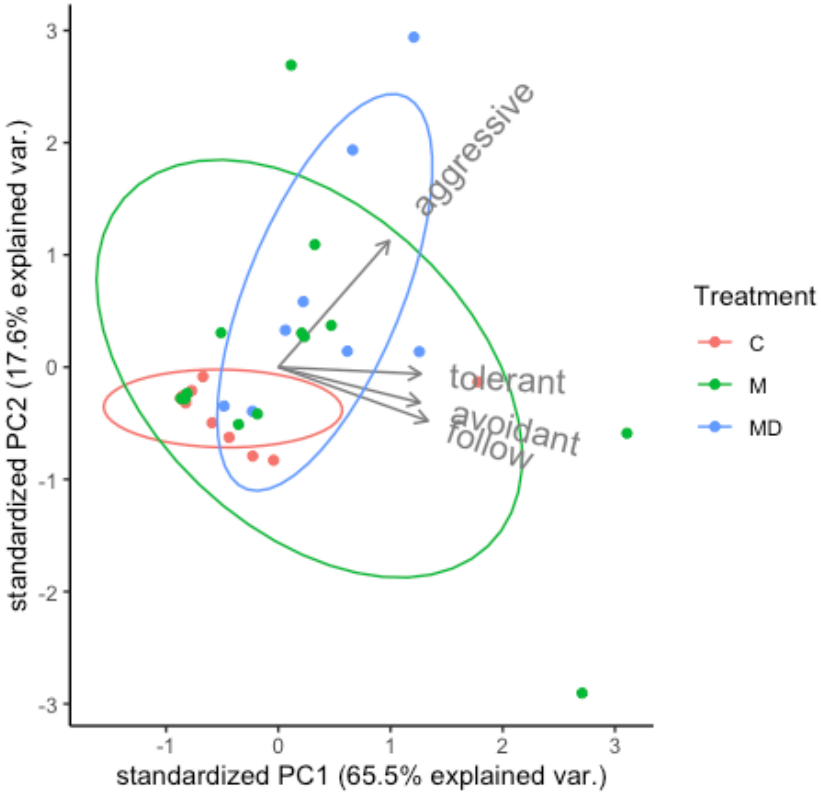

Figure 3. PCA of social behaviors by type, aggressive, tolerant, avoidant and follow. Each point represents an observation nest. Counts of behaviors averaged per observation for each nest. Ellipses represent 0.68 normal probabilities for each treatment.

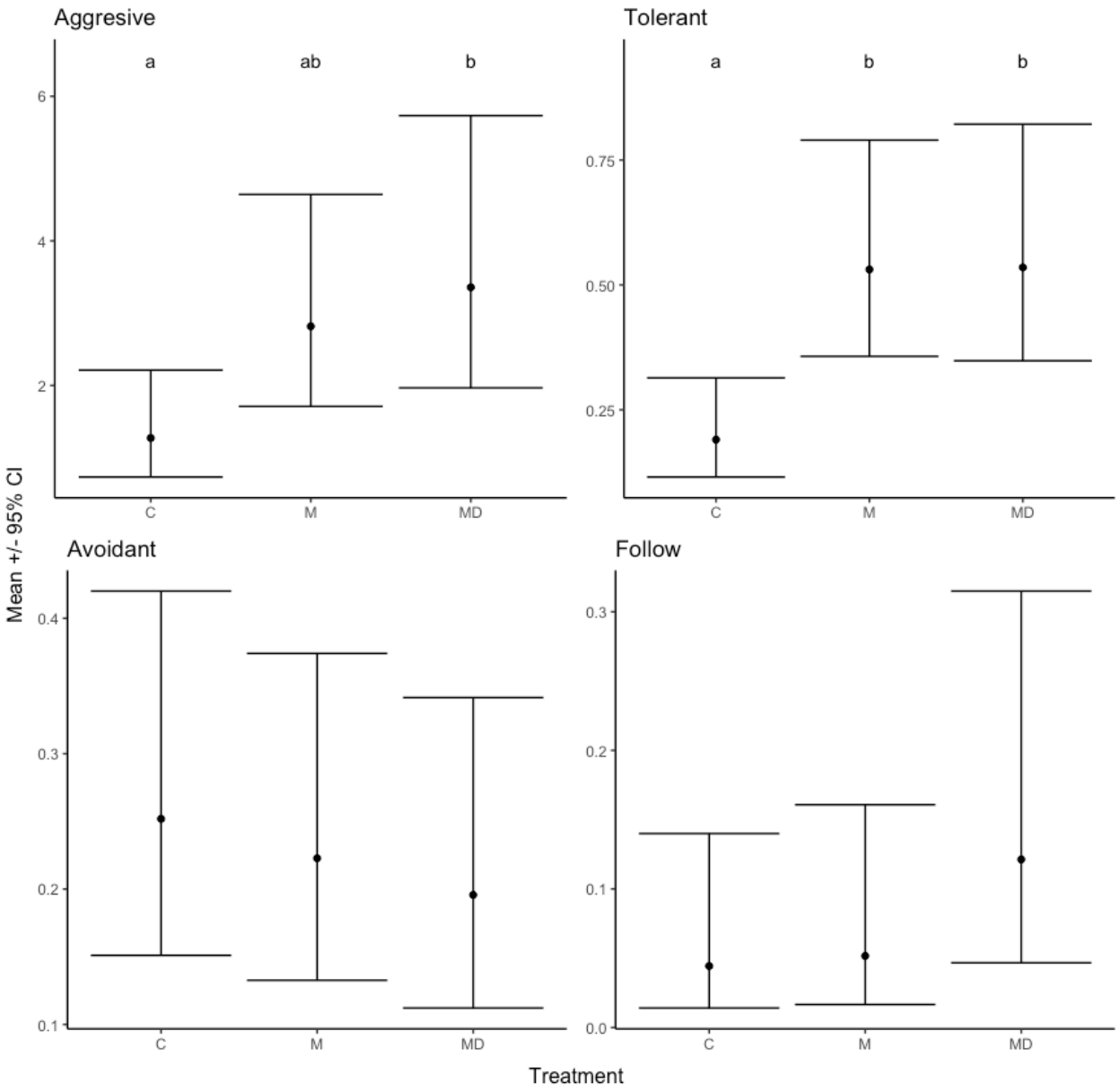

Figure 4. Estimated marginal means of categories of behavior per observation per individual bee. Differing letters identify significant differences from post-hoc Tukey tests. Treatments C = control, M = mother removed, MD = mother and DED removed.

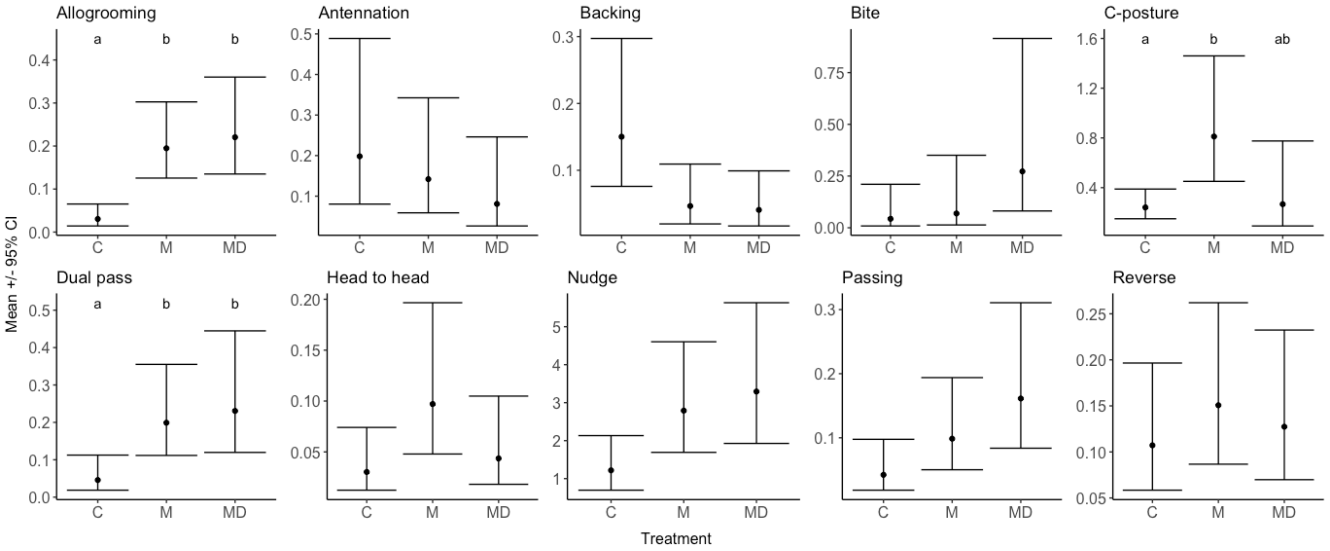

Figure 5. Frequencies of interactive behaviors. Estimated marginal means of interactive behaviors as frequencies per individual bee, per observation. Differing letters distinguish significantly different results of pairwise comparisons of estimates from GLMM with post-hoc Tukey tests. Treatments C = control, M = mother removed, MD = mother and DED removed.

Chapter III: Behavioural Genetics of Cooperation in *Ceratina calcarata*

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Abstract

Simple social systems have the potential to both advance our understanding of how complex sociality may have evolved, and our understanding of how changes in social environment may influence gene expression and cooperation. Recently studies of incipiently and primitively eusocial Hymenoptera have greatly expanded empirical evidence for the role of social environment in shaping behaviour and gene expression. Here we present brain gene expression profiles of foragers of the small carpenter bee, *Ceratina calcarata.* We conducted an experiment in the field to compare gene expression profiles among foraging mothers, worker-like dwarf eldest daughters (DEDs) in the presence and absence of mothers, and regular daughters. Our analysis found significant differences in gene expression associated with age, size and social environment. These groups were also each associated with distinct gene co-expression modules. We also found that a subset of differentially expressed genes were associated with division of labour, including foraging, in both primitively and advanced eusocial insects. Our results offer strong support for the role of social environment in producing differences in social behaviour and associated distinct gene expression profiles, depending on age, size and social environment.

Introduction

Sociogenomic study of animal societies is critical to understanding the evolution of social behaviour (Robinson et al. 2005, Rehan and Toth 2015, Shell and Rehan 2018b). Studies of gene

expression in complex insect societies have provided insight into how changes in expression of conserved genes are associated with cooperative task specialization (Ament et al. 2012, Berens et al. 2015, Mikheyev and Linksvayer 2015, Khamis et al. 2015, Morandin et al. 2016, Sun et al. 2019). These studies suggest that multiple phenotypes involved in task specialization share 'genetic-toolkits', or conserved genes that are co-opted for novel social functions (Toth and Robinson 2007b, Rittschof and Robinson 2016). Within Hymenoptera cooperative roles are not only linked to the expression of key genes (Toma et al. 2000, Ben-Shahar 2003, Ingram et al. 2016), but are also associated with genes co-expressed in modules (Chandrasekaran et al. 2011, Khamis et al. 2015). The expression of genes associated with particular cooperative roles and behavioural states are also sensitive to changes in the social environment (Arsenault et al. 2018, Manfredini et al. 2021). While these studies of complex societies suggest the importance of social environment to gene expression and associated behaviours, study of simple animal societies, lacking castes, is critical to understanding the evolution of insect social behaviour in its simplest stages.

The diversity of social behaviours displayed by the Hymenoptera (Michener 1974) has made them valuable models for study of the molecular mechanisms underlying social behaviour (Robinson et al. 2005, Schwarz et al. 2007, Kocher and Paxton 2014). Social behaviour in Hymenoptera ranges from solitary to advanced eusocial species with fully obligate castes (Wilson 1971, Michener 1974). The role of social environment in producing cooperation in primitively social species is critical to understanding how cooperation may have evolved, as these species lack pre-imaginal determination of caste roles (Michener 1974, Litte 1979, 1981, Strassmann 1981, Yanega 1989, Kukuk and May 1991, Richards

and Packer 1994). In particular, study of species that are facultatively social, capable of solitary and social organization, have begun to advance our understanding of the proximate causes of cooperation (Rehan and Toth 2015, Kronauer and Libbrecht 2018, Shell and Rehan 2018b). For instance transcriptomic studies of *Ceratina australensis, Megalopta genalis* and *Polistes canadensis* have all revealed significant differences in gene expression between individuals exhibiting different behavioural states, suggesting that even in simple social systems evolution has acted to produce highly distinct phenotypes preceding the evolution of advanced eusociality (Ferreira et al. 2013, Jones et al. 2017, Rehan et al. 2018).

Parental care, ancestral to many eusocial lineages, and sibling care offer compelling examples of simple social behaviours that may play a role in the evolution of sociality at its earlies stages (Alexander 1974, Tallamy and Wood 1986, West-Eberhard 1996). From an evolutionary-developmental perspective the close association of parents and offspring presents an opportunity for phenotypic plasticity to emerge even in simple social systems, through parental control of development (Toth and Robinson 2007b, Toth and Rehan 2017). Moreover, nest loyalty and mutual tolerance involved in extended parental care are necessary preadaptations for the more complex social behaviours found in eusocial insects (Wilson 1971, Tallamy and Wood 1986). Maternal manipulation is found across diverse taxa, including both vertebrates and invertebrates (Chandrashekara and Gadagkar 1991, Stacey and Koenig 1992, Briga et al. 2012). By differentially allocating resources among offspring mothers may determine their life history options and direct fitness, potentially limiting them to persuing

alternative cooperative strategies to gain inclusive fitness benefits (Eickwort 1969, Michener and Brothers 1974, Charnov 1978).

Within the Hymenoptera maternal manipulation is particularly influential on phenotype, as adult body size is directly attributable to larval provisions (Michener and Brothers 1974, Packer and Knerer 1985). Within many Hymenoptera size is decisive in division of labour, as larger individuals are frequently winners in the repeated agonistic encounters that determine social hierarchies (Kukuk and May 1991, Boomsma and Eickwort 1993, Richards and Packer 1994, Hogendoorn and Velthuis 1999, Smith et al. 2009, Kapheim et al. 2011b, Withee and Rehan 2016). Maternal manipulation, both of nutrition and through behavioural coercion, is thus a primary mechanism by which cooperation may be produced in subsocial Hymenoptera (Michener and Brothers 1974, Kapheim et al. 2016).

While prolonged subsocial behaviour is relatively rare in invertebrates, particularly care for adult offspring, it is common to several species of *Ceratina* small carpenter bees (Sakagami 1977, Michener 1990, Rehan 2020). *Ceratina calcarata* is not only subsocial, but displays cooperative social behaviours, as mothers frequently produce a smaller daughter, the dwarf eldest daughter (DED), who engages in sibling care (Rehan and Richards 2010a). This DED is the first of the brood to emerge in the late summer, and forages simultaneously with her mother while her adult siblings remain within the nest (Johnson 1988, Rehan and Richards 2010a, 2010b, Rehan et al. 2014). DED foragers are thus thought to act as insurance against loss of mothers as a second round of feeding and increased fat stores are required for overwinter survival until the spring dispersal period (Rehan and Richards 2010a, Mikát et al. 2017, Shell and Rehan 2018a). The smaller size of the worker-like DEDs suggests that maternal

manipulation through nutrition is critical in inducing cooperative behaviour (Rehan and Richards 2010a). While the smaller size of DEDs is determined by larval provisioning, the proximate mechanism of increased cooperative behaviour of DEDs in the absence of mothers is not well known (Alexander 1974, Michener and Brothers 1974, Lawson et al. 2017). Maternal presence in *C. calcarata* nests has been shown to have significant effects on both behaviour and gene expression (Arsenault et al. 2018). Experience has also been found to play a role in both winning and losing status in conflicts and gene expression patterns of *C. calcarata* (Withee and Rehan 2016, 2017). Critically, division of labour in *C. calcarata* is associated with distinct gene regulatory modules and highly conserved genes associated with caste differentiation in eusocial insects (Shell and Rehan 2019). Increased foraging behaviour of DEDs in orphaned nests of *C. calcarata* presents an opportunity to investigate the proximate mechanisms of cooperation, by comparing brain gene expression among phenotypes with and without mothers (Mikát et al. 2017, Huisken et al. 2021).

Here we examine the effects of social environment on cooperation in *C. calcarata* through removal of nest mothers combined with brain gene expression analysis of mothers, foraging DEDs and regular daughters. From the results of our transcriptomic analysis we compared differentially expressed genes (DEGs) among DEDs, regular daughters and mothers, and among DEDs under mother removal treatments. Gene ontology enrichment terms for these differentially expressed genes were compared to find putative regulatory processes underlying social behaviours. Maternal absence is expected to result in increased foraging among DEDs, and significant correlated changes in gene expression (Rehan et al. 2014, Kapheim et al. 2016, Arsenault et al. 2018, Shell and Rehan 2019). Secondarily, we conduct

comparative analysis of foraging females from this study with other social insect species (Rehan et al. 2014, Withee and Rehan 2017, Arsenault et al. 2018). These results reveal the role of social environment in producing cooperative behaviours and determine the underlying gene expression and regulatory pathways that regulate cooperative behaviour in an incipiently social bee.

Methods

Sample collection and foraging data

In April of 2020, artificial nests of cut raspberry branches (*Rubus idaeus*) mounted to bamboo stakes mounted on bamboo with zip-ties were deployed at 10 sites across the Greater Toronto Area (GTA). Occupied nests were identified by newly excavated nest entrances in the central pith of stems. In June two sites in the GTA, The Langstaff Eco Park (43°48'21.9"N 79°29'03.1"W) and Tommy Thompson Park (43°39'02.2"N 79°19'16.6"W) were chosen for the experiment. Foraging behaviour was observed across the season to compare foraging behaviour in control and removal nests. Four plots of 30 nests were established at the two sites (n = 240). Beginning June 14th sites were attended sequentially, from 8am-4pm on days without precipitation or overcast weather. Sites were attended once a week at each site, until August when sites were attended more frequently, using all days with suitable weather. During the late summer adult females were captured from their nests while foraging for analysis of brain gene expression. Beginning August 13th, treatments were applied and foraging bees were collected in the field sequentially as they emerged to forage. Foraging bees were captured by securing clear plastic cups over openings of occupied nests before 8am. Captured bees were anesthetized on ice and marked on the thorax with an enamel paint pen, identified to sex, head width was measured to identify DEDs, and their wing wear score to determine relative age (Rehan and Richards 2010a). Removed bees were collected in microcentrifuge tubes containing RNAlater and

labeled with nest of origin and identifying mark and placed on ice in a cooler. At the end of each field day collected samples were stored at -20°. Observation and collecting were ended September 6th, and a total of 103 bees was collected in RNAlater. All nests were collected and dissected and nest occupants recorded and preserved in 100% EtOH for other research.

RNA extraction and bioinformatic methods

RNA was extracted from brains of individual bees using the QIAGEN RNeasy Kit and standard protocol. A total of five mothers, three foraging DEDs from control nests, three from removal nests, as well as three foraging regular daughters (a total of 14) were sent to Genome Québec for library preparation and Illumina NovaSeq 6000 sequencing using 150 base paired-end reads, with 50M reads per library.

All analysis were conducted in R 3.6.2 and using BLAST+ (Camacho et al. 2009). Reads were aligned to the *C. calcarata* genome using STAR (Dobin et al. 2013, Rehan et al. 2016) with an average of 93% reads uniquely mapped. Analysis of differentially expressed genes (DEGs) between foraging mothers, DEDs, both in presence and absence of mothers, and regular daughters was performed using DESeq2 (Love et al. 2014). DEGs were compared to the current social insect literature for association with foraging and caste differentiation. Gene ontology (GO) term enrichment was determined in topGO v2.44.0 (Alexa and Rahnenfuhrer 2016) with GO annotation from *Apis mellifera* orthologs. Lists of DEGs were compared to publicly available studies of five bees (Harrison et al. 2015, Khamis et al. 2015, Jones et al. 2017, Rehan et al. 2018, Saleh and Ramírez 2019), using BLAST+, with greater than 65% identity and $< 1 \times 10^{-15}$ e-value matches retained (Camacho et al. 2009). A complementary weighted gene co-expression network analysis WGCNA (Langfelder and Horvath 2008) was also performed to find modular gene co-expression correlated with each phenotype and under each treatment (Shell and Rehan 2019).

Results

Alignment and Differential Gene Expression

RNA sequencing of brain samples produced and average of 53.23 MB of sequence data per sample. Comparison of gene expression among phenotypes resulted in 1094 DEGs (FDR < 0.05; Table 1, full list Table S1). Our multivariate analysis accounted for 74% of variation in the data across the first four principle components (PCs). It showed that regular daughters were associated with higher scores on PC1, while treatment DEDs were associated with higher scores on PC2 (Fig. 1). Mothers and regular daughters had more negatively scores on PC1 (Fig. 1). Treatment DEDs had more positivem scores for PCs 2-3.

A subset of DEGs were uniquely upregulated among specific subsets of the four categories of adult females examined (Fig. 2). Foraging regular daughters showed the highest number of these uniquely upregulated genes, followed by mothers, DEDs with mother removed, and control DEDs (Fig. 2). Regular daughters and DEDs with mother removed showed the greatest overlap in upregulated DEGs followed by mothers and regular daughters (Fig. 2). Control DEDs were distinguished by 244 DEGs compared to all other groups (Table S1). Overexpressed genes included *ache1* and *ddi1* (Table 1). Overall, DEGs expressed in control DEDs were enriched for GO terms including RNA-dependent DNA biosynthetic process (GO:0006278) and DNA metabolic process (GO:0006259; Table S2). These DEDs were negatively associated with a single co-expression module, dark turquoise, enriched for circadian sleep/wake cycle (GO:0042745) among other GO terms (Tables 2, S4).

Dwarf eldest daughters with mother removed were distinguished by 584 DEGs compared to other groups, including upregulated rhythmically expressed gene 5 (*Reg-5*), circadian clock-controlled protein daywake (*dyw*), and UDP-glucosyltransferase (*egt*) (Table S1). In contrast to control DEDs, those with mother removed expressed DEGs with GO enrichment for the largest number of terms, including methylation (GO:0032259), DNA integration (GO:0015074), generation of precursor

metabolites and energy (GO:0006091), and ATP biosynthetic processes (GO:0006754; Table S2). Dwarf eldest daughters in the absence of mothers were also associated with a larger number of gene co-expression modules (Table 2). These include positive association with the yellow module, enriched for response to light stimulus (GO:0009416), and the light yellow module, associated with response to external stimulus (GO:0009605), among other GO terms (Table 2; S4). Comparison between control DEDs and DEDs with mothers removed showed the least differential expression, with only seven genes differentially expressed (Table S1). These genes were:L trypsin-1 (*tryp1*), retrovirus-related polyprotein from transposon 17.6 (*pol*) and ecdysteriod UDP-glucosyltransferase (*egt*) and four proteins of unknown function (Table S1). These genes were up regulated in DEDs with mothers present, with the exception of *egt*, downregulated in the absence of mothers.

Mothers were distinguished by 874 DEGs compared to other groups (Table S1). Overexpressed genes included royal jelly protein apisimin, and AMP phosphotransferase AK3 (Table 1). Differentially expressed genes expressed in mothers were enriched for GO terms including transmembrane transport (GO:0055085), establishment of localization (GO:0051234), cation transport (GO:0006812) and carboxylic acid metabolic process (GO:0019752) and oxoacid metabolic processes (GO:0043436; Table S2). Mothers were associated with four gene co-expression modules including steel blue and grey, both enriched for response to carbohydrate (GO:0009743; Tables 2, S4).

Regular daughters were distinguished by 941 DEGs relative to other groups, the largest compliment of genes (Table S1). Over expressed genes included *harbi1* and *olpB* (Table 1). Regular daughters expressed DEGs GO enriched for cellular protein metabolic process (GO:0044267) and carbohydrate metabolic processes (GO:0005975), as well as macromolecule modification (GO:0043412; Table S2). Regular daughters were associated with the most gene co-expression modules, including black, turquoise and dark green, enriched for GO terms sensory perception of smell and taste (GO:0007608; GO:0050909) and peptide pheromone export (GO:0000770; Table 2, S4).

Comparative analysis

Our comparison with studies of DEGs in differing behavioural categories across five bees, *Ceratina australensis, Euglossa dilemma, Bombus terrestris, A. mellifera,* and *M. genalis* found overlapping gene expression patterns (Table 3; Table S6). Genes upregulated in *C. calcarata* mothers were similarly upregulated in predisperal and social primaries in *C. australensis*, in dominants of *E. dilemma*, non-reproductive females of *B. terrestris* and nurses and workers of *A. mellifera* (Table 3; Table S6). These include *ilp* and *pban* (Table 3). In contrast upregulated genes of regular daughters of *C. calcarata* overlapped primarily with genes upregulated in social secondaries of *C. australensis*, and workers of *A. mellifera,* including *spon1* and *hamlet* (Table 3; Table S6). Upregulated genes of control DEDs also overlapped with genes upregulated in social secondaries of *C. australensis,* and in non-reproductive workers of *B. terrestris* (Table 3; Table S6). These included ankyrin repeat and kinase domain containing 1, *ankk1* (Table 3). Up regulated genes in treatment DEDs overlapped with those in both predispersal and social secondaries of *C. australensis*, and with non-reproductive workers of *B. terrestris*, also including insulin like growth factor binding protein acid labile subunit (*igfal*) and *moody* (Table 3).

Discussion

Differential gene expression

This study revealed that despite engaging the same foraging behaviour differences both age and social environment have different but significant impacts on gene expression in *C. calcarata.* Mothers tended to show differences in gene expression known to be related to foraging. These include *ache1*, associated with the transition from nursing to foraging in *A. mellifera* (Shapira et al. 2001). Similarly mothers also showed greater expression of *pban*, coding for a pheromone regulating neuropeptide associated with increased foraging in *A. mellifera* (Brockmann et al. 2009).

I found differential expression among groups of multiple genes related to division of labour in advanced eusocial species, including *ioIG,* hexamerine, *ilp* and a putative gene coding for a royal jelly protein apisimin all involved in queen vs. worker differentiation in *A. mellifera* (Evans and Wheeler 1999, Bíliková et al. 2002, Wheeler et al. 2006, Begna et al. 2011, Wang et al. 2013, Saleh and Ramírez 2019). Mothers also showed upregulation of *jhamt*, involved in juvenile hormone synthesis. Juvenile hormone (JH) is of particular interest as higher titers of JH are implicated in higher levels within the social hierarchy and reproductive dominance in many primitively eusocial and advanced eusocial Hymenoptera (Robinson et al. 1992, Tibbetts and Huang 2010b, Smith et al. 2013, Jones et al. 2021). Hexamerin is of particular interest as it is associated with caste differentiation through modulation of JH in a fire ant *Solenopsis invicta,* and termite *Reticulitermes flavipes* (Zhou et al. 2007, Hawkings et al. 2019). Mothers also showed greater expression of *klf10*, a family of transcription factors associated with delay of transition from nurse to worker in *A. mellifera* by queen mandibular pheromone (Grozinger et al. 2003).

Regular daughters were the most distinct in terms of both uniquely expressed genes, and in overall gene expression patterns as captured in our multivariate analysis (Figs. 1 and 2). Regular daughters tended to express genes related to reproduction and feeding/olfaction and protein and carbohydrate production, in keeping with their status as foraging for provisions to survive overwintering. Genes related to reproduction include *GnRH-R* and *Hr39* involved in neuropeptide and hormone signalling, including JH and ecdysteriod (Loof et al. 2001, Wang et al. 2012). Genes of interest also included those related to olfaction and hunger, in particular neuropeptide receptor *CCHa1-R,* signalling between brain and gut and olfaction during starvation in *D. melanogaster* (Farhan et al. 2013, Li et al. 2013). This study reveals that genes associated with foraging in regular daughters are distinct from those of mothers. This is corroborated by regular daughters having a greater number of significantly co-expressed modules, potentially associated with a greater repertoire of behaviours

including their both preparation for overwintering and subsequent mating (Rehan and Richards 2010a, Rehan et al. 2014).

Control DEDs were the least distinct in terms of uniquely differentially expressed genes and the fewest co-expressed modules (two). Of particular interest is that DEDs in the absence of mothers tended to express genes related to circadian rhythms including both *Reg-5* and *dyw*. regulated by the cyclic expression of the *period* (*per*) gene in *Drosophila melanogaster* (Van Gelder and Krasnow 1996). The regulation of circadian rhythms by the *per* gene network is found in highly diverse species, and has been associated with division of labour in *A. mellifera* (Bloch et al. 2001). In contrast *dyw,* while also interacting with *per*, codes for a JH binding protein that responds to changes in temperature through the sensory system in to supress siesta behaviour in *D. melanogaster* (George and Stanewsky 2021). The importance of circadian rhythms and response to light and temperature in DEDs in the absence of mothers is further supported by their association with yellow and light yellow modules, enriched for GO terms related to response to light and external stimulus (Table 2). Accordingly, DEDs in the presence of mothers were negatively associated with the dark turquoise module enriched for circadian rhythms (Table 2). The expression of these genes suggests that foraging of DEDs in the absence of mothers may be related to changes in the regulation of circadian rhythms and response to temperature.

Our results suggest mothers, daughters and DEDs are all distinguished by expression of distinct genes while engaging in foraging behaviour. Interestingly DEDs in the presence/absence of mothers were distinguished by as few as seven genes, including the down regulation of a single gene *egt*, a suppressor of ecdysteriods essential for development, in DEDs in the absence of mothers (Ahn et al. 2012). Suggesting that differences in foraging frequency are highly sensitive to social environment and may be significantly altered by behavioural responses to circadian rhythms and abiotic changes in temperature.

Comparative analysis

In keeping with our analysis of differential gene expression, our comparative analysis found similarities between *C. calcarata* age and behavioural categories and distinct gene expression patterns in behavioural categories of other social insects. Interestingly many DEGs expressed by mothers and regular daughters are found to play a critical role in foraging behaviour and caste differentiation in several of the considered eusocial insect species, especially with the honey bee *A. mellifera* and the ants *S. invicta* and *F. exsecta* (Brockmann et al. 2009, Choi and Meer 2012). In particular the gene *pban*, coding for a pheromone induced neuropeptide, is not only associated with increased foraging in *A. mellifera* (Brockmann et al. 2009) but in the synthesis of ant trail pheromones in *S. invicta* (Choi and Meer 2012). This suggests a possible role for chemical communication in division of labour in *C. calcarata* foraging behaviour. This is corroborated by gene co-expression modules enriched for GO terms related to peptide pheromone export (GO:0000770; Table 2). These results support the toolkit hypothesis that conserved genes associated with advanced eusociality underpin simpler forms of division of labour within *C. calcarata*. Regular daughters also showed differential expression of genes known to play a role in foraging and caste differentiation, as well as those related to reproduction, in many of the eusocial species considered (Table 2). Those related to reproduction include *GnRH-R* and *Hr39* involved in neuropeptide and hormone signalling, including JH and ecdysteriod, and *A. mellifera* reproductive status and behavioural associated gene networks (Loof et al. 2001, Wang et al. 2012).

Both DEDs, regardless of social environment, expressed fewer behaviourally relevant DEGs orthologs in eusocial insects than mothers or regular daughters (Table 3). Of genes with known roles in foraging only control DEDs showed upregulation of *ache1*, also upregulated in mothers, associated with the transition from nursing to foraging in *A. mellifera* (Shapira et al. 2001). Also in contrast to the upregulation of genes directly related to foraging and caste differentiation in social insects, DEDs in the absence found in mothers tended to express behaviourally relevant genes related to circadian rhythms (Table 2). The foraging behaviour of DEDs thus appears to be associated less with genes known to play

a conserved role in eusocial lineages but instead with a highly conserved circadian rhythms known from a diversity of species, including both insects and mammals (Van Gelder and Krasnow 1996, Tei et al. 1997, Bloch et al. 2001).

Conclusion

Study of foraging behaviour in *Ceratina calcarata* represents a distinct opportunity to characterize a cooperative behaviour and the influence of social environment on behavior and corollary gene expression. Our results suggest that foraging behaviour is highly sensitive to social environment, and that distinct patterns of gene expression may underly foraging depending on age, size and social environment. We further found that genes related to foraging may be associated with those related to foraging and caste in eusocial species, including advanced eusocial Hymenoptera, but may also be related gene networks broadly conserved in insects and even mammals. This, along with the modular nature of gene expression in *C. calcarata*, tends to suggest the existence toolkit genes that may be coopted for novel social functions in evolution. These genes appear to be related to key developmental hormones, including JH and ecdysteroid, with possible roles for processes of circadian rhythms and chemical communication. These show the critical need for further study of the role of social environment influences on behaviour and associated gene expression in simple social systems.

Figures and Tables Chapter III

Table 1. The five most overexpressed genes in each group with contrasts. DC = DEDs in the presence of mothers, DT = DEDs in the absence of mothers, M= mothers, RC = Regular daughters.

C. calcarata gene ID	*C. calcarata* DEG contrast	Gene symbol	Gene annotation	log_2(fold change)	p-value (adjusted)
Ccalc.v3.14914	DC > R	*ACHE1*	Acetylcholinesterase	8.51	0.00103
Ccalc.v3.18753	DC > R	*ddi1*	DNA damage-inducible protein 1	7.61	0.03150
Ccalc.v3.05352	DC > M	*olpB*	Cell surface glycoprotein 1	6.97	0.00015
Ccalc.v3.05716	DC > DT	*TRYP1*	Trypsin-1	6.17	0.01146
Ccalc.v3.07657	DC > R	*tcf21*	Transcription factor 21	5.33	0.00475
Ccalc.v3.05352	DT > M	*olpB*	Cell surface glycoprotein 1	6.25	0.00077
Ccalc.v3.09104	DT > M	*Ccp84Ab*	Cuticle protein	4.53	0.00377
Ccalc.v3.14895	DT > M	*TcasGA2*	Venom carboxylesterase-6	4.50	0.04700
Ccalc.v3.05489	DT > M	*TM-C1B*	Pupal cuticle protein C1B	4.36	0.00025
Ccalc.v3.04501	DT > M	*SgAbd-1*	Endocuticle structural glycoprotein	4.00	0.00000
Ccalc.v3.23828	M > DC	*pol*	Retrovirus-related Pol polyprotein from type-1 retrotransposable element R1	8.70	0.00240
Ccalc.v3.01947	M > DC	*Apisimin*	Apisimin	7.38	0.02084
Ccalc.v3.00322	M > R	*LINE-1*	Reverse transcriptase homolog	7.19	0.00200
Ccalc.v3.14593	M > DT	*Tf2-9*	Transposon Tf2-9 polyprotein	7.04	0.00747
Ccalc.v3.17477	M > DT	*AK3*	GTP:AMP phosphotransferase AK3	7.01	0.00042
Ccalc.v3.19308	R > M	*harbi1*	Putative nuclease HARBI1	8.63	0.01657
Ccalc.v3.14896	R > M	*TcasGA2*	Venom carboxylesterase-6	8.59	0.00000
Ccalc.v3.05352	R > M	*olpB*	Cell surface glycoprotein 1	7.31	0.00001
Ccalc.v3.23828	R > DC	*pol*	Retrovirus-related Pol polyprotein from type-1 retrotransposable element R1	6.72	0.04152
Ccalc.v3.17477	R > DT	*AK3*	GTP:AMP phosphotransferase AK3	6.67	0.00156

Table 2. Gene co-expression modules. Modules significantly correlated with mothers, regular daughters and DEDs in the presence/absence of mothers, with selected GO enrichment terms. Full list of genes and GO terms available in supplementary Tables S3 and S4.

Module	Group	Correlation	p-value	GO Terms
Steel blue	Mothers	0.56	0.04	Glycerolipid metabolic process (GO:0046486) Response to carbohydrate (GO:0009743) Response to nutrient levels (GO:0031667)
Salmon		0.68	0.007	Dephosphorylation (GO:0016311) Regulation of neuron death (GO:1901214) Regulation of peptidyl-serine phosphorylation (GO:0033139)
Dark orange		0.81	0.0004	cGMP biosynthetic process (GO:0006182) cGMP metabolic process (GO:0046068) Aromatic amino acid family catabolic process (GO:0000949)
Dark orange		0.81	0.0004	cGMP biosynthetic process (GO:0006182) Regulation of nervous system process (GO:0031644) Regulation of sensory perception of pain (GO:0051930)
Grey	Mothers	0.57	0.03	mRNA processing (GO:0006397) Cellular response to glucose stimulus (GO:0071333) Response to carbohydrate (GO:0009743)
	DED mother removed	-0.58	0.03	
Light yellow	DED mother removed	0.59	0.03	ncRNA metabolic process (GO:0034660) Cellular glucose homeostasis (GO:0001678) Response to external stimulus (GO:0009605)
Yellow		0.56	0.04	Arginine catabolic process (GO:0006527) Arginine catabolic process to glutamate (GO:0019544) Response to light stimulus (GO:0009416)
Purple		0.63	0.01	Negative regulation of peptide hormone secretion (GO:0090278) Neurotransmitter secretion (GO:0007269) Regulation of cAMP-dependent protein kinase (GO:2000479)
Dark turquoise	DED mother present	-0.55	0.04	Circadian sleep/wake cycle (GO:0042745) Mating behavior, sex discrimination (GO:0048047) Regulation of trans-synaptic signaling (GO:0099177)
	Regular daughters	0.82	0.0003	
Saddle brown	Regular daughters	-0.6	0.02	Cholesterol storage (GO:0010878) Neurotransmitter receptor transport (GO:0099637) Age-dependent general metabolic decline (GO:0007571)

Black		0.6	0.02	Regulation of lipoprotein metabolic process (GO:0050746) Sensory perception of smell (GO:0007608) Regulation of mRNA processing (GO:0050684)
Turquoise		0.59	0.03	Flavin-containing compound metabolic process (GO:0042726) Fatty acid transport (GO:0015908) Peptide pheromone export (GO:0000770)
Dark green		0.6	0.02	Regulation of dendrite development (GO:0050773) Positive regulation of insulin receptor (GO:0046628) Response to peptide hormone (GO:0043434)
Midnight blue		0.44	0.04	Ecdysteroid secretion (GO:0045457) Sensory perception of taste (GO:0050909) Sensory perception of light stimulus (GO:0050953)

Table 3. Selected annotated genes. Putative orthologs (greater than 65% identity and < 1 x 10^{-15} e-value) from the four behavioural groups examined in *Ceratina calcarata* from this study, with differential expression patterns in relevant groups in considered studies. *C. australensis* PD = predispersal, Sol. = solitary, 1st = social primary, 2nd = social secondary, *Bombus terrestris* NRW = non-reproductive worker, RW = reproductive worker.

Group	Gene symbol	*Ceratina australensis*	*Euglossa dilemma*	*Bombus terrestris*	*Apis mellifera*	*Megalopta genalis*
Mother	*cad87A*	PD > 1st				
	slc30a2:	PD > 1st				
	pban	1st > 2nd				
	lrrc20	Soc 1st > 2nd		NRW > RW		
	klf10	PD > Sol.			Nurse > Worker	
	ilp		Dominant > Guard		Worker > Queen	
Regular daughter	*hr39*	2nd & PD > Sol.				
	inx2	2nd & PD > Sol. & 1st				
	spon1	2nd & PD > Sol. & 1st				
	hamlet	2nd & PD > Sol. & 1st				
	nbl1	PD > Sol.				
	pnlip					Workers > Queen
	apn1					Workers > Queen
	gld					Workers > Queen
DED control	*ankk1*			NRW > RW		
	PNP	2nd & PD > Sol. & 1st				
	Ptchd3	2nd & PD > Sol. & 1st				
DED treatment	*igfals*	2nd & PD > Sol. & 1st		NRW > RW		
	ankk1			NRW > RW		
	moody	2nd & PD > Sol. & 1st				
	cmtm4	PD > Sol.				

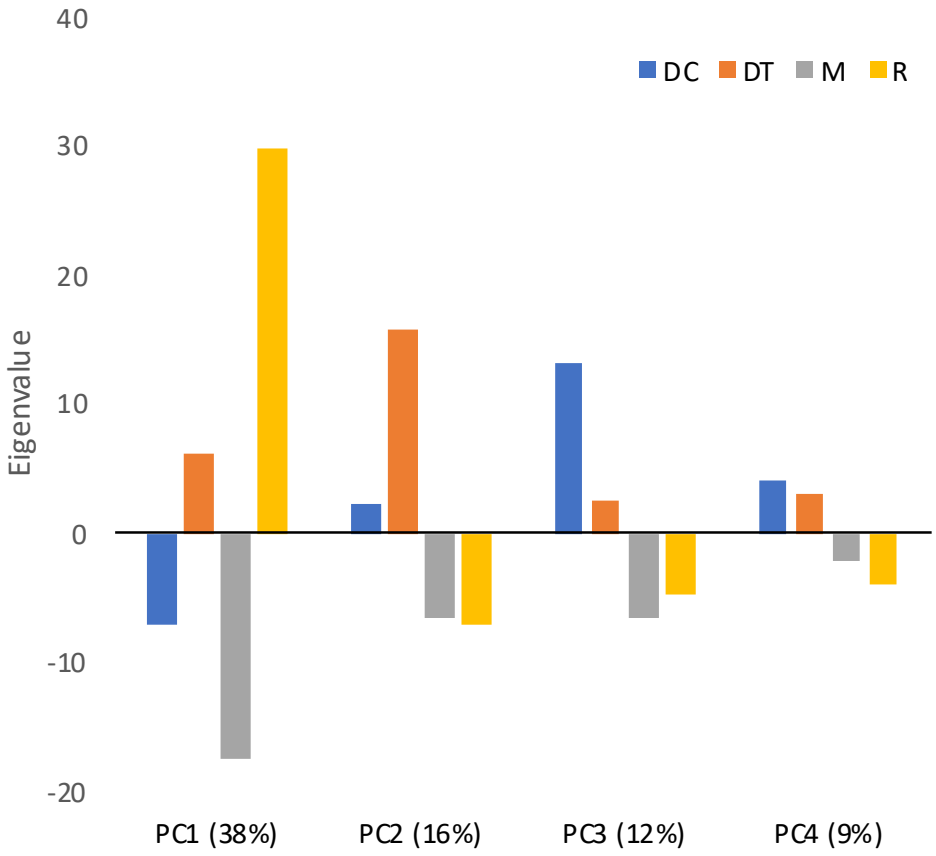


Fig. 1. Principle component analysis (PCA) of gene expression in foraging females of each behavioural group, across the first four principle components (PC1-PC4). DC =. Control DEDs with mother present, DT = DEDs with mother removed, M = mothers, R = regular daughters.

DEDs mother removed

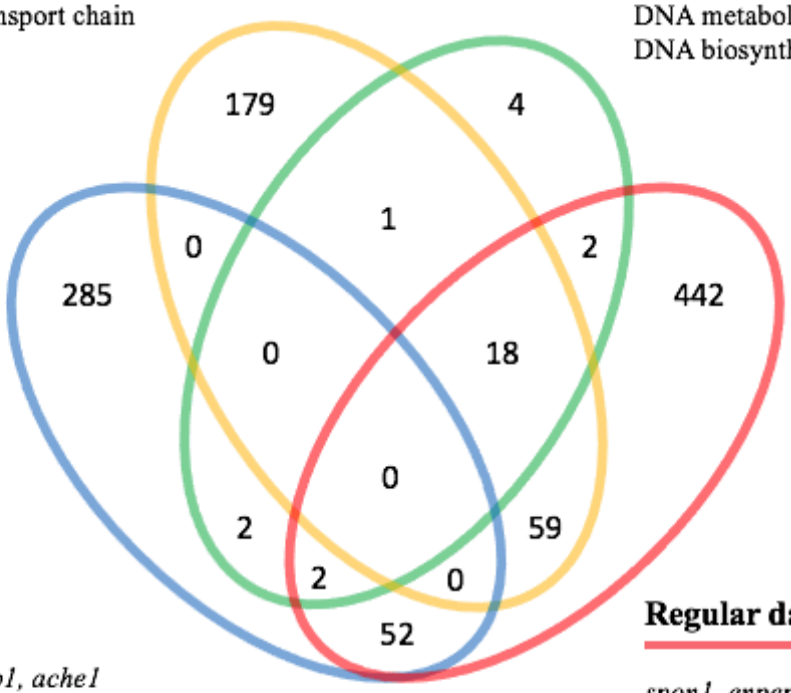
upf2, rop, Reg-5, dyw

Generation of precursor metabolites and energy
Electron transport chain
Methylation

DEDs control

ache1, ddi1

RNA-dependent DNA biosynthetic process
DNA metabolic process
DNA biosynthetic process



Mothers

apisimin, fpb1, ache1
pban, ankk1, Reg-5, ioIG

Transmembrane transport
Cation transport carboxylic acid metabolic process

Regular daughters

spon1, enpep, CCHa1-R, Hr39

Cellular protein metabolic process
Carbohydrate metabolic process
Macromolecule modification

Fig. 2. Counts of uniquely up regulated genes within each female behavioural category, with associated annotated genes, followed by enriched GO terms.

Chapter IV: General Discussion

Thorough studies of the proximate mechanisms involved in the evolution of sociality at its simplest stages are advancing rapidly with the availability of next generation sequencing techniques, as is our increasing appreciation for model organisms that are factitively social. The aim of this thesis was to understand how social environment influences cooperation and conflict in simple social systems, and associated gene expression to understand regulatory pathways underpinning these behaviours. Study of behaviour and gene expression in eusocial Hymenoptera have suggested many potential pathways for the evolution of sociality, but simple social systems offer compelling examples of how solitary ancestors of social insects may have evolved cooperation. Through the study of simple societies we may begin to account for the diversity of potential pathways to eusociality, as well as offer comparisons to cooperation as observed in a more diverse range of taxa, including vertebrates. *Ceratina calcarata* is one of a small number of bees that have been studied as model organisms for how sociality may have evolved from solitary ancestry (Shell and Rehan 2018b). *Ceratina calcarata* mothers engage in extended care of adult offspring, but also frequently produce a worker-like daughter, who feeds siblings (Johnson 1988, Rehan and Richards 2010a, 2010b, Rehan et al. 2014, Mikát et al. 2017). By considering an incipiently social species such as *C. calcarata,* my research has provided key insights into the role of social environment, the interaction of maternal care and sibling cooperation, on both behaviour and gene expression.

In chapter II I found that social environment influences both intranidal and foraging behavior in social groups of *C. calcarata.* To do this, I analyzed data from an experiment manipulating the presence of mothers and DED in observation nests. I predicted that aggression would increase in the absence of mothers, and that foraging would decrease in the absence of both mothers and DEDs. My results found that behaviour in nests with mothers and both mothers and DED removed were associated with higher aggression, and aggression was significantly greater in the absence of both. Unexpectedly, I

found that overall activity within nests increased significantly in the absence of mothers, and that maternal presence lowered overall activity within nests. This suggests that DEDs do not substitute for mothers, but may moderate aggression. Also, unexpectedly I found that foraging behaviour was lowest in nests lacking mothers but where DEDs were still present. This may be explained by a lack of stimulus to forage directed from mothers to DEDs along with the quiescence of regular daughters waiting to receive provisions from DEDs. In the absence of both mothers and DEDs, foraging behaviour was observed in regular daughters indicating that they are capable of foraging when lacking provisions provided by mothers and/or DEDs (Rehan et al. 2014; Mikát et al. 2017).

The unexpected overall increase in activity under both removal treatments implies that behaviour within nests is reorganized in the absence of mothers, and is not preserved by the presence of DEDs, even as they act as alloparents (Rehan and Richards 2010a, Mikát et al. 2017). Similar results have been observed in *Ropalidia* paper wasps when removal of a dominant wasp leads to loss of distinct reproductive and foraging roles (Chandrashekara and Gadagkar 1991). The dramatic change in activity in the absence of mothers, and the increase in aggression in the absence of mothers and DEDs, suggests that size-matched regular daughters may engage in agonistic contests to determine a foraging role (Smith and Parker 1976). While previous studies suggest that DEDs are both the primary forager in the absence of mothers, and that they forage as frequently as mothers, these results suggest that ordinary siblings may contribute to intranidal activities and the overall social environment that may also be important to fitness in the absence of mothers (Mikát et al. 2017).

These results demonstrated that aggressive behaviours are frequent within nests likely functioning to maintain division of labour as in group living vertebrates (Reyer et al. 1986, Creel et al. 1992, Reeve 1992, Mulder and Langmore 1993). The presence of such social hierarchies based in agonistic conflicts is also commonly found in primitively eusocial paper wasps and sweat bees (West-Eberhard 1969, Brothers and Michener 1974, Michener and Brothers 1974, Dropkin and Gamboa 1981, Buckle 1982, 1985, Reeve and Gamboa 1987, Pratte 1989, Michener 1990). Through removal

experiments, I have shown that *C. calcarata* is highly sensitive to changes in social environment. Further research is required to understand how variation in response thresholds, age, and experience may result cooperative food sharing among size matched siblings (Calabi 1988, Breed and Page 1989, Page and Robinson 1991, Fewell and Page 2000). These results shed light on how changes in social environment influence individual differences and result in mutual tolerance, cooperation and conflict, and bring us closer to understanding why some individuals cooperate, and others do not.

In chapter III, I outlined the results of an experiment conducted in the field to examine the effects of social environment on foraging behaviour in *C. calcarata* and associated gene expression. I removed individuals from nests and analysed brain gene expression of mothers, foraging DEDs and regular daughters. Using transcriptomic analysis, I compared differentially expressed genes (DEGs) among DEDs, regular daughters, mothers, and of DEDs under mother removal treatments. In addition to characterizing gene expression under these conditions, I also conducted a comparative analysis with other social insect species to detect conserved genes across lineages and independent origins of hymenopteran sociality. This experiment explored the role of social environment in producing cooperative behaviours, and determined the underlying gene expression associated with cooperative behaviour in an incipiently social bee.

My results revealed that social environment has a significant influence on gene expression, along with differences in age. Despite engaging in the same foraging behaviour, each category of females showed distinct gene expression profiles. While mothers were associated with genes known to be related to foraging and caste differentiation, regular daughters upregulated genes related to reproduction, feeding/olfaction, and protein and carbohydrate production. Regular daughters were the most distinct in terms of gene expression, in keeping with their status in engaging in both foraging for provisions, and preparation for reproduction after overwintering. Control DEDs were the least distinct in terms of gene expression, and overlapped considerably with mothers. While control DEDs and DEDs in the absence of mothers had few differentially expressed genes, their overall expression

patterns only overlapped slightly, suggesting that changes in the expression of a small number of genes may be critical to differences in behaviour. DEDs with mothers removed were associated with the expression of genes involved in a highly conserved gene regulatory network producing circadian rhythms (Van Gelder and Krasnow 1996, Tei et al. 1997, Bloch et al. 2001).

Both mothers and regular daughters differentially expressed genes which are known to be related to both foraging behaviour and caste differentiation in eusocial insect species, in particular with *A. mellifera*, and some ant species. Several of these conserved genes are integral to the modulation or response to JH in social insects, pointing to the centrality of JH on the phenotypic plasticity underlying social behaviour. The expression of *pban* in mothers also suggests a potential role for chemical communication between foraging mothers and offspring (Brockmann et al. 2009).

In conclusion my results suggest that behaviour in *C. calcarata* is highly sensitive to social environment, in terms of both behaviour and gene expression. Differences in cooperative behaviour are particularly influenced by mothers, suggesting that maternal manipulation of offspring development is at the core of the evolution of social behaviour offspring (Michener and Brothers 1974, Crespi and Ragsdale 2000, Kapheim et al. 2016). I also found support for the presence of genetic toolkits, with a central role for diverse conserved genes being coopted in the convergent evolution of social behaviour (Toth and Robinson 2007b, Rittschof and Robinson 2016).

Developing our experimental methods and our understanding of social behaviour in *C. calcarata* has the potential to answer many questions at the core of sociobiology. Understanding how social behaviours are not only influenced by developmental differences, but are flexible and depend on social context is critical to a fuller description of social behaviour in simple social systems.

References

Ahn, S.-J., H. Vogel, and D. G. Heckel. 2012. Comparative analysis of the UDP-glycosyltransferase multigene family in insects. Insect Biochemistry and Molecular Biology 42:133–147.

Alexander, R. D. 1974. The evolution of social behavior. Annual Review of Ecology and Systematics 5:325–383.

Ament, S. A., Y. Wang, C.-C. Chen, C. A. Blatti, F. Hong, Z. S. Liang, N. Negre, K. P. White, S. L. Rodriguez-Zas, C. A. Mizzen, S. Sinha, S. Zhong, and G. E. Robinson. 2012. The transcription factor ultraspiracle influences honey bee social behavior and behavior-related gene expression. PLoS Genetics 8.

Amsalem, E., O. Malka, C. Grozinger, and A. Hefetz. 2014. Exploring the role of juvenile hormone and vitellogenin in reproduction and social behavior in bumble bees. BMC Evolutionary Biology 14:1–13.

Arneson, L., and W. T. Wcislo. 2003. Dominant-subordinate relationships in a facultatively social, nocturnal bee, *Megalopta genalis* (Hymenoptera: Halictidae). Journal of the Kansas Entomological Society 76:183–193.

Arsenault, S. V., H. G. Brendan, and S. M. Rehan. 2018. The effect of maternal care on gene expression and DNA methylation in a subsocial bee. Nature Communications 9:1–9.

Barchuk, A. R., A. S. Cristino, R. Kucharski, L. F. Costa, Z. L. Simões, and R. Maleszka. 2007. Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera*. BMC Developmental Biology 7:70.

Begna, D., Y. Fang, M. Feng, and J. Li. 2011. Mitochondrial proteins differential expression during honeybee (*Apis mellifera* L.) queen and worker larvae caste determination. Journal of Proteome Research 10:4263–4280.

Belle, J. S. de, and M. B. Sokolowski. 1987. Heredity of rover/sitter: alternative foraging strategies of *Drosophila melanogaster* larvae. Heredity 59:73–83.

Ben-Shahar, Y. 2003. cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honey bee division of labor. Journal of Experimental Biology 206:2507–2515.

Ben-Shahar, Y., N. L. Dudek, and G. E. Robinson. 2004. Phenotypic deconstruction reveals involvement of manganese transporter *malvolio* in honey bee division of labor. Journal of Experimental Biology 207:3281.

Ben-Shahar, Y., A. Robichon, M. B. Sokolowski, and G. E. Robinson. 2002. Influence of gene action across different time scales on behavior. Science 296:741–744.

Berens, A. J., J. H. Hunt, and A. L. Toth. 2015. Comparative transcriptomics of convergent evolution: different genes but conserved pathways underlie caste phenotypes across lineages of eusocial insects. Molecular Biology and Evolution 32:690–703.

Bíliková, K., J. Hanes, E. Nordhoff, W. Saenger, J. Klaudiny, and J. Šimúth. 2002. Apisimin, a new serine–valine-rich peptide from honeybee (*Apis mellifera* L.) royal jelly: purification and molecular characterization. FEBS Letters 528:125–129.

Bloch, G., D. P. Toma, and G. E. Robinson. 2001. Behavioral rhythmicity, age, division of labor and period expression in the honey bee brain. Journal of Biological Rhythms 16:444–456.

Boomsma, J. J., and G. C. Eickwort. 1993. Colony structure, provisioning and sex allocation in the sweat bee *Halictus ligatus* (Hymenoptera: Halictidae). Biological Journal of the Linnean Society 48:355–377.

Brand, N., and M. Chapuisat. 2012. Born to be bee, fed to be worker? The caste system of a primitively eusocial insect. Frontiers in Zoology 9:1–9.

Branstetter, M. G., B. N. Danforth, J. P. Pitts, B. C. Faircloth, P. S. Ward, M. L. Buffington, M. W. Gates, R. R. Kula, and S. G. Brady. 2017. Phylogenomic insights into the evolution of stinging wasps and the origins of ants and bees. Current Biology 27:1019–1025.

Breed, M. D., and R. E. Page Jr, editors. 1989. Genetic basis for division of labor in an insect society. Pages 61–80 The Genetics of Social Evolution. Westview Press, Boulder.

Breed, M. D., J. M. Silverman, and W. J. Bell. 1978. Agonistic behavior, social interactions, and behavioral specialization in a primitively eusocial bee. Insectes Sociaux 25:351–364.

Briga, M., I. Pen, and J. Wright. 2012. Care for kin: within-group relatedness and allomaternal care are positively correlated and conserved throughout the mammalian phylogeny. Biology Letters 8:533–536.

Brockmann, A., S. P. Annangudi, T. A. Richmond, S. A. Ament, F. Xie, B. R. Southey, S. R. Rodriguez-Zas, G. E. Robinson, and J. V. Sweedler. 2009. Quantitative peptidomics reveal brain peptide signatures of behavior. Proceedings of the National Academy of Sciences 106:2383–2388.

Brooks, M. E., K. Kristensen, K. J. van Benthem, A. Magnusson, C. W. Berg, A. Nielsen, H. J. Skaug, M. Mächler, and B. M. Bolker. 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. The R Journal 9:378–400.

Brothers, D. J., and C. D. Michener. 1974. Interactions in colonies of primitively social bees: III. Ethometry of division of labor in *Lasioglossum zephyrum* (Hymenoptera: Halictidae). Journal of comparative physiology 90:129–168.

Buckle, G. R. 1982. Queen-worker behavior and nesmate [sic] interactions in young colonies of *Lasioglossum zephyrum*. Insectes Sociaux 29:125–137.

Buckle, G. R. 1985. Increased queen-like behaviour of workers in large colonies of the sweat bee *Lasioglossum zephyrum*. Animal Behaviour 33:1275–1280.

Calabi, P. 1988. Behavioral flexibility in Hymenoptera: a re-examination of the concept of caste. Pages 237–253 *in* J. C. Trager, editor. Advances in Myrmecology. E.J. Brill, New York.

Caldji, C., B. Tannenbaum, S. Sharma, D. Francis, P. M. Plotsky, and M. J. Meaney. 1998. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proceedings of the National Academy of Sciences of the United States of America 95:5335–5340.

Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, and T. L. Madden. 2009. BLAST+: architecture and applications. BMC Bioinformatics 10:421.

Cameron, S. A., and M. C. Jost. 1998. Mediators of dominance and reproductive success among queens in the cyclically polygynous Neotropical bumble bee *Bombus atratus* Franklin. Insectes Sociaux 45:135–149.

Cardinal, S., and B. N. Danforth. 2011. The antiquity and evolutionary history of social behavior in bees. PLoS ONE 6.

Chandrasekaran, S., S. A. Ament, J. A. Eddy, S. L. Rodriguez-Zas, B. R. Schatz, N. D. Price, and G. E. Robinson. 2011. Behavior-specific changes in transcriptional modules lead to distinct and predictable neurogenomic states. Proceedings of the National Academy of Sciences 108:18020–18025.

Chandrashekara, K., and R. Gadagkar. 1991. Behavioural castes, dominance and division of labour in a primitively eusocial wasp. Ethology 87:269–283.

Charnov, E. L. 1978. Evolution of eusocial behavior: offspring choice or parental parasitism? Journal of Theoretical Biology 75:451–465.

Chen, X., Y. Hu, H. Zheng, L. Cao, D. Niu, D. Yu, Y. Sun, S. Hu, and F. Hu. 2012. Transcriptome comparison between honey bee queen- and worker-destined larvae. Insect Biochemistry and Molecular Biology 42:665–673.

Choi, M.-Y., and R. K. V. Meer. 2012. Ant trail pheromone biosynthesis is triggered by a neuropeptide hormone. PLOS ONE 7:e50400.

Corona, M., R. A. Velarde, S. Remolina, and A. Moran-Lauter. 2007. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proceedings of the National Academy of Sciences 104:7128–7133.

Costa, J. T. 2006. The Other Insect Societies. Belknap Press of Harvard University Press, Cambridge, Mass.

Craig, R. 1979a. Parental Manipulation, Kin Selection, and the Evolution of Altruism. Evolution 33:319–334.

Craig, R. 1979b. Parental manipulation, kin selection, and the evolution of altruism. Evolution 33:319–334.

Creel, S., N. Creel, D. E. Wildt, and S. L. Monfort. 1992. Behavioural and endocrine mechanisms of reproductive suppression in Serengeti dwarf mongooses. Animal Behaviour 43:231–245.

Crespi, B. J., and J. E. Ragsdale. 2000. A skew model for the evolution of sociality via manipulation: why it is better to be feared than loved. Proceedings of the Royal Society of London. Series B: Biological Sciences 267:821–828.

Daugherty, T. H. F., A. L. Toth, and G. E. Robinson. 2011. Nutrition and division of labor: Effects on foraging and brain gene expression in the paper wasp *Polistes metricus*. Molecular Ecology 20:5337–5347.

Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T. R. Gingeras. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21.

Dropkin, J. A., and G. J. Gamboa. 1981. Physical comparisons of foundresses of the paper wasp, *Polistes metricus* (Hymenoptera: Vespidae). The Canadian Entomologist 113:457–461.

Duffy, J. E., J. E. Duffy, C. L. Morrison, and R. Ríos. 2000. Multiple origins of eusociality among sponge-dwelling shrimps (*Synalpheus*). Evolution 54:503–516.

Eberhard, M. J. W. 1975. The evolution of social behavior by kin selection. The Quarterly Review of Biology 50:1–33.

Eickwort, K. 1969. Separation of the castes of *Polistes exclamans* and notes on its biology (Hym.: Vespidae). Insectes Sociaux 16:67–72.

Emlen, S. T. 1984. Cooperative breeding in birds and mammals. Pages 305–39 *in* J. R. Krebs and N. B. Davies, editors. Behavioural Ecology: an Evolutionary Approach. 2nd ed. Blackwell Science, Cambridge, Mass.

Enquist, M., T. Ljungberg, and A. Zandor. 1987. Visual assessment of fighting ability in the cichlid fish *Nannacara anomala*. Animal Behaviour 35:1262–1264.

Evans, J. D., and D. E. Wheeler. 1999. Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera*. Proceedings of the National Academy of Sciences of the United States of America 96:5575–5580.

Evans, J. D., and D. E. Wheeler. 2000. Expression profiles during honeybee caste determination. Genome biology 2:research0001. 1.

Farhan, A., J. Gulati, E. Groβe-Wilde, H. Vogel, B. S. Hansson, and M. Knaden. 2013. The CCHamide 1 receptor modulates sensory perception and olfactory behavior in starved *Drosophila*. Scientific Reports 3:2765.

Feil, R., F. Hofmann, and T. Kleppisch. 2005. Function of cGMP-dependent protein kinases in the nervous system. Reviews in the Neurosciences 16:23–41.

Ferreira, P. G., S. Patalano, R. Chauhan, R. Ffrench-Constant, T. Gabaldón, R. Guigó, and S. Sumner. 2013. Transcriptome analyses of primitively eusocial wasps reveal novel insights into the evolution of sociality and the origin of alternative phenotypes. Genome Biology 14:R20.

Fewell, J. H., and R. E. Page. 2000. Colony-level selection effects on individual and colony foraging task performance in honeybees, *Apis mellifera L*. Behavioral ecology and sociobiology 48:173–181.

Fish, E. W., D. Shahrokh, R. Bagot, C. Caldji, T. Bredy, M. Szyf, and M. J. Meany. 2006. Epigenetic programming of stress responses through variations in maternal care. Annals of the New York Academy of Sciences 1036:167–180.

Fitzpatrick, M. J., Y. Ben-Shahar, H. M. Smid, L. E. M. Vet, G. E. Robinson, and M. B. Sokolowski. 2005. Candidate genes for behavioural ecology. Trends in Ecology & Evolution 20:96–104.

Fuchikawa, T., K. Beer, C. Linke-Winnebeck, and R. Ben-David. 2017. Neuronal circadian clock protein oscillations are similar in behaviourally rhythmic forager honeybees and in arrhythmic nurses. Open Biology 7.

Fujiwara, M., P. Sengupta, and S. L. McIntire. 2002. Regulation of body size and behavioral state of C-elegans by sensory perception and the EGL-4 cGMP-dependent protein kinase. Neuron 36:1091–1102.

Gadagkar, R. 1980. Dominance hierarchy and division of labour in the social wasp *Ropalida marginata (Lep.)*. Current Science:772–775.

Gadagkar, R., C. Vinutha, A. Shanubhogue, A. P. Gore, and W. D. Hamilton. 1988. Pre-imaginal biasing of caste in a primitively eusocial insect. Proceedings of the Royal Society of London. Series B. Biological Sciences 233:175–189.

George, R., and R. Stanewsky. 2021. Peripheral sensory organs contribute to temperature synchronization of the circadian clock in *Drosophila melanogaster*. Frontiers in Physiology 12:622545.

Gibbs, J., S. G. Brady, K. Kanda, and B. N. Danforth. 2012. Phylogeny of halictine bees supports a shared origin of eusociality for *Halictus* and *Lasioglossum* (Apoidea: Anthophila: Halictidae). Molecular Phylogenetics and Evolution 65:926–939.

Greenberg, L., and G. R. Buckle. 1981. Inhibition of worker mating by queens in a sweat bee, *Lasioglossum zephyrum*. Insectes Sociaux 28:347–352.

Grozinger, C. M., N. M. Sharabash, C. W. Whitfield, and G. E. Robinson. 2003. Pheromone-mediated gene expression in the honey bee brain. Proceedings of the National Academy of Sciences 100:14519–14525.

Hamilton, W. D. 1964. The genetical evolution of social behaviour. II. Journal of theoretical biology 7:17–52.

Harrison, M. C., R. L. Hammond, and E. B. Mallon. 2015. Reproductive workers show queenlike gene expression in an intermediately eusocial insect, the buff-tailed bumble bee *Bombus terrestris*. Molecular Ecology 24:3043–3063.

Hawkings, C., T. L. Calkins, P. V. Pietrantonio, and C. Tamborindeguy. 2019. Caste-based differential transcriptional expression of hexamerins in response to a juvenile hormone analog in the red imported fire ant (*Solenopsis invicta*). PLOS ONE 14:e0216800.

Hemelrijk, C. K. 2000. Towards the integration of social dominance and spatial structure. Animal Behaviour 59:1035–1048.

Hines, H. M., J. H. Hunt, T. K. O'Connor, and J. J. Gillespie. 2007. Multigene phylogeny reveals eusociality evolved twice in vespid wasps. Proceedings of the National Academy of Sciences of the United States of America 104:3295–3299.

Hofmann, F. 2005. The biology of cyclic GMP-dependent protein kinases. The Journal of biological chemistry 280:1.

Hogendoorn, K., and H. H. W. Velthuis. 1999. Task allocation and reproductive skew in social mass provisioning carpenter bees in relation to age and size. Insectes Sociaux 46:198–207.

Hölldobler, B., and E. O. Wilson. 1990. The Ants. Belknap Press of Harvard University Press, Cambridge, MA.

Huisken, J. L., W. A. Shell, H. K. Pare, and S. M. Rehan. 2021. The influence of social environment on cooperation and conflict in an incipiently social bee, *Ceratina calcarata*. Behavioral Ecology and Sociobiology 75:74.

Hunt, J. H., and C. A. Nalepa. 1994. Nourishment, evolution and insect sociality. Pages 1–19 *in* J. H. Hunt and C. A. Nalepa, editors. Nourishment and Evolution in Insect Societies. Westview Press, Boulder.

Ingram, K. K., D. M. Gordon, D. A. Friedman, M. Greene, J. Kahler, and S. Peteru. 2016. Context-dependent expression of the foraging gene in field colonies of ants: the interacting roles of age, environment and task. Proceedings of the Royal Society B: Biological Sciences 283:20160841.

Ingram, K. K., P. Oefner, and D. M. Gordon. 2005. Task-specific expression of the foraging gene in harvester ants. Molecular Ecology 14:813–818.

Jandt, J. M., E. A. Tibbetts, and A. L. Toth. 2014. *Polistes* paper wasps: a model genus for the study of social dominance hierarchies. Insectes Sociaux 61:11–27.

Jeanson, R., R. M. Clark, C. T. Holbrook, S. M. Bertram, J. H. Fewell, and P. F. Kukuk. 2008. Division of labour and socially induced changes in response thresholds in associations of solitary halictine bees. Animal Behaviour 76:593–602.

Johnson, M. D. 1988. The relationship of provision weight to adult weight and sex ratio in the solitary bee, *Ceratina calcarata*. Ecological Entomology 13:165–170.

Jones, B. M., C. J. Kingwell, W. T. Wcislo, and G. E. Robinson. 2017. Caste-biased gene expression in a facultatively eusocial bee suggests a role for genetic accommodation in the evolution of eusociality. Proceedings of the Royal Society B: Biological Sciences 284:20162228.

Jones, B. M., B. E. R. Rubin, O. Dudchenko, K. M. Kapheim, E. S. Wyman, B. G. St. Hilaire, W. Liu, L. R. Parsons, S. R. Jackson, K. Goodwin, S. M. Davidson, C. J. Kingwell, A. E. Webb, M. Fernández Otárola, M. Pham, A. D. Omer, D. Weisz, J. Schraiber, F. Villanea, W. T. Wcislo, R. J. Paxton, B. G. Hunt, E. L. Aiden, and S. D. Kocher. 2021. Convergent selection on juvenile hormone signaling is associated with the evolution of eusociality in bees. preprint, Evolutionary Biology.

Jones, B. M., W. T. Wcislo, and G. E. Robinson. 2015. Developmental transcriptome for a facultatively eusocial bee, *Megalopta genalis*. G3: Genes, Genomes, Genetics 5:2127–2135.

Kapheim, K. M. 2017. Nutritional, endocrine, and social influences on reproductive physiology at the origins of social behavior. Current Opinion in Insect Science 22:62–70.

Kapheim, K. M., S. P. Bernal, A. R. Smith, P. Nonacs, and W. T. Wcislo. 2011a. Support for maternal manipulation of developmental nutrition in a facultatively eusocial bee, *Megalopta genalis* (Halictidae). Behavioral Ecology and Sociobiology 65:1179–1190.

Kapheim, K. M., T.-Y. Chan, A. R. Smith, W. T. Wcislo, and P. Nonacs. 2016. Ontogeny of division of labor in a facultatively eusocial sweat bee *Megalopta genalis*. Insectes Sociaux 63:185–191.

Kapheim, K. M., P. Nonacs, A. R. Smith, and R. K. Wayne. 2015. Kinship, parental manipulation and evolutionary origins of eusociality. Proceedings of the Royal Society B 282.

Kapheim, K. M., A. R. Smith, K. E. Ihle, and G. V. Amdam. 2012. Physiological variation as a mechanism for developmental caste-biasing in a facultatively eusocial sweat bee. Proceedings: Biological Sciences 279:1437–1446.

Kapheim, K. M., A. R. Smith, K. E. Ihle, G. V. Amdam, P. Nonacs, and W. T. Wcislo. 2011b. Physiological variation as a mechanism for developmental caste-biasing in a facultatively eusocial sweat bee. Proceedings of the Royal Society B: Biological Sciences 279:1437–1446.

Kaun, K. R., and M. B. Sokolowski. 2008. cGMP-dependent protein kinase: linking foraging to energy homeostasis. Genome 52:1–7.

Keller, L., and H. K. Reeve. 1994. Partitioning of reproduction in animal societies. Trends in Ecology and Evolution 9:98–102.

Khamis, A. M., A. R. Hamilton, Y. A. Medvedeva, T. Alam, I. Alam, M. Essack, B. Umylny, B. R. Jankovic, N. L. Naeger, M. Suzuki, M. Harbers, G. E. Robinson, and V. B. Bajic. 2015. Insights into the transcriptional architecture of behavioral plasticity in the honey bee *Apis mellifera*. Scientific Reports 5:11136.

Kocher, S. D., and C. M. Grozinger. 2011. Cooperation, conflict, and the evolution of queen pheromones. Journal of chemical ecology 37:1263–1275.

Kocher, S. D., and R. J. Paxton. 2014. Comparative methods offer powerful insights into social evolution in bees. Apidologie 45:289–305.

Konopka, R. J., and S. Benzer. 1971. Clock mutants of *Drosophila melanogaster*. Proceedings of the National Academy of Sciences 68:2112–2116.

Kramer, J., J. Thesing, and J. Meunier. 2015. Negative association between parental care and sibling cooperation in earwigs: a new perspective on the early evolution of family life? Journal of Evolutionary Biology 28:1299–1308.

Kronauer, D. J., and R. Libbrecht. 2018. Back to the roots: the importance of using simple insect societies to understand the molecular basis of complex social life. Current Opinion in Insect Science 28:33–39.

Kukuk, P. F., and B. P. May. 1991. Colony dynamics in a primitively eusocial halictine bee *Lasioglossum (Dialictus) zephyrum* (Hymenoptera: Halictidae). Insectes Sociaux 38:171–188.

Lawson, S. P., S. L. Helmreich, and S. M. Rehan. 2017. Effects of nutritional deprivation on development and behavior in the subsocial bee *Ceratina calcarata* (Hymenoptera: Xylocopinae). The Journal of Experimental Biology 220:4456–4462.

Li, S., T. Torre-Muruzabal, K. C. Søgaard, G. R. Ren, F. Hauser, S. M. Engelsen, M. D. Pødenphanth, A. Desjardins, and C. J. P. Grimmelikhuijzen. 2013. Expression Patterns of the drosophila neuropeptide CCHamide-2 and its receptor may suggest hormonal signaling from the gut to the brain. PLOS ONE 8:e76131.

Linksvayer, T. A., and M. J. Wade. 2005. The evolutionary origin and elaboration of sociality in the aculeate Hymenoptera: maternal effects, sib-social effects, and heterochrony. The Quarterly Review of Biology 80:317–336.

Litte, M. 1979. *Mischocyttarus flavitarsis* in Arizona: social and nesting biology of a polistine wasp. Zeitschrift für Tierpsychologie 50:282–312.

Litte, M. 1981. Social biology of the polistine wasp *Mischocyttarus labiatus:* survival in a Colombian rain forest. Smithsonian Contributions to Zoology.

Loof, A. D., G. Baggerman, M. Breuer, I. Claeys, A. Cerstiaens, E. Clynen, T. Janssen, L. Schoofs, and J. V. Broeck. 2001. Gonadotropins in insects: an overview. Archives of Insect Biochemistry and Physiology 47:129–138.

Manfredini, F., C. Martinez-Ruiz, Y. Wurm, D. W. Shoemaker, and M. J. F. Brown. 2021. Social isolation and group size are associated with divergent gene expression in the brain of ant queens. Genes, Brain and Behavior:e12758.

Michener, C. D. 1974. The Social Behaviour of the Bees: a Comparative Study. Harvard University Press, Cambridge, MA.

Michener, C. D. 1990. Castes in xylocopine bees. Pages 123–126 *in* W. Engels, editor. Social insects: an Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Berlin ;

Michener, C. D., and D. J. Brothers. 1974. Were workers of eusocial Hymenoptera initially altruistic or oppressed? Proceedings of the National Academy of Sciences of the United States of America 71:671–674.

Mikát, M., C. Franchino, and S. M. Rehan. 2017. Sociodemographic variation in foraging behavior and the adaptive significance of worker production in the facultatively social small carpenter bee, *Ceratina calcarata*. Behavioral Ecology and Sociobiology 71:135.

Mikheyev, A. S., and T. A. Linksvayer. 2015. Genes associated with ant social behavior show distinct transcriptional and evolutionary patterns. eLife 4:e04775.

Morandin, C., M. M. Y. Tin, S. Abril, C. Gómez, L. Pontieri, M. Schiøtt, L. Sundström, K. Tsuji, J. S. Pedersen, H. Helanterä, and A. S. Mikheyev. 2016. Comparative transcriptomics reveals the

conserved building blocks involved in parallel evolution of diverse phenotypic traits in ants. Genome Biology 17:43.
Mulder, R. A., and N. E. Langmore. 1993. Dominant males punish helpers for temporary defection in superb fairy-wrens. Animal Behaviour 45:830–833.
Packer, L. 1986. Multiple-foundress associations in a temperate population of *Halictus ligatus* (Hymenoptera; Halictidae). Canadian Journal of Zoology 64:2325–2332.
Packer, L. 2006. Use of artificial arenas to predict the social organisation of halictine bees: data for fourteen species from Chile. Insectes Sociaux 53:307–315.
Packer, L., and G. Knerer. 1985. Social evolution and its correlates in bees of the subgenus *Evylaeus* (Hymenoptera; Halictidae). Behavioral Ecology and Sociobiology 17:143–149.
Padilla, M., E. Amsalem, N. Altman, A. Hefetz, and C. M. Grozinger. 2016. Chemical communication is not sufficient to explain reproductive inhibition in the bumblebee *Bombus impatiens*. Royal Society Open Science 3:160576.
Page, R. E., and G. E. Robinson. 1991. The genetics of division of labour in honey bee colonies. Pages 117–169 Advances in Insect Physiology. Elsevier.
Pardi, L. 1948. Dominance order in *Polistes* wasps. Physiological Zoology 21:1–13.
Pratt, A. E., D. K. McLain, and G. R. Lathrop. 2003. The assessment game in sand fiddler crab contests for breeding burrows. Animal Behaviour 65:945–955.
Pratte, M. 1989. Foundress association in the paper wasp *Polistes dominulus* Christ. (Hymen. Vesp.). Effects of dominance hierarchy on the division of labour. Behaviour:208–219.
Queller, D. C. 1996. The origin and maintenance of eusociality: the advantage of extended parental care. Page *in* S. Turillazzi and M. J. West-Eberhard, editors. Natural History and Evolution of Paper-Wasps. Oxford University Press, New York.
Ratnieks, F. L., K. R. Foster, and T. Wenseleers. 2006. Conflict resolution in insect societies. Annual Review of Entomology 51:581–608.
Reeve, H. K. 1992. Queen activation of lazy workers in colonies of the eusocial naked mole-rat. Nature 358:147–149.
Reeve, H. K., and G. J. Gamboa. 1987. Queen regulation of worker foraging in paper wasps: a social feedback control system (*Polistes fuscatus,* Hymenoptera: Vespidae). Behaviour 102:147–167.
Rehan, S. M. 2020. Small carpenter bees (*Ceratina*). Page *in* C. Stark, editor. Encyclopedia of Social Insects. Springer International Publishing.
Rehan, S. M., A. J. Berens, and A. L. Toth. 2014. At the brink of eusociality: transcriptomic correlates of worker behaviour in a small carpenter bee. BMC Evolutionary Biology 14:260.
Rehan, S. M., K. M. Glastad, S. P. Lawson, and B. G. Hunt. 2016. The genome and methylome of a subsocial small carpenter bee, *Ceratina calcarata*. Genome Biology and Evolution 8:1401–1410.
Rehan, S. M., K. M. Glastad, M. A. Steffen, C. R. Fay, B. G. Hunt, and A. L. Toth. 2018. Conserved genes underlie phenotypic plasticity in an incipiently social bee. Genome Biology and Evolution 10:2749–2758.
Rehan, S. M., R. Leys, and M. P. Schwarz. 2012. A mid-cretaceous origin of sociality in xylocopine bees with only two origins of true worker castes indicates severe barriers to eusociality. PloS one 7:e34690.
Rehan, S. M., and M. H. Richards. 2010a. Nesting biology and subsociality in *Ceratina calcarata* (Hymenoptera: Apidae). The Canadian Entomologist 142:65–74.
Rehan, S. M., and M. H. Richards. 2010b. The influence of maternal quality on brood sex allocation in the small carpenter bee, *Ceratina calcarata*. Ethology 116:876–887.
Rehan, S. M., and M. H. Richards. 2013. Reproductive aggression and nestmate recognition in a subsocial bee. Animal Behaviour 85:733–741.

Rehan, S. M., and A. L. Toth. 2015. Climbing the social ladder: the molecular evolution of sociality. Trends in Ecology & Evolution 30:426–433.

Reyer, H.-U., J. P. Dittami, and M. R. Hall. 1986. Avian helpers at the nest: are they psychologically castrated? Ethology 71:216–228.

Richards, M. H., and L. Packer. 1994. Trophic aspects of caste determination in *Halictus ligatus,* a primitively eusocial sweat bee. Behavioral Ecology and Sociobiology 34:385–391.

Rittschof, C. C., and G. E. Robinson. 2016. Behavioral genetic toolkits: toward the evolutionary origins of complex phenotypes. Current topics in developmental biology 119:157–204.

Robinson, G. E. 2002. Sociogenomics takes flight. Science 297:204–205.

Robinson, G. E., R. D. Fernald, and D. F. Clayton. 2008. Genes and Social Behavior. Science (Washington) 322:896–900.

Robinson, G. E., C. M. Grozinger, and C. W. Whitfield. 2005. Sociogenomics: social life in molecular terms. Nature Reviews Genetics 6:257–270.

Robinson, G. E., C. Strambi, A. Strambi, and Z.-Y. Huang. 1992. Reproduction in worker honey bees is associated with low juvenile hormone titers and rates of biosynthesis. General and Comparative Endocrinology 87:471–480.

Rodrigues, V., P. Y. Cheah, K. Ray, and W. Chia. 1995. *malvolio,* the *Drosophila* homologue of mouse NRAMP-1 (Bcg), is expressed in macrophages and in the nervous system and is required for normal taste behaviour. The EMBO journal 14:3007–3020.

Rutte, C., M. Taborsky, and M. W. G. Brinkhof. 2006. What sets the odds of winning and losing? Trends in Ecology & Evolution 21:16–21.

Sakagami, S. F. 1977. Some presumably presocial habits of Japanese *Ceratina* bees, with notes on various social types in Hymenoptera. Insectes Sociaux 24:319–343.

Sakagami, S. F., and Y. Maeta. 1984. Multifemale nests and rudimentary castes in the normally solitary bee *Ceratina japonica* (Hymenoptera: Xylocopinae). Journal of the Kansas Entomological Society 57:639–656.

Sakagami, S. F., and Y. Maeta. 1987. Sociality, induced and/or natural, in the basically solitary small carpenter bees *(Ceratina).* Pages 1–16 *in* Y. Ito, J. Brown, and J. Kikkawa, editors. Animal Societies: Theories and Facts. Japanese Scientific Societies Press.

Sakagami, S. F., and Y. Maeta. 1989. Compatibility and incompatibility of solitary life with eusociality in two normally solitary bees *Ceratina japonica* and *Ceratina okinawana* (Hymenoptera, Apoidea), with notes on the incipient phase of eusociality. Japanese Journal of Entomology 57:417–439.

Sakagami, S. F., Y. Maeta, K. Saito, and K. Hyodo. 1993. Diapause and non-delayed eusociality in a univoltine and basically solitary bee, *Ceratina japonica* (Hymenoptera, Anthophoridae): I. Diapause Termination by cooling and application of juvenile hormone analog. Japanese Journal of Entomology 61:443–457.

Saleh, N. W., and S. R. Ramírez. 2019. Sociality emerges from solitary behaviours and reproductive plasticity in the orchid bee *Euglossa dilemma*. Proceedings of the Royal Society B: Biological Sciences 286:20190588.

Schwarz, M. P., M. H. Richards, and B. N. Danforth. 2007. Changing paradigms in insect social evolution: insights from halictine and allodapine bees 52:127–150.

Schwarz, M. P., and R. E. Woods. 1994. Order of adult eclosion is a major determinant of reproductive dominance in the allodapine bee *Exoneura bicolor*. Animal Behaviour 47:373–378.

Shapira, M., C. K. Thompson, H. Soreq, and G. E. Robinson. 2001. Changes in neuronal acetylcholinesterase gene expression and division of labor in honey bee colonies. Journal of Molecular Neuroscience 17:1–12.

Shell, W. A., and S. M. Rehan. 2018a. The price of insurance: costs and benefits of worker production in a facultatively social bee. Behavioral Ecology 29:204–211.

Shell, W. A., and S. M. Rehan. 2018b. Behavioral and genetic mechanisms of social evolution: insights from incipiently and facultatively social bees. Apidologie 49:13–30.

Shell, W. A., and S. M. Rehan. 2019. Social modularity: conserved genes and regulatory elements underlie caste-antecedent behavioural states in an incipiently social bee. Proceedings of the Royal Society B 286:20191815.

Sherman, P. W., J. U. M. Jarvis, and R. D. Alexander, editors. 1991. The Biology of the Naked Mole-rat. Princeton University Press, Princeton NJ.

Smith, A. R., K. M. Kapheim, S. O'Donnell, and W. T. Wcislo. 2009. Social competition but not subfertility leads to a division of labour in the facultatively social sweat bee *Megalopta genalis* (Hymenoptera: Halictidae). Animal Behaviour 78:1043–1050.

Smith, A. R., K. M. Kapheim, B. Pérez-Ortega, C. S. Brent, and W. T. Wcislo. 2013. Juvenile hormone levels reflect social opportunities in the facultatively eusocial sweat bee *Megalopta genalis* (Hymenoptera: Halictidae). Hormones and Behavior 63:1–4.

Smith, A., M. Simons, V. Bazarko, and M. Seid. 2019. The influence of sociality, caste, and size on behavior in a facultatively eusocial bee. Insectes Sociaux 66:153–163.

Smith, C. R., A. L. Toth, A. V. Suarez, and G. E. Robinson. 2008. Genetic and genomic analyses of the division of labour in insect societies. Nature Reviews Genetics 9:735–748.

Smith, I. P., F. A. Huntingford, R. J. A. Atkinson, and A. C. Taylor. 1994. Strategic decisions during agonistic behaviour in the velvet swimming crab, *Necora puber* (L.). Animal Behaviour 47:885–894.

Smith, J. M., and G. A. Parker. 1976. The logic of asymmetric contests. Animal Behaviour 24:159–175.

Stacey, P. B., and W. D. Koenig. 1990. Cooperative Breeding In Birds: Long Term Studies of Ecology and Behaviour. Cambridge University Press, New York.

Stacey, P. B., and W. D. Koenig. 1992. Stacey, P. B. and Koenig, W. D. 1990. Cooperative Breeding In Birds: Long Term Studies of Ecology and Behaviour. Cambridge University Press.

Steitz, I., C. Kingwell, R. J. Paxton, and M. Ayasse. 2018. Evolution of caste-specific chemical profiles in halictid bees. Journal of Chemical Ecology 44:827–837.

Strassmann, J. E. 1981. Wasp reproduction and kin selection: reproductive competition and dominance hierarchies among *Polistes annularis* foundresses. The Florida Entomologist 64:74–88.

Sullivan, J., and J. Strassmann. 1984. Physical variability among nest foundresses in the polygynous social wasp, *Polistes annularis*. Behavioral Ecology and Sociobiology 15:249–256.

Sumner, S., E. Bell, and D. Taylor. 2018. A molecular concept of caste in insect societies. Current Opinion in Insect Science 25:42–50.

Sumner, S., J. J. M. Pereboom, and W. C. Jordan. 2006. Differential gene expression and phenotypic plasticity in behavioural castes of the primitively eusocial wasp, *Polistes canadensis*. Proceedings of the Royal Society B: Biological Sciences 273:19–26.

Sun, P., G. Li, J. Jian, L. Liu, J. Chen, S. Yu, H. Xu, C. Lei, X. Zhou, and Q. Huang. 2019. Transcriptomic and functional analyses of phenotypic plasticity in a higher termite, *Macrotermes barneyi* Light. Frontiers in Genetics 10.

Szathmáry, E., and J. M. Smith. 1995. The major evolutionary transitions. Nature 374:227–232.

Taborsky, M. 1984. Broodcare helpers in the cichlid fish *Lamprologus brichardi:* their costs and benefits. Animal Behaviour 32:1236–1252.

Tallamy, D. W., and T. K. Wood. 1986. Convergence patterns in subsocial insects. Annual Review of Entomology 31:369–390.

Tei, H., H. Okamura, Y. Shigeyoshi, C. Fukuhara, R. Ozawa, M. Hirose, and Y. Sakaki. 1997. Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. Nature 389:512–516.

Tepedino, V. J., and P. F. Torchio. 1982. Phenotypic variability in nesting success among *Osmia lignaria propinqua* females in a glasshouse environment: (Hymenoptera: Megachilidae). Ecological Entomology 7:453–462.

Tibbetts, E. A., and Z. Y. Huang. 2010a. The challenge hypothesis in an insect: juvenile hormone increases during reproductive conflict following queen loss in *Polistes* wasps. The American Naturalist 176:123–130.

Tibbetts, E. A., and Z. Y. Huang. 2010b. The challenge hypothesis in an insect: juvenile hormone increases during reproductive conflict following queen loss in *Polistes* wasps. The American Naturalist 176:123–130.

Tibbetts, E. A., and M. J. Sheehan. 2012. The effect of juvenile hormone on *Polistes* wasp fertility varies with cooperative behavior. Hormones and Behavior 61:559–564.

Toma, D. P., G. Bloch, D. Moore, and G. E. Robinson. 2000. Changes in period mRNA levels in the brain and division of labor in honey bee colonies. Proceedings of the National Academy of Sciences of the United States of America 97:6914–6919.

Toth, A. L., and S. M. Rehan. 2017. Molecular evolution of insect sociality: an eco-evo-devo perspective. Annual Review of Entomology 62:419–442.

Toth, A. L., and G. E. Robinson. 2007a. Evo-devo and the evolution of social behavior. Trends in Genetics 23:334–341.

Toth, A. L., and G. E. Robinson. 2007b. Evo-devo and the evolution of social behavior. Trends in Genetics 23:334–341.

Toth, A. L., J. F. Tooker, S. Radhakrishnan, and R. Minard. 2014. Shared genes related to aggression, rather than chemical communication, are associated with reproductive dominance in paper wasps (*Polistes metricus*). BMC Genomics 15:75.

Toth, A. L., K. Varala, M. T. Henshaw, S. L. Rodriguez-Zas, M. E. Hudson, and G. E. Robinson. 2010. Brain transcriptomic analysis in paper wasps identifies genes associated with behaviour across social insect lineages. Proceedings of the Royal Society B: Biological Sciences 277:2139–2148.

Toth, A. L., K. Varala, T. C. Newman, F. E. Miguez, S. K. Hutchison, D. A. Willoughby, J. F. Simons, M. Egholm, J. H. Hunt, and M. E. Hudson. 2007. Wasp gene expression supports an evolutionary link between maternal behavior and eusociality. Science 318:441–444.

Van Gelder, R., and M. Krasnow. 1996. A novel circadianly expressed *Drosophila melanogaster* gene dependent on the period gene for its rhythmic expression. The EMBO Journal 15:1625–1631.

Wade, M. J. 1985. Soft selection, hard selection, kin selection, and group selection. The American Naturalist 125:61–73.

Wade, M. J. 2001. Maternal effect genes and the evolution of sociality in haplo-diploid organisms. Evolution 55:453–458.

Wang, Y., S. V. Azevedo, K. Hartfelder, and G. V. Amdam. 2013. Insulin-like peptides (AmILP1 and AmILP2) differentially affect female caste development in the honey bee (*Apis mellifera* L.). Journal of Experimental Biology 216:4347–4357.

Wang, Y., S. D. Kocher, T. A. Linksvayer, C. M. Grozinger, R. E. Page Jr, and G. V. Amdam. 2012. Regulation of behaviorally associated gene networks in worker honey bee ovaries. Journal of Experimental Biology 215:124–134.

Wcislo, W. T. 1997. Social interactions and behavioral context in a largely solitary bee, *Lasioglossum (Dialictus) figueresi* (Hymenoptera, Halictidae). Insectes Sociaux 44:199–208.

West, M. J. 1967. Foundress associations in polistine wasps: dominance hierarchies and the evolution of social behavior. Science 157:1584–1585.

West-Eberhard, M. J. 1969. The Social Biology of Polistine Wasps. Museum of Zoology, University of Michigan, Michigan.

West-Eberhard, M. J. 1987. Flexible strategy and social evolution. Animal societies. Theories and facts.

West-Eberhard, M. J. 1989. Phenotypic plasticity and the origins of diversity. Annual Review of Ecology and Systematics 20:249–278.

West-Eberhard, M. J. 1996. Wasp societies as microcosms for the study of development and evolution. Page *in* M. J. West-Eberhard and S. Turillazzi, editors. Natural history and evolution of paper-wasps. Oxford University Press, New York.

Wheeler, D. A., C. P. Kyriacou, M. L. Greenacre, Q. Yu, J. E. Rutila, M. Rosbash, and J. C. Hall. 1991. Molecular transfer of a species-specific behavior from *Drosophila simulans* to *Drosophila melanogaster*. Science 251:1082–1085.

Wheeler, D. E., N. Buck, and J. D. Evans. 2006. Expression of insulin pathway genes during the period of caste determination in the honey bee, *Apis mellifera*. Insect Molecular Biology 15:597–602.

Whitfield, C. W., A.-M. Cziko, and G. E. Robinson. 2003. Gene expression profiles in the brain predict behavior in individual honey bees. Science 302:296–299.

Widowski, T. M., T. E. Ziegler, A. M. Elowson, and C. T. Snowdon. 1990. The role of males in the stimulation of reproductive function in female cotton-top tamarins, *Saguinus o. oedipus*. Animal Behaviour 40:731–741.

Wilson, E. O. 1971. The Insect Societies. Belknap Press of Harvard University Press, Cambridge, Mass.

Wilson, E. O. 1975. Sociobiology. Belknap, Cambridge, Massachusets.

Wilson, E. O., and B. Hölldobler. 2005. Eusociality: origin and consequences. Proceedings of the National Academy of Sciences 102:13367–13371.

Withee, J. R., and S. M. Rehan. 2016. Cumulative effects of body size and social experience on aggressive behaviour in a subsocial bee. Behaviour 153:1365–1385.

Withee, J. R., and S. M. Rehan. 2017. Social aggression, experience, and brain gene expression in a subsocial bee. Integrative and Comparative Biology 57:640–648.

Yanega, D. 1989. Caste determination and differential diapause within the first brood of *Halictus rubicundus* in New York (Hymenoptera: Halictidae). Behavioral Ecology and Sociobiology 24:97–107.

Zhou, X., M. R. Tarver, and M. E. Scharf. 2007. Hexamerin-based regulation of juvenile hormone-dependent gene expression underlies phenotypic plasticity in a social insect. Development 134:601–610.

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Statement of Student Contribution to Published Thesis Chapter

The undersigned graduate student Jesse Huisken and supervisor Sandra Rehan wish to acknowledge Jesse Huisken's contribution to the second chapter of his Master thesis. This chapter, "The influence of social environment on cooperation and conflict in an incipiently social bee, *Ceratina calcarata"* has been published in Behavioural Ecology and Sociobiology (75:74). This article was coauthored with Wyatt Shell and Hannah Pare, and their statements granting permission to include the article as a chapter in his thesis are also included here. Jesse conducted a review of video to produce the data set for this study, developed an ethogram and for the study organism, developed statistical methods and conducted all statistical analyses, wrote and was first author of the article.

Sincerely,

Sandra Rehan, Assistant Professor of Molecular Evolution

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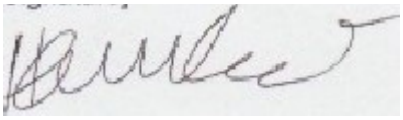

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