SOCIAL MECHANISMS REGULATING REPRODUCTIVE DIVISION OF LABOR IN BUMBLE BEES (*Bombus impatiens*)

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Abstract

Investigations into mechanisms regulating the reproductive division of labor in social insects can have wide implications regarding the evolution of social behavior. In social insects, there has been a hypothesized transition from aggressive regulation of the reproductive division of labor to pheromonal regulation of the reproductive division of labor. Bumble bees are prime candidate organisms to explore this transition because both aggression and chemical communication are used by workers and queens to regulate this competition, but the relative contributions of each mechanisms seem to vary between species. In this thesis, I investigate the physiological, behavioral and genetic mechanisms underlying the reproductive division of labor in the bumble bee species, Bombus impatiens, and compare those to the well-studied species, Bombus terrestris.
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Chapter 1.
Introduction

With their complex and dynamic social behaviors and the development of new genomic tools and resources, bumble bees are emerging as an outstanding model system to study the sociogenomic mechanisms mediating social behaviors. The genus *Bombus* contains over 250 species (Williams et al. 2008), are economically important for pollination purposes and are a principal model system for a variety of studies in social behavior and ecology (Goulson 2010). They have unique thermoregulatory adaptations (Heinrich 1975) which allow them to fly in cold weather, an adaptation that has allowed them to inhabit much of the northern hemisphere. The bumble bee colony cycle begins in the early spring when a queen (mated in the previous fall) emerges from diapause and searches for a suitable nesting site to found her colony. The queen lays her first brood of eggs, foraging and provisioning the larvae until they pupate. When the first workers emerge after a period of 16-25 days (Heinrich 2004), the queen switches to laying eggs exclusively, while the newly emerged workers take on the tasks of nursing and foraging. Colony size differs between species, but usually grows to several hundred workers. In some species, near the end of the colony cycle (late summer/fall), cooperation between the queen and the workers breaks down, and they compete over the production of males. As the colony senesces, adult males and gynes (unmated queens) emerge and leave the colony to mate, starting the cycle over again. Because of this dynamic transition from a solitary queen to a cooperative, eusocial group with a clear reproductive division of labor and finally to a period of intense competition and conflict, bumble bees serve as an excellent model system in which to study the proximate mechanisms that regulate complex social behavior, and how these systems evolved (van Honk and Hogeweg 1981, Owen and Plowright 1982, Doorn 1987, Duchateau and Velthuis 1988).

Ultimate mechanisms mediating reproductive division of labor in bumble bees.
Social behavior is widespread in Hymenoptera, which includes ants, bees and wasps, with eusociality being the most advanced grouping. The characteristics of eusociality include overlapping generations, cooperative care of young and reproductive division of labor (Michener 1969, Wilson 1971). In colonies of social insects, one or a handful of individuals (queens) dominate the reproductive output of the group composed of (mostly) sterile
workers, which forgo their own reproduction to rear the offspring of the queen. According to kin selection theory workers improve their inclusive fitness by acting altruistically to help raise their brothers and sisters even at the detriment to their personal fitness. If relatives successfully mate (especially if they are closely related) workers gain an indirect fitness benefit, as their genes will be represented in the next generation (Hamilton 1972). By gaining indirect fitness through their kin, workers are selected to act in an altruistic manner, by rearing larvae, foraging and defending (sometimes to death) the colony. However, kin selection also predicts conflict to arise in some social groups.

In Hymenoptera, sex determination is haplodiploid (Trivers and Hare 1976): males are haploid and females are diploid and females can often lay unfertilized eggs than develop into males (Bourke 1988). In colonies where queens only mate once (monandrous) workers are more related to each other ($r=0.75$) than their would-be daughters ($r=0.5$), favoring the rearing of sisters. However, workers are significantly more related to their sons ($r=0.5$) than their brothers ($r=0.25$), resulting in the preferential rearing of sons (Bourke and Ratnieks 2001). This dichotomy results in distinct colony phases, where workers act altruistically for much of the colony cycle, but will compete with the queen for male parentage (Trivers and Hare 1976). These phases are especially well characterized in the bumble bee, *Bombus terrestris*, making them an appropriate representative species to investigate colony dynamics in a monandrous social society (Payne et al. 2003). Three phases exist in the colony cycle, each of them are markedly different and characterized by diagnostic behaviors. The colony cycle begins with a **cooperative phase** where diploid female workers assist the queen in brood care and forage for resources, aggression does not occur, and workers do not lay eggs. During the second phase, known as the **switch point**, queens begin to produce haploid males (Doorn and Heringa 1986, Duchateau and Velthuis 1988). The last phase of the colony cycle is called the **competition phase** and is characterized by worker oviposition, mutual aggression between queens and workers, and reciprocal oophagy (egg eating) (Duchateau and Velthuis 1988). The onset of the competition phase is thought to be triggered by a variety of factors including the initiation of gyne production and loss of queen dominance. The competition phase arises late in colony development, so the colony will soon die off leaving only the newly mated queens to start colonies in the upcoming spring. The competition point is not sensitive to changing colony dynamics, as many factors including colony size have no effect on the initiation of
worker reproduction (Alaux et al. 2005). Although workers actively compete for male parentage, studies using microsatellite analysis have shown that even though 63.8% of workers develop their ovaries and 38.4% of them lay eggs, only 5.0% of males were produced by workers (Alaux et al. 2004b).

In colonies with queens mated by multiple males (polyandrous), workers police and destroy any eggs laid by their sisters (Ratnieks 1988, Ratnieks and Visscher 1989). This policing occurs because workers are less related to nephews ($r<0.125$) than brothers ($r=0.25$), making it more favorable for them to help rear brothers (Trivers and Hare 1976, Peters 1999, Bourke and Ratnieks 2001). This allows the queen to dominate reproduction for the entire colony cycle, and competition for reproduction is rare.

**Proximate mechanisms mediating reproductive division of labor in bumble bees.** Conflict between the queen and workers can be mediated by aggression, coercion and chemical communication (Ratnieks et al. 2006a). It has been hypothesized that ancestral groups used aggression to regulate this conflict and the evolution of pheromones (originally used as sex attractants in solitary species) would allow for larger, more harmonious social groups (Kocher and Grozinger 2011). However, the majority of social insect species examined primarily use aggression to regulate reproductive division of labor, with pheromonal regulation only playing a major role in honey bees, ants and termites (Reeve and Gamboa 1983, Premnath et al. 1996, Vargo and Hulsey 2000, Matsuura et al. 2010).

**Aggression.** Aggression is commonly observed in many species, especially those where there is little adult morphological differentiation. In primitively eusocial wasps and queenless ants, morphological caste differentiation is lacking and all individuals are totipotent (able to reproduce) (Reeve and Gamboa 1983, Premnath et al. 1996, Monnin and Peeters 1999, Gobin et al. 1999). Aggression by the dominant reproductive individual can inhibit reproductive development in the subordinate individuals, and once dominance is established, the frequency of aggressive behaviors will fall (Sledge et al. 2001). These social interactions create a dominance and reproductive hierarchy where the dominant, queen-like individual remains at the top and is the principle egg layer (Reeve and Gamboa 1983, Röseler 1991, Premnath et al. 1996, Kocher and Grozinger 2011). Subordinate females may still be able to lay eggs, though those eggs will be immediately eaten by the dominant female.
Physical contact and aggressive behavior play an important role in establishing reproductive division of labor in bumble bees. In *B. terrestris*, aggression is associated with the onset of the competition phase. During the competition phase and when queenless, workers display distinct behaviors towards other workers and the queen (Duchateau 1989, Amsalem and Hefetz 2010, 2011). These behaviors include: 1) **Attack**, where one bee physically contacts another bee an aggressive manner, this may result in “biting, pushing, dragging, struggling or attempted stinging” 2) **Humming**, characterized by short bursts of wing vibrations directed at another bee and 3) **Darting**, where one bee makes a sudden and directed movement towards another bee without making actual contact (Duchateau 1989, Amsalem and Hefetz 2010, 2011). Aggression during the competition phase causes brood production of the colony to fall greatly because workers participate in oophagy and larvaphagy (Duchateau and Velthuis 1988). When queenless, worker aggression begins a short time after group establishment and peaks on days three and four (Doorn 1989, Amsalem and Hefetz 2010), resulting in the formation of dominance hierarchies (Doorn and Heringa 1986, Doorn 1987). In these queenless groups, aggression by the most dominant worker (α-worker) can inhibit ovarian activation in subordinate workers (Bloch and Hefetz 1999a, Cnaani et al. 2007, Amsalem et al. 2013). The α-worker will also monopolize reproduction in groups smaller than five workers, and the frequency of aggressive behavior increases proportionally with group size. However, the α-worker cannot attain the same level of reproductive dominance when groups are comprised of ten workers (Amsalem and Hefetz 2011). Aggression may also be modulated by the presence of developing brood. When pairs of queenless workers were exposed to developing brood, they exhibited fewer aggressive behaviors, suggesting that developing brood may trigger rearing behaviors and therefore disrupt formation of dominance hierarchies (Sibbald and Plowright 2012, 2013).

Surprisingly, physical contact is necessary to trigger any ovary activation at all. In *B. terrestris*, isolated workers, paired workers separated by double mesh, and paired workers separated by single mesh exhibited low levels of ovarian activation and were not able to lay.
eggs, while paired workers housed together formed a dominance hierarchy, activated their ovaries and laid eggs (Amsalem and Hefetz 2010).

Chemical communication. Chemical communication is used by a variety of organisms to mediate social behavior. Pheromones are chemical signals that cause physiological or behavioral changes to the receiver of the signal, and are used for mate attraction in most insects (Karlson and Lüscher 1959). In social insects, queen pheromones regulate the physiology and behavior of many thousands of individual workers. Queen pheromones regulate colony organization, foraging, defense, brood care and reproductive dominance (Blum 1996). These complex signals likely evolved from ancestral signals that communicated reproductive state and fecundity in solitary insects and which were co-opted to signal both fecundity and reproductive dominance (Blum 1969).

The best-studied pheromone regulating reproductive division of labor is the queen pheromone of honey bees (Apis mellifera). Queen pheromone acts as both a releaser pheromone, triggering immediate changes in behavior such as short-range attraction (the 'retinue response'), mid-range attraction of swarming workers, and long-range attraction of male bees (drones). It also functions as a primer pheromone, where it inhibits worker ovary activation (Winston et al. 1989, Wossler and Crewe 1999, Keeling et al. 2003, Hoover et al. 2003, Grozinger et al. 2007) and slows down behavioral maturation (Pankiw et al. 1998a). Honey bee queens produce this complex blend in the Dufour’s, mandibular and tergite glands (Winston and Slessor 1992). Quantitative and qualitative differences in gland chemical composition are associated with differences in queen reproductive state and worker responses (Kocher et al. 2009, Peso et al. 2012, Niño et al. 2013). Queen pheromones are not limited to honey bees, as they are also present in wasps and ants (Vargo and Hulsey 2000, Sledge et al. 2001, D’Ettorre 2004, Eliyahu et al. 2011), though many of these chemical signatures are also found in egg laying workers, supporting the theory that queen pheromones were originally fertility signals and later co-opted as indicators of dominance.

Although less is known about chemical communication in bumble bees, chemical profiles of Bombus hypnorum vary with reproductive state (sterile or fertile) and caste, suggesting the presence of caste, fertility, and/or dominance pheromones. Group-specific odor bouquets
(cuticular and gland extracts as well as headspace volatiles) are found in queens, dominant workers, subordinate workers and foragers (Ayasse et al. 1995). In B. terrestris, cuticular hyrdocarbons (CHC) amounts differ significantly between nest searching queens, fertile queens and workers prior to the competition point, and CHC amounts in workers steadily increase with ovarian development after the competition phase. The increase in CHCs in workers with developed ovaries and fertile queens suggests an honest fertility signal (Sramkova et al. 2008). An honest fertility signal would mean that workers are “choosing” to forgo reproduction, rather than a signal that would physiologically prevent them from reproducing. Workers and queens may also mark their eggs during oviposition, as eggs from these two groups differ in their chemical profile, and these compounds correspond to those chemical founds in the Dufour’s gland. Eggs from different colonies also have unique profiles allowing the bees to differentiate between colonies (Ayasse et al. 1999).

*Bombus terrestris* queens are thought to inhibit worker reproduction via pheromone signaling. Queen fertility may be linked to the inhibition early in the colony cycle as workers exhibit high rates of JH biosynthesis and aggression in the presence of a virgin queen, suggesting a change in pheromone emission or behavior after the queen has mated (Bloch et al. 1996). However, in pre- and post-competition phase queens, chemical profiles are similar and the ability to inhibit JH synthesis in workers is unchanged (Bloch et al. 1996, Amsalem et al. 2009). This indicates that another factor may be contributing to the loss of dominance during the competition phase. There may be a dilution of the pheromone as the queen ages, or the queen may be unable to signal (pheromonal or otherwise) her reproductive dominance to each worker when the colony has become too large.

The queen pheromone is not volatilized, as workers separated from a queen by a double mesh screen reproduced before the competition phase began (Alaux et al. 2004a). Transferring wax and workers from queenright compartments to queenless compartments had no effect on worker aggression or egg laying (Lopez-Vaamonde and Brown 2007). Furthermore, workers that were able to contact the queen, but seek refuge in a queen-excluded compartment (QEC) still reproduced before the competition phase occurred in queenright colonies (Alaux et al. 2004a). These results suggest that the queen pheromone is a contact pheromone, which must be transmitted directly from the queen and cannot be transmitted through workers, or wax. Furthermore, the pheromone seems to serve as an
honest fertility signal, which does not directly inhibit worker reproduction, rather workers may “weigh” their options and choose to reproduce when the queen is ineffective or unable to destroy their eggs.

The glandular source of a possible queen pheromone in *Bombus terrestris* remains to be determined. Because the pheromone produced in the mandibular glands of queen honey bees serves as both a sex and social pheromone, researchers investigated whether the *B. terrestris* queen pheromone was also produced in the mandibular gland of *B. terrestris* (van Honk et al. 1980, Röseler et al. 1981). In van Honk et al. (1980) workers from colonies headed by a queen whose mandibular glands were removed tended to reproduce earlier than control queens, though they still suggest that, “the queen has other means to impose her dominance on her workers than just the pheromones from the mandibular glands. These might include pheromones from another source, or more likely, behavioral means.”

Röseler et al. (1981) investigated the effect of mandibular gland extracts and cuticular extractions on juvenile hormone (JH) synthesis in the corpora allata (reduced JH titers in the hemolymph are associated with inhibition of oogenesis in workers (Röseler 1977)). Workers exhibit high JH titers in the queenless condition and low titers in the presence of an active queen (Bloch et al. 1996). Röseler et al. (1981) found that JH synthesis in the corpora allata was inhibited by extracts from the queen mandibular gland, cuticular extracts and active queens. However, subsequent studies have refuted the theory that the mandibular gland is a source for the queen pheromone as mandibular gland extracts had no inhibitory effects on JH biosynthesis in workers (Bloch and Hefetz 1999b). The other four glands examined (hypopharyngeal, salivary, Dufour’s and tarsal) also had no effect on JH biosynthesis. Interestingly, total body extracts and cuticular washes effectively inhibited JH biosynthesis in workers when applied to virgin queens. This suggests that the pheromone may be a multi-component blend derived from multiple glands and spread over the cuticle of the queen. The chemical composition of this pheromone has not been identified, however a recent study showed that conserved, saturated hydrocarbons produced by the queen acted to inhibit reproduction or secondarily caused resorption in already activated ovaries in workers from various social Hymenoptera. Specifically, ovarian activation in *B. terrestris* workers was inhibited by the linear alkane, n-C25 (Van Oystaeyen et al. 2014). However, workers in this study were not naïve, they were introduced to the hydrocarbon in their colony of origin after the queen was removed, which may indicate that the cue was learned
rather than a true representation of the pheromone. Also, oocyte resorption (one of the parameters used as indication of reproductive inhibition in the study) is not indicative of reproductive inhibition as this phenomenon is also seen in active egg laying queens (Duchateau and Velthuis 1989). Resorption may, in fact, indicate that ovarian activation is occurring.

Interestingly, workers appear to signal their subordinate status in queenless groups. Sterile workers possess extra compounds in their Dufour’s gland (octyl esters) which are not found in queens or workers with developed ovaries (Amsalem et al. 2009). These esters signal sterility to dominant workers and queens, rendering subordinate workers safe from attacks during the competition phase. In fact, when placed in pairs, the behaviorally dominant worker’s aggression directed towards the behaviorally subordinate workers was negatively correlated with the proportion of octyl esters in the behaviorally subordinate worker (Amsalem and Hefetz 2010).

Brood pheromones may also be involved in maintaining reproductive division of labor within bumble bee colonies. In B. impatiens workers reared without brood, aggression was more likely to co-occur with oviposition, while the same relationship was not found in workers reared with brood (Sibbald and Plowright 2012). Ovarian activation in worker pairs provided with brood was lower than ovarian activation in workers where brood was absent (Sibbald and Plowright 2013). Developing brood may signal to workers that the queen is still functioning and it would be selectively advantageous for them to continue to rear sisters.

Genomics. With the development of new genomic tools and resources in bumble bees, we can now investigate the underlying genes regulating reproductive dominance, aggression and chemical communication in these species. In addition to identifying genes that are differentially expressed between reproductively dominant and subordinate individuals, we can also identify the genes that trigger the production of aggression and pheromones in signaling individuals as well as the responses of receiver individuals to these social cues. By comparing these processes within and across species, we can determine if reproductive dominance is regulated by a conserved set of genes, suggesting that core "genetic toolkits" mediate social dominance across bees, Hymenoptera or all social insects (Toth and
Furthermore, we can determine if aggression and pheromones modulate the same "neurogenomic social pathways" in receiving individuals, thus providing support for the hypothesis that pheromones evolved to co-opt existing pathways that were regulated by aggression in ancestral species (Bloch and Grozinger 2011).

The genes that regulate responses to queen pheromones and production of queen pheromones have been examined in honey bees. Exposure to queen mandibular pheromone (QMP) affects expression levels of thousands of genes in worker brains in both controlled laboratory environments as well as in the field (Grozinger et al. 2003). QMP specifically activates genes associated with nursing (brood care) and represses genes associated with foraging, which is consistent with QMP’s ability to slow down worker behavioral maturation from nursing to foraging (Pankiw et al. 1998a). Gene expression patterns within mandibular glands of virgin queens, mated queens, queenright workers, and queenless workers were also examined, which demonstrated that expression was most strongly correlated with caste and social context (queenless vs queenright), and surprisingly less correlated with reproductive status (Malka et al. 2014), suggesting that mandibular gland gene expression is associated with dominance rather than fertility (Malka et al. 2008).

The genes regulating aggressive behavior have also been identified in honey bees. There are significant and overlapping gene expression differences between European honey bees versus more aggressive Africanized honey bees, between aggressive forager bees and more docile nurse bees, and between bees exposed to alarm pheromone (which elicits defensive behavior) and unexposed bees (Alaux et al. 2009b). Interestingly, there is significant overlap between the genes found to be associated with aggression in honey bees and genes differentially expressed between reproductively dominant and subordinate Polistes metricus wasps (Toth et al. 2014). However, there was no significant overlap between the genes regulated by queen pheromone in honey bees and the genes regulated by reproductive dominance in wasps, suggesting that molecular mechanisms mediating reproductive dominance in honey bees (advanced eusocial, pheromones) and wasps (primitively eusocial, aggression) differ.

In bumble bees, these genes and molecular mechanisms regulating dominance have not been thoroughly examined. Using a candidate gene approach, it was demonstrated that
expression of Krüppel homolog 1 (Kr-h1) is downregulated in the brain of honey bee and bumble bee workers by the presence of a queen, or by the presence of a dominant worker in bumble bees (Grozinger and Robinson 2007, Shpigler et al. 2010), suggesting a conserved genetic mechanism that regulates dominance. Expression of vitellogenin, an egg yolk protein, in the fat bodies also appears to be regulated by aggression and dominance: it is upregulated in workers compared to queens, and also upregulated in aggressive and aggressed workers compared to passive workers (Amsalem et al. 2014). Thus, Kr-h1 and vg are suitable biomarkers for dominance, making them appropriate candidate genes to investigate the social cues mediating the reproductive division of labor in bumble bees.

**Aims of thesis.**

In this thesis, I investigate the mechanisms that mediate reproductive dominance in *Bombus impatiens*, the common Eastern bumble bee. There are major differences in colony development between *B. impatiens* and *B. terrestris*: *B. impatiens* colonies tend to be larger (Duchateau and Velthuis 1988, Cnaani 1992, Cnaani et al. 2002), workers are less aggressive (Cnaani et al. 2002, 2007) and the competition phase appears to be limited; only 9-11% (Cnaani et al. 2002, Jandt and Dornhaus 2011) of *B. impatiens* workers activated ovaries at the end of the colony cycle, compared to 50-64% of *B. terrestris* workers (Duchateau & Velthuis 1989; Alaux et al. 2004). This suggests that *B. impatiens* queens exhibit a greater reliance on pheromone cues to maintain the reproductive division of labor and inhibit ovarian activation, making comparisons between the two species especially intriguing. Furthermore, comparisons between these two *Bombus* species are more likely to provide insights into the mechanisms mediating the transition from aggression to pheromonal regulation of reproductive dominance than comparisons between more distantly related taxa, such as bees and wasps.

In these studies, I investigated possible role of volatile and contact pheromones in mediating reproductive division of labor in *B. impatiens*, to determine if pheromones play a larger role in this species than in *B. terrestris*. In Chapter 2, I investigated the role of queen-produced volatile and brood-produced volatile/contact cues in mediating worker reproduction in *B. impatiens*. To verify previous studies, which suggested that *B. impatiens* queens can inhibit ovarian activation in workers throughout the colony cycle, I collected workers at various points during colony development, including after gyne production was
initiated (which is when the competition phase is predicted to occur). To examine the role a volatile pheromone may play in the control of reproduction, I investigated the effect of volatiles from two different sources ("reduced" and full colonies) on callow worker groups. Finally, I developed the mesh partition experiment to observe if various stimuli (volatiles, indirect contact via antennation, and visual cues) affected reproductive capacity of workers. In Chapter 3, I examined the effect of queen behavior and contact pheromones on worker behavior (specifically aggression), physiology (ovary activation), and gene expression of Kr-h1 and vg.

**Conclusion.**

Investigations into mechanisms regulating the reproductive division of labor in social insects can have wide implications regarding the evolution of social behavior. Bumble bees are prime candidate organisms to explore this transition because both aggression and chemical communication are used by workers and queens to regulate this competition, but the relative contributions of each mechanisms seem to vary between species (i.e., *B. terrestris* appears to primarily use aggression while *B. impatiens* is hypothesized to primarily use pheromones). This thesis lays the groundwork for future genomic studies to investigate the mechanisms mediating the evolution of complex social behavior.
Chapter 2.

Investigating the role of volatile queen and brood pheromones in mediating social behavior in bumble bees (*Bombus impatiens*)

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Abstract

Reproductive division of labor is a hallmark of eusociality, but proximate mechanisms underlying it have only been identified in some species. Bumble bees are widely used models for the study of social behavior, because in the best-studied species, *Bombus terrestris*, cooperative and competitive phases during the colony cycle regulated by behavior and pheromones. When isolated from the queen, workers become reproductively active and lay haploid eggs that develop into males. We investigated if volatile compounds produced by the queen or the developing brood plays a role in mediating reproductive dominance in *Bombus impatiens*. We exposed groups of three callow (<24 hours old) workers to volatiles produced by queenright colonies (with a fully functioning queen), with and without developing brood, under three different types of experimental designs and examined the effects on worker ovary activation. In all treatments workers in direct contact with the queen did not activate their ovaries, while workers held under queenless conditions, regardless of the volatile, visual or limited contact cues they were exposed to, activated their ovaries. Furthermore, workers with direct contact with brood or volatiles from worker/brood groups also fully activated their ovaries. These data suggest that reproductive inhibition is dependent on direct queen contact in *B. impatiens*.
Introduction

In colonies of social insects, one or a handful of individuals (queens) dominate the reproductive output of the group composed of (mostly) sterile workers. Proximate mechanisms that regulate reproductive dominance include coercion, aggression and pheromones (Ratnieks, Foster, and Wenseleers 2006). It has been hypothesized that aggression was originally used to mediate this conflict in the ancestral state, and the evolution of queen pheromones or brood pheromones would allow for the development of larger, harmonious social groups (Kocher and Grozinger 2011). There is some evidence for the transition from aggression to pheromone communication (Monnin and Peeters 1999, Cuvillier-Hot et al. 2004), though it has not been extensively studied. Previous studies have suggested that *Bombus terrestris* bumble bees exhibit significantly greater aggression and competition over reproduction than *Bombus impatiens* bumble bees (Cnaani et al. 2002, 2007), suggesting that *B. impatiens* may have evolved pheromones to regulate the reproductive division of labor. This makes *B. impatiens* an especially interesting model to investigate the transition from aggression to pheromone communication. Here, we examine if volatile pheromones produced by *B. impatiens* queens or brood inhibit worker reproduction.

In *B. terrestris*, queens dominate reproduction early in the colony cycle, but a period of competition arises near the end of the colony cycle, during which workers activate their ovaries and compete with the queen over the production of males, which are haploid and develop from unfertilized eggs (Duchateau and Velthuis 1988, 1989, Doorn 1989, Bourke and Ratnieks 2001). The distinct cooperation and competition phases are supported by kin selection theory. Workers are significantly more related to their sisters than their daughters so cooperation is favored during the early colony stages (Bourke and Ratnieks 2001). Once gyne (new queens) production is imminent, workers begin to compete over male production, marking the start of the competition phase (Alaux et al. 2006). Workers favor production of sons over production of brothers or nephews during this competition phase because they are more closely related to their sons.

In *B. terrestris*, both pheromone cues and aggressive behavior mediate reproductive dominance (van Honk and Hogeweg 1981, Duchateau and Velthuis 1988, Alaux et al. 2004a, Amsalem and Hefetz 2011). Aggressive behavior erupts between and among workers and
the queen during the competition phase, and also between workers when isolated from the queen (queenless). These aggressive behaviors result in the formation of a dominance hierarchy in queenless conditions (Doorn and Heringa 1986) where one female exhibits the majority of the aggressive behaviors, has the highest ovarian development, and can inhibit reproduction in subordinate workers (Bloch and Hefetz 1999a, Cnaani et al. 2007, Amsalem and Hefetz 2011, Amsalem et al. 2013).

Queen pheromone can also inhibit worker reproduction, and the mandibular gland of the queen was initially thought to be the source of this pheromone (van Honk et al. 1980, Röseler et al. 1981). However, subsequent studies demonstrated that extracts of mandibular glands had no inhibitory effect on juvenile hormone biosynthesis (low titers of juvenile hormone in the hemolymph are associated with inhibition of oogenesis) (Röseler 1977, Bloch and Hefetz 1999b). Full body extracts and cuticular extracts did inhibit rates of juvenile hormone biosynthesis in workers, suggesting that the source of the putative pheromone is the queen, though these extracts had no effect on worker aggression (Bloch & Hefetz 1999a). A recent study showed that a saturated hydrocarbon, the linear alkane n-C25, can inhibit reproduction in B. terrestris workers (Van Oystaeyen et al. 2014). However, workers in this study were not naïve, they were introduced to the hydrocarbon in their colony of origin after the queen was removed, which may indicate that the cue was learned rather than a true representation of the pheromone. Also, oocyte resorption (one of the parameters used as indication of reproductive inhibition in the study) is not indicative of reproductive inhibition as this phenomenon is also seen in active egg laying queens (Duchateau and Velthuis 1989). It may, in fact, indicate ovarian activation. Worker pheromone emission may appease worker aggression during formation of the dominance hierarchy. In B. terrestris, subordinate workers in queenless groups produce "sterility signals" to indicate that they are out of the competition for reproduction, which reduces the amount of aggression directed at them from dominant workers (Amsalem et al. 2009, Amsalem and Hefetz 2010). Although contact pheromones seem to be important regulators of reproduction, the presence of volatile pheromones in B. terrestris has been refuted. Workers were able to reproduce prior to the competition phase when separated from the queen by a double mesh screen (Alaux et al. 2004).
The mechanisms mediating reproductive dominance in *Bombus impatiens*, the common Eastern bumble bee, have not been investigated. There are major differences in colony development between *B. impatiens* and *B. terrestris*: *B. impatiens* colonies tend to be larger (Duchateau and Velthuis 1988, Cnaani 1992, Cnaani et al. 2002), workers are less aggressive (Cnaani et al. 2002, 2007) and the competition phase appears to be limited; only 9-11% (Cnaani et al. 2002, Jandt and Dornhaus 2011) of *B. impatiens* workers activated ovaries at the end of the colony cycle, compared to 50-64% of *B. terrestris* workers (Duchateau & Velthuis 1989; Alaux et al. 2004). This suggests that *B. impatiens* queens exhibit a greater reliance on pheromone cues to maintain the reproductive division of labor and inhibit ovarian activation than in *B. terrestris*.

Here, we investigate the role of queen- and brood-produced volatile/contact cues in mediating worker reproduction in *B. impatiens*. To verify previous studies, which suggested that *B. impatiens* queens can inhibit ovarian activation in workers throughout the colony cycle, we analyzed worker ovarian activation at various points during colony development, including after gyne production is initiated (which is when the competition phase is predicted to occur). To examine the role a volatile pheromone may play in the control of reproduction, we investigated the effect of volatiles from two different sources (full colonies and reduced colonies, which had a small number of workers) on worker ovarian activation. We developed the mesh partition experiment to observe if stimuli from a reduced colony (volatiles, indirect contact via antennation, and visual cues) affected ovarian activation in workers. Finally, to determine whether a brood pheromone might be playing a role in worker reproduction we compared worker ovarian activation in groups supplemented with developing brood and those without. We hypothesize that *B. impatiens* queens produce a volatile pheromone that acts to inhibit worker reproduction.

**Materials and Methods**

**General bumble bee rearing**

For all experiments, colonies of *B. impatiens* were obtained from Koppert Biological Systems, Howell Michigan, USA (n=19). Colonies were approximately two weeks old (counted from the first worker emergence) with less than 30 workers. Colonies were maintained in nest boxes (Koppert) at a constant temperature of 30°C and 50%–60% humidity, and supplied *ad libitum* with a sugar solution and honey bee collected pollen (Koppert). Workers were
sampled from queenright colonies to determine ovarian activation at different time-points during the colony cycle. Callow workers (<24 h) of approximately the same size were collected from queenright colonies and randomly assigned to different treatment groups in the experiments (reduced colony, full colony and mesh partition) described below. All workers were immediately frozen 10 days after establishment and stored at -80°C until dissection. Eggs were only counted in queenless groups in the manipulative experiments to ensure they were worker laid.

Assessment of ovary activation and egg laying
Ovaries were dissected under a stereomicroscope in double distilled water and the terminal oocyte from the largest three ovarioles was measured with an eyepiece micrometer. Mean terminal oocyte length for each bee was used as an index of ovarian activation (Amsalem and Hefetz 2010, 2011). Eggs were counted in each queenless group immediately after workers were collected. Eggs were not counted in groups with a queen (reduced colonies) because it would be impossible to differentiate worker eggs from queen eggs; however, workers in these groups had inactivated ovaries and therefore could not have laid eggs.

Ovarian activation in queenright colonies
We sampled five colonies at the age of 3-4 weeks and sampled from the eight additional colonies either once (7.5-9 or weeks), in the case of three colonies, or twice (5 weeks and 7.5-9 weeks) in the case of five colonies. Colonies were sampled only when there would be at least 50 workers remaining after sampling, to ensure proper colony function (which is why colonies were initially sampled when they were 3-4 weeks old). Approximately thirty workers (n=24-37) were collected at each sampling point. All workers collected late in the colony cycle (7.5-9 weeks) were from colonies where gynes (newly emerged queens) had already emerged. Ovaries were ranked according to Duchateau & Velthuis (1989), with a rank of 4 (2.31-2.91mm) considered a mature ovary with ready-to-lay-eggs.

Exposure to "reduced colony" volatiles
A "reduced colony" was created by placing a mated, laying queen from a source colony into an airtight mason jar with brood (3-5 developing pupae) from her colony, along with three callow workers. Reduced colonies were then attached to airtight jars contain three additional callow workers via PVC tubing (VWR, USA). Callow workers were assigned to the
two groups randomly. Air was drawn from the callow groups at 200 μl/sec using a lab air pump (Gast Manufacturing, Benton Harbor, Michigan, USA) causing air from the reduced colony to flow into these groups (Figure 1A). We employed a double tube system to prevent a buildup of pressure in the jars. For the control trials, groups of callow workers were exposed to the volatiles from three queenless workers and developing brood from the same colony. In total we had 15 paired groups: 7 controls and 8 treatments.

**Exposure to full colony volatiles**

Twelve groups of three randomly selected callow workers were collected from queenright colonies and placed in airtight mason jars. These groups were then connected to full queenright colonies via PVC tubing (VWR, USA). Air was drawn from the worker groups by a lab air pump (Gast Manufacturing, Benton Harbor, Michigan, USA) at 200 μl/sec, causing air to flow from the full colonies into the worker groups. We employed a double tube system to prevent a buildup of pressure in the jars. Twelve control groups of three callow workers were also established, which received air from an empty nest box.

**Exposure to stimuli from “reduced colonies”**

A wooden cage (18.4cm x 19.7cm) was separated into two chambers by a mesh sheet, dividing each cage into two equal halves, which allowed indirect contact via antennation, transfer of volatiles and visual cues through the mesh but did not allow passing of either workers or queen (Figure 1B). One side of the cage housed three callow workers and the other side housed a “reduced colony” with three callow workers, a queen, and developing brood. For the controls, one chamber housed three callow workers and the other chamber housed three callow workers with developing brood from a queenright colony (both groups were queenless). In total we established 12 paired groups: six controls and six treatments.

**Selection of developing brood**

Developing brood in the pupal stage was collected from queenright colonies. Queens were provided with developing brood from their colony of origin, and because the design was paired, queenless controls were also provided brood from the corresponding colony.
Statistical analyses

Statistical analyses were done in JMP Pro 10. Ovarian activation in all cases deviated from the normal distribution (Shapiro-Wilk test p<0.05), so these data were analyzed using non-parametric tests. Ovarian activation was compared between workers receiving “full colony volatiles” and workers receiving “air from an empty nest box” using the Wilcoxon rank sum test. Ovarian activation in workers exposed to “reduced colony volatiles” and “reduced colony stimuli” and ovarian activation in their respective controls was compared using the Wilcoxon signed rank test because of the paired design. Number of eggs laid in all experiments was compared using a Student's t- test after testing for deviation from the normal distribution (Shapiro-Wilk test p>0.05).

Results

Ovarian activation in queenright colonies

Early in the colony cycle (3-4 weeks after the first worker emergence) ovarian activation in workers was low; 1.7% of workers from the sample group had mature ovaries (n=5 colonies). Midway through colony development (5 weeks) levels of ovarian activation increased; 17.2% of workers had mature ovaries (n=5 colonies). Levels of ovarian activation are highest at 7.5 weeks; 45% of workers had mature ovaries (n=3 colonies). Workers in 9 week old colonies had low levels of ovarian activation; 0.67% of workers had mature ovaries (n= 5 colonies). See Figure 2 and Supplementary Figure 1 for a graphical description. Aggressive interaction between queens and workers was not witnessed (data not shown).

Effects of exposure to volatiles from “reduced colonies”

Workers exposed to air from reduced colonies (consisting of a queen, three workers, and brood) had significantly larger oocytes than workers within the reduced colony, which had direct contact with the queen and brood (Wilcoxon signed rank test: n=8, p=0.008; Figure 3A). Workers exposed to air from reduced colonies had fully activated ovaries (2.31+/−0.215mm) while workers in the reduced colonies were completely reproductively inhibited (0.557+/-0.134mm). In the control groups, workers with direct contact with brood and workers exposed to the volatiles from these worker/brood groups did not have significant differences in oocyte size and both had fully activated ovaries (2.41+/−0.293mm and 2.135+/−0.358mm, Wilcoxon signed rank test: n=7 p=0.203, Figure 3B). Worker groups exposed to air from reduced colonies had no significant differences in total eggs laid.
compared to worker groups exposed to air from workers and brood (Student’s t-test: n=7,8 t=2.16, p=0.186; Figure 4).

**Effects of exposure to volatiles from full colonies**

Ovarian activation in workers exposed to volatiles from full colonies did not differ significantly from that of the control group, which consisted of workers exposed to volatiles from an empty cage (Wilcoxon rank sum: z=-1.50, p=0.126; Figure 5). Differences in total eggs laid from these two groups were not statistically significant (Student's t-test: n=12, t=2.07, p=0.294; Figure 6).

**Effects of exposure to stimuli (volatiles, visual cues, and limited physical contact) from “reduced colonies”**

By separating workers and reduced colonies via a mesh, workers were exposed to the volatiles of reduced colonies, as well as allowed visual and potentially partial physical contact with workers and queens from the reduced colonies. Workers exposed to various stimuli (volatiles, antennation and visual cues) from reduced colonies had significantly larger terminal oocytes than workers in the reduced colonies (Wilcoxon signed rank test: n=6, p=0.031; Figure 7A). Workers exposed to stimuli from reduced colonies had fully activated ovaries (2.72+/−0.109mm) while workers in the reduced colonies were completely reproductively inhibited (0.560+/−0.109mm). For the control treatments, workers housed alone and workers housed with brood both fully activated their ovaries (2.44+/−0.314mm, 1.98+/−0.374mm) and there was no significant difference in the average terminal oocyte size (Wilcoxon signed rank test: n=6, p=0.219; Figure 7B). Worker egg laying was not significantly different in workers exposed to stimuli from workers and developing brood and workers exposed to stimuli from a reduced colony (Student's t-test: n=6, t=2.23, p=0.086; Figure 8).

**Discussion**

Our results demonstrate that levels of worker ovary activation fluctuate in *B. impatiens* colonies, with low levels of worker ovary activation early in the colony cycle, increasing midway through the colony cycle and then decreasing at the end of the colony cycle. Thus, the presence of an active queen and brood midway through colony development is not sufficient to completely inhibit worker ovary activation. Using three different experimental
designs, we also demonstrate that *B. impatiens* worker ovary activation is not inhibited by volatiles produced by the queen or brood; rather, direct contact with the queens is required to fully inhibit ovary activation.

In both *B. impatiens* and *B. terrestris*, levels of worker ovary activation fluctuate during the colony cycle. In *B. impatiens*, we found that levels are highest approximately 7.5 weeks from the first worker emergence, with an average of 45% of workers displaying mature ovaries (Figure 1). In *B. terrestris*, Duchateau and Velthius (1989) found that >50% of the workers had mature ovaries after the competition phase, which they found to be \(~4\) weeks (30.4+/−4.6 days) after the eusocial phase began (when the first brood of workers emerges). Alaux et al. (2004) found 63.8% of *B. terrestris* workers had activated ovaries (stages 3-4) at the end of the colony cycle (when the last adult bee had emerged). Interestingly, in our study, only 0.67% workers in old *B. impatiens* colonies (approximately 9 weeks) had activated ovaries. Previous studies also found that only 9%- 11% of *B. impatiens* workers from ~8-9 week old (56-64 day) colonies had activated ovaries (Cnaani et al. 2002, Jandt and Dornhaus 2011). This reduction in the later stages of the colony cycle may be caused by dominant workers inhibiting ovary activation in subordinate workers, as has been observed in *B. terrestris* (van Honk and Röseler 1981, Bloch and Hefetz 1999a, Amsalem and Hefetz 2011).

However, despite worker ovary activation levels rising mid-way through the colony cycle, it is debatable if this corresponds to a competition phase. The competition point is defined as the first time workers oviposit or display aggressive behavior (Duchateau and Velthuis 1988). In *B. terrestris*, Alaux et al. (2004) found that 38.4% of *B. terrestris* workers laid eggs by the end of the colony cycle. In *B. impatiens*, worker oviposition in a queenright colony has not been observed and worker aggression was only seen in one instance (Cnaani et al. 2002). This study and others have shown that *B. impatiens* workers do activate their ovaries, though this activation does not result in overt aggression or active egg-laying behavior from workers. Thus, rising worker ovary activation levels may be intrinsic to the colony cycle; as the colony get larger, the queen may not be able to signal reproductive dominance to all workers, causing workers to activate their ovaries. In fact, at the end of the colony cycle, workers that resided further from the queen developed larger oocytes than workers who
remained closer to the queen (Jandt and Dornhaus 2011). However, this still does not explain why workers refrain from laying eggs, even with activated ovaries.

While queens clearly inhibit worker ovary activation early in the colony cycle and in reduced colony groups, there is no indication that a volatile queen pheromone or a brood pheromone causes this inhibition. In our manipulative experiments (full colony volatiles, reduced colony volatiles, mesh partition) workers in direct contact with the queen were always reproductively inhibited. Volatile cues from the full colonies, reduced colonies or control groups (provisioned with developing brood) did not cause inhibition of ovary activation. Furthermore, additional stimuli (visual, limited contact with workers) from reduced colonies did not inhibit ovary activation. Previous studies in *B. impatiens* found that aggression and oviposition were less likely to co-occur in workers housed with brood and workers housed without brood tended to be more aggressive and have higher ovarian development, suggesting the presence of a brood pheromone (Sibbald and Plowright 2012, 2013). Though, co-occurrence of aggression and oviposition may not indicate presence of a brood pheromone, as all groups with brood in Sibbald & Plowright (2012) laid eggs (one group without brood did not lay eggs) and actually displayed more aggression than groups without brood. In Sibbald & Plowright (2013) workers were isolated for a period of 12 days before introduction to other workers or brood, which has been found to delay egg laying drastically and increase chemical secretions in *B. terrestris* (Amsalem et al. 2009). In our study, workers who were housed with developing brood displayed no difference in ovarian activation compared to queenless workers. All workers were introduced to brood at <24hr of age and were collected after 10 days. This suggests that a volatile or contact brood pheromone does not exist, in contrast to honey bees where a brood pheromone inhibits worker ovarian activation (Jay 1972, Le Conte et al. 1990, Pankiw et al. 1998b).

Given that direct contact with the queen is necessary to inhibit ovary activation, a contact pheromone or behavioral interactions are likely to mediate queen reproductive dominance in *B. impatiens*. This hypothesis is somewhat supported by the increased levels of ovary activation observed in large colonies (5-7.5 weeks old), where the queen no longer can directly interact with each worker, though we cannot ignore the drop in ovary activation at 9 weeks. Thus reproductive division of labor in both *B. impatiens* and *B. terrestris* are heavily dependent on direct contact with the queen (Alaux et al. 2004).
observed in earlier studies, aggression between queens and workers was not witnessed (data not shown). Lack of aggression still stands as a contrasting feature of the natural history between these two species. Aggression in B. impatiens may have been replaced with chemical signaling of fertility or sterility to maintain the dominance hierarchy without costly aggression, similar to signals found in in ponerine ants and B. terrestris bumble bees (Cuvillier-Hot et al. 2004, Amsalem and Hefetz 2010)

These studies suggest that there are key differences and similarities in social organization and the mechanisms that regulate reproductive dominance in B. impatiens and B. terrestris. B. impatiens workers activate their ovaries in the presence of the queen, but do not lay eggs. Like B. terrestris, inhibition of ovarian activation in B. impatiens requires direct contact with the queen, though the lack of aggression displayed by the queen suggests the existence of a contact pheromone that mediates her reproductive dominance. Additional studies are needed to test the presence of a contact pheromone. Reproductive division of labor is one of the core tenets of eusocial behavior; investigations into the mechanisms that regulate this process can provide insights into the genes, neurophysiological, and communication mechanisms that underpin the switch from solitary to social behavior in insects.
Figure 1: Experimental systems to test effect of queen- and brood-produced volatiles.  
A. Reduced colony volatiles. Three workers were exposed to volatiles from a reduced colony system consisting of an active queen, three workers and developing brood from the same colony as the queen. B. Mesh partition. An active queen, three workers and developing brood from the same colony as the queen were placed in the front compartment and three callow workers were placed in the rear compartment. Compartments were separated by mesh, allowing worker-worker contact. Trials for both experimental systems lasted 10 days.
Figure 2: Worker ovarian activation in queenright colonies. Approximately 30 workers were collected from 13 queenright colonies at different time points during colony development. In five of the colonies, workers were collected at two different time points. Worker ovarian activation was assessed using methods described above. The number of replicates (colonies) is presented at the bottom of the bar.
Figure 3: Ovarian activation in the reduced colony volatile experiment. A. Worker oocytes in reduced colonies (workers+queen+brood) are significantly smaller than worker oocytes that are exposed to volatiles from reduced colonies (Wilcoxon signed rank test, n=8, p=0.008). B. Oocyte size is not significantly different between workers reared with developing brood and workers exposed to volatiles from workers with developing brood (Wilcoxon signed rank test, n=7, p=0.203). The number of replicates (3 workers per group) is presented at the bottom of the bar.
Figure 4. Total eggs laid in the reduced colony volatile experiment. Worker oviposition was not significantly different in workers exposed to volatiles from workers and developing brood and workers exposed to volatiles from a reduced colony (workers+queen+brood) (Student's t-test: t=2.16, p=0.186). The number of replicates (3 workers per group) is presented at the bottom of the bar.
Figure 5. Ovarian activation in the full colony volatile experiment. Workers exposed to volatiles from full colonies had no significant difference in ovary size when compared to workers exposed to volatiles from an empty cage (Wilcoxon rank sum: z=-1.50, p=0.126). The number of replicates (3 workers per group) is presented at the bottom of the bar.
Figure 6: Total eggs laid in the full colony volatile experiment. Workers exposed to volatiles from full colonies had no significant difference in total eggs laid when compared to workers exposed to volatiles from an empty cage (Student’s t-test: t=2.07, p=0.294). The number of replicates (3 workers per group) is presented at the bottom of the bar.
Figure 7: Ovarian activation in the mesh partition experiment. 

A. Ovarian activation in workers in reduced colonies (which had direct contact with the queen and brood) was inhibited, while workers that were exposed to various stimuli from the reduced colony through the mesh (volatiles, antennation and visual cues) had fully developed ovaries (Wilcoxon signed rank test, n=6, p=0.031).

B. Both workers housed with brood and workers that were exposed to various stimuli from the worker/brood groups through the mesh (volatiles, antennation and visual cues) were able to fully activate their ovaries with no statistically significant difference in the average terminal oocyte size (Wilcoxon signed rank test, n=6, p=0.219). The number of replicates (3 workers per group) is presented at the bottom of the bar.
Figure 8: Total eggs laid in the mesh partition experiment. Worker oviposition was not significantly different in workers exposed to stimuli from workers and developing brood and workers exposed to stimuli from a reduced colony (workers+queen+brood) (Student's t-test: t=2.23, p=0.086). The number of replicates (3 workers per group) is presented at the bottom of the bar.
Supplementary figures

Supplementary Figure 1: Worker ovarian activation in queenright colonies. Approximately 30 workers were collected from 13 queenright colonies at different time points during colony development. Letters indicate colony of origin. Colonies I, J, F, G and H were sampled twice as indicated above.
Chapter 3.
Evaluating the role of social cues in mediating queen-worker conflict in bumble bees

(_Bombus impatiens_)

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Abstract
Bumble bees are widely used models for the study of social behavior, and in the best-studied species, _Bombus terrestris_, there are distinct cooperative and competitive phases regulated by both pheromones and behavior. The common Eastern bumble bee, _Bombus impatiens_, tends to be less aggressive, suggesting a higher reliance on pheromone communication. Here, we investigated whether visual cues, contact cues or volatile cues produced by the queen impacted worker behavior, physiology and gene expression patterns associated with reproductive dominance. We evaluated workers that were reared in chambers with an uncaged queen, caged queen, or no queen. Workers were found to have an innate attraction to queens, making contact with uncaged queens and caged queens significantly more often than empty cages. Workers housed with caged queen exhibited slightly less aggression than workers housed in queenless conditions, but levels of ovarian activation were the same in both groups and significantly higher than workers housed with a free queen. There were also significant effects of treatment on gene expression. Expression of vitellogenin in α-worker heads was highest in queenless treatments, intermediate in caged treatments and lowest in uncaged queen treatments. Expression of _Kr-h1_ in α-worker heads was significantly higher in highest in queenless and caged treatments compared to uncaged queen treatments.
Introduction

Proximate mechanisms that regulate the conflict for reproduction in eusocial insects include coercion, aggression and pheromones (Ratnieks et al. 2006b). It has been hypothesized that aggression was originally used to mediate this conflict in the ancestral state, and the subsequent evolution of queen pheromones allowed for the development of larger, more harmonious social groups (Kocher and Grozinger 2011). However, the mechanisms that underlie this transition from aggressive behavior to chemical communication have not been extensively studied (but see (Toth et al. 2014). Bombus terrestris bumble bees exhibit significantly greater aggression and competition over reproduction than Bombus impatiens bumble bees (Cnaani et al. 2002, 2007), suggesting that B. impatiens may be more dependent on pheromones to regulate the reproductive division of labor. Our previous studies demonstrated that volatile cues from B. impatiens colonies could not inhibit worker reproduction and direct contact with the queen was necessary to inhibit worker reproduction (Chapter 2). Here we further investigate the cues produced by the queen, which act to mediate worker reproduction, aggression and gene expression patterns associated with reproductive dominance.

In B. terrestris colonies, aggression is associated with the onset of the competition phase, a point in colony development where workers compete with the queen to produce male offspring. The competition phase occurs near the end of the colony cycle and is characterized by worker oviposition, mutual aggression between queens and workers along with reciprocal oophagy (egg eating) (Duchateau and Velthuis 1988, Duchateau 1989, Amsalem and Hefetz 2010, 2011). Workers also exhibit aggressive behaviors when placed in queenless conditions, resulting in the formation of dominance hierarchies (Doorn and Heringa 1986, Doorn 1987). Aggression begins a short time after isolation from the queen and peaks after three or four days (Doorn 1989, Amsalem and Hefetz 2010). These aggressive behaviors inhibit ovarian activation in subordinate workers (Bloch and Hefetz 1999a, Cnaani et al. 2007, Amsalem et al. 2013).

Pheromone signaling also appears to play a role in mediating queen reproductive dominance in B. terrestris (van Honk and Hogeweg 1981, Duchateau and Velthuis 1988, Alaux et al. 2004a). The queen pheromone is not volatilized; rather it appears that workers must have direct contact with the queen to be inhibited (Alaux et al. 2004a). Though it is not
known where the pheromone is produced, total body extracts and cuticular washes effectively inhibited juvenile hormone biosynthesis (low JH titers in the hemolymph are associated with inhibition of oogenesis in workers) in workers when applied to virgin queens (Röseler 1977, Bloch and Hefetz 1999b). This suggests that the pheromone may be derived from multiple glands and spread over the cuticle of the queen. The chemical composition of this pheromone has not been identified, however a recent study showed that ovarian activation in *B. terrestris* workers was inhibited by the linear alkane, n-C$_{25}$ (Van Oystaeyen et al. 2014).

The mechanisms that regulate the reproductive division of labor in *B. impatiens* are less well known. During the competition phase only 9-11% (Cnaani et al. 2002, Jandt and Dornhaus 2011) of workers activate their ovaries at the end of the colony cycle, compared to 50-64% of *B. terrestris* workers (Duchateau & Velthuis 1989; Alaux et al. 2004). *B. impatiens* workers are also less aggressive in the queenless condition and rarely ever exhibit aggression in the presence of an active, laying queen (Cnaani et al. 2002; Cnaani et al. 2007; personal observations). In one study, aggression levels were linked to the presence of developing brood. When pairs of queenless workers were exposed to developing brood, they exhibited fewer aggressive behaviors and lower ovarian activation (Sibbald and Plowright 2013). However, workers were isolated for a period of 12 days before introduction to other workers or brood, which has been found to delay egg laying drastically and increase chemical secretions in *B. terrestris* (Amsalem et al. 2009). Our previous studies have also shown that queenless workers exposed to developing brood display no differences in ovarian activation compared to queenless workers without brood (Padilla et al. in preparation). Reduced aggression and ovary activation during the colony cycle suggests that *B. impatiens* queens exhibit a greater reliance on pheromone cues to maintain the reproductive division of labor and inhibit ovarian activation than in *B. terrestris*.

Gene expression analysis can be used to study the impact of social cues, and can be a more sensitive measure than behavioral assays, which are difficult to study in a controlled setting. Pheromones and aggression both regulate gene expression in social insects. In honey bees, queen mandibular pheromone (QMP), brood pheromone and alarm pheromone modulate gene expression patterns associated with task specialization, reproduction and aggression.
in worker bee brains (Grozinger et al. 2003, Alaux et al. 2009a, 2009b). In *B. terrestris*, expression levels of *Krüppel homolog 1* and *vitellogenin* are linked to aggression and dominance. Expression of *Kr-h1* is downregulated in workers in the presence of a queen or a dominant worker (Shpigler et al. 2010), and expression of *vitellogenin* is upregulated in aggressive and aggressed workers compared to passive workers (Amsalem et al. 2014). Thus, *Kr-h1* and *vg* are both sensitive to social context, making them appropriate candidate genes to investigate the social cues mediating the reproductive division of labor in bumble bees.

In this study, we investigated the role of social cues and social interactions in mediating queen reproductive dominance in *Bombus impatiens*. Our previous studies (Chapter 2) demonstrated that direct contact with the queen is necessary to inhibit worker ovary activation. Here, we examined what aspect of "direct contact" was most relevant: the ability of the workers to visually inspect the queen, the ability of the workers to contact the queen with their antennae (and therefore be exposed to contact pheromones), or the ability of the queen to freely behaviorally interact with the workers. Thus, we reared groups of *B. impatiens* workers with (1) an uncaged queen (2) a caged queen and (3) no queen. In the caged queen groups we were able to investigate the effects of visual and chemical cues produced by the queen, as well as worker-initiated contact with the queen. We examined the effects of these treatments on worker attraction to the queen, aggression, ovary activation, and gene expression patterns associated with reproductive dominance. We hypothesize if *B. impatiens* queen dominance is mediated by factors other than queen-initiated behavioral interactions, workers in the presence of a caged queen (relative to queenless workers) will exhibit reduced or delayed worker-worker aggression and ovary activation, and gene expression patterns consistent with the subordinate state.

**Materials and methods**

**General bumble bee rearing**

Colonies of *B. impatiens* were obtained from Koppert Biological Systems (Howell, Michigan, USA) at two weeks old with less than 30 workers. Colonies were maintained in nest boxes provided by Koppert at a constant temperature of 30°C and 50%–60% humidity, and supplied *ad libitum* with a sugar solution and fresh pollen. Callow workers (<24 h) of approximately the same size were collected from queenright colonies and randomly
assigned to treatments described below. All workers were paint marked to indicate colony of origin, and to track individuals during observations. All workers were collected on dry ice and stored at -80°C until dissection. Developing brood in the pupal stage was collected from the queenright colonies and placed with the queen inside the cage as well as outside the cage with the workers.

Treatments
Groups of 3 callow workers were placed in a wooden rearing chamber (size) with developing brood (approximately 3-4 pupal stage workers). In treatment 1, workers were left queenless. In treatment 2, a laying queen was placed in the group (uncaged queen). In treatment 3, a laying queen was placed in a cage with brood and placed in the group (caged queen) (Figure 1). All workers were collected 10 days post establishment.

Queen-worker interactions.
Worker-initiated contact events with the queen were recorded in 5 minute intervals for 45 minutes at 12:00pm on days 2, 3 and 4 post group establishment. We avoided recording incidental contact, and only recorded worker antennation and attempted antennation with the caged queen and the uncaged queen. For workers housed with an empty cage, contact with the empty cage was recorded. Queen-worker interactions were measured in 39 groups (queenless workers: n=14, caged queen: n=16, uncaged queen: n=14).

Worker-worker aggression
Discrete aggressive behaviors were recorded in ten-minute intervals at three fixed time points (7:00am, 11:00am and 3:00pm) on days 3 and 4 post group establishment; behaviors recorded as in Amsalem & Hefetz (2011), Amsalem & Hefetz (2010) and Duchateau (1989). The behaviors were 1) Attacking, where one bee physically contacts another bee in an aggressive manner, this may result in "biting, pushing, dragging, struggling or attempted stinging" 2) Humming, characterized by short bursts of wing vibrations directed at another bee and 3) Darting, where one bee makes a sudden and directed movement towards another bee without making actual contact. Observations were performed on days 3 and 4 because aggression in B. terrestris workers was found to be highest on these days in Amsalem & Hefetz (2011). The most aggressive bee was selected by determining the bee with the highest number of aggressive behaviors over the 60 min of observation time.
Worker-worker aggression was measured in 39 groups (queenless workers: n=13, caged queen: n=14, uncaged queen: n=12). Aggression between queens and workers was never observed.

**Ovary activation**

Ovaries were dissected under a stereomicroscope in double distilled water and the terminal oocyte from the largest three ovarioles was measured with an eyepiece micrometer. Mean terminal oocyte length for each bee from each treatment group was used as an index of ovarian activation (Duchateau and Velthuis 1989, Amsalem and Hefetz 2010, 2011). Ovarian activation was measured in 18 groups (queenless workers: n=7, caged queen: n=6, uncaged queen: n=5).

**Expression levels of vitellogenin and Krüppel homolog 1**

To quantify expression of Kr-h1 and vg in worker heads we first designed primers using known gene sequences. These genes (Table 1) were identified using the NCBI/blast home page ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)). Primer design for vg (Fwd: 5’ CAGCCGCAATATGATACCT 3’; Rev: 5’ CCCTCCGTTCGAAGTGATAA 3’) and Kr-h1 (Fwd: 5’ GAATTGCCAAATCGAGAGGA 3’; Rev: 5’ GAGGGGTATGCTGATCAGGA 3’) was performed using the Primer3 v 0.4.0 ([http://frodo.wi.mit.edu/](http://frodo.wi.mit.edu/)). To control for PCR efficiency and individual differences across samples, two housekeeping genes were used: Arginine kinase (AK) (Fwd: 5’ TGTCGGATATCTACCGCCTG 3’; rev: 5’ TTGGTGGATGCTTGTACGTC 3’) and Phospholipase A2 (PLA2) (Fwd: 5’ GGTCACACCGAACCCATT 3’; rev: 5’ TCGCAACACTTCGTCATTCC 3’) (Hornáková et al. 2010). RNA was extracted from the heads of individual workers using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA quantity and quality were assayed with a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington DE). cDNA was synthesized according to the manufacturer’s instructions using 200 ng of RNA with Reverse Transcriptase (Invitrogen, Carisbad, CA, USA). Two microliters of diluted cDNA were used together with 5 µl SYBR-Green (Bioline, Luckenwalde, Germany), 0.2 mM of each gene specific primer and 2.6 µl DEPC-water for the gene expression assay. Expression levels of vg and Kr-h1 were determined using quantitative real-time PCR on an ABI Prism®7900 sequence detector with SYBR Green detection method (Applied Biosystems, Foster City, CA, USA). Triplicate reactions were performed for each of the
samples and averaged for use in statistical analysis. Quantification was based on the number of PCR cycles required to cross a threshold of fluorescence intensity (Ct), using the 2-ΔCt technique. The geometric mean of the two reference genes was used as a control. Negative control samples (cDNA reaction without RT enzyme) and a water control were also present on each plate. PCR product quality and specificity was verified using melt curve analysis.

**Statistical analyses**

Statistical analyses were done in JMP Pro 10. Aggression, contact, gene expression and ovarian activation species comparison data all deviated from the normal distribution (Shapiro-Wilk test p<0.05), so these data were analyzed using non-parametric tests. Ovarian activation did not deviate from the normal distribution (Shapiro-Wilk test p>0.05) so an ANOVA was performed followed by a Students t-test.

**Results**

**Queen-worker contact**

The number of times workers initiated contact (antennated) uncaged queens was significantly higher than the number of times workers initiated contact with caged queens (Wilcoxon multiple comparisons; z=3.37, p=0.0007) and empty cages (Wilcoxon multiple comparisons; z=4.671, p<0.0001) (Figure 2). Workers contacted caged queens significantly more than empty cages (Wilcoxon multiple comparisons, z=-3.895, p<0.0001)(Figure 10).

**Worker-worker aggression**

Each group of workers was observed for a total of 60 minutes over a two-day period. The amount of aggressive behavior was significantly higher in queenless groups than groups with uncaged queens (Wilcoxon multiple comparisons; z=1.70, p=0.013). There were no significant differences between queenless groups and groups with caged queens (Wilcoxon multiple comparisons; z=-0.955, p=0.089) or between groups with caged queens and uncaged queens (Wilcoxon multiple comparisons; z=-2.48, p=0.339) though there is a trend of reduced aggression in groups with caged queens compared to queenless groups (Figure 11).
When the behavior of the most aggressive female alone was considered, these females displayed significantly more aggressive behavior in queenless groups compared to groups with uncaged queens (Wilcoxon multiple comparisons; z=-2.433, p=0.015). There were no significant differences between the behaviors of the most aggressive females from queenless and caged queen groups (Wilcoxon multiple comparisons; z=1.56, p=0.1179) or caged queen groups compared to the uncaged queen groups (Wilcoxon multiple comparisons; z=-1.165, p=0.244) though there is a trend of reduced aggression in caged queen groups compared to the queenless groups. Aggression performed by the most aggressive females strongly tracked whole group aggression: these workers performed 76.4 +/- 4.8% of the total aggression in the caged queen groups, 73.0 +/−5.6% of the total aggression in the queenless groups and 70.7 +/−8.9% in the uncaged queen groups (Figure 12).

Comparison of Bombus impatiens and Bombus terrestris aggression

Worker aggression data for B. terrestris was obtained from Amsalem & Hefetz (2011) and was compared to worker aggression data from this study. Both studies grouped three callow workers together and observed the same aggressive behaviors for a total of 60 minutes on days 3 and 4 post establishment. Aggressive behaviors of B. terrestris groups were significantly higher than B. impatiens groups (Wilcoxon rank sums; z=2.69, p=0.007)(Figure 13). Data sets were collected at a different time by different researchers.

Worker ovarian activation

There was a significant effect of treatment on worker ovary activation (one-way ANOVA; f2,15=4.468, p=0.030 ). Worker ovary activation was higher in the queenless and caged queen groups relative to the uncaged queen groups (p<0.025) and there was no significant different between queenless workers and workers housed with the caged queen (p=0.769)(Figure 14).

Relative RNA expression levels of vg and Kr-h1

Relative RNA expression of vg and Kr-h1 was analyzed for the α-worker in each treatment group. α–workers were the individuals with a combination of the highest ovarian activation and total aggressive behaviors on days 3 and 4. For both genes there was a significant effect of treatment. For vg, expression was significantly higher in α-worker heads from the
queenless treatments compared to the caged and uncaged treatments (Wilcoxon multiple comparisons: \( z=2.482, p=0.013; \ z=-2.082, \ p=0.005 \)). Expression was also significantly higher in \( \alpha \)-workers from the caged treatment compared to the uncaged treatment (Wilcoxon multiple comparisons: \( z=-2.162, p=0.031 \)) (Figure 15). For \( Kr-h1 \), expression was significantly higher in \( \alpha \)-worker heads from the queenless and caged treatments compared to the uncaged treatments (Wilcoxon multiple comparisons: \( z=2.482, p=0.013; \ z=-2.162, \ p=0.031 \). No significant differences were found between \( \alpha \)-workers from the queenless and caged treatments. (Wilcoxon multiple comparisons \( z=1.201, p=0.229 \))(Figure 16).

**Discussion**

Our results demonstrate that workers are attracted to caged queens, and the presence of a caged queens results in worker-worker aggression levels that are intermediate between queenless workers and workers housed with an uncaged queen. However, worker ovarian activation is only inhibited in the presence of an uncaged queen. Gene expression of \( vg \) was found to track aggression closely; it was elevated in queenless workers, intermediate in the presence of a caged queen and low in the presence of an uncaged queen. Expression of \( Kr-h1 \) was found to track reproductive dominance; it was high in the presence of a caged queen and in queenless worker groups, and low in the presence of a caged queen. These data suggest that exposure to queen volatile cues, queen visual cues and worker-initiated queen contact (caged queens) reduces aggression and modulates expression of \( vg \) in workers; though ovarian activation and expression of \( Kr-h1 \) were unaffected by these variables.

The intermediate levels of aggression observed in the caged queen groups may be due to reduced or delayed aggression. Aggression is dynamic in queenless groups, peaking during dominance establishment and then reducing after. In *B. terrestris*, the amount of aggressive behaviors peak on days 3 and 4 post isolation from the queen, and reduce thereafter (Doorn 1989, Amsalem and Hefetz 2010). This dynamic is also seen in other social species, including *Polistes* wasps and permanently queenless ants (Monnin and Peeters 1999, Sledge et al. 2001). In the caged queen treatments aggression was intermediate, indicating that these queens may have delayed or reduced the formation of the dominance hierarchy. Though it seems as if the hierarchy was in fact delayed, considering that there was no difference in ovarian activation compared to workers from the queenless treatments. If the
formation of a dominance hierarchy was delayed, we would not be able verify this hypothesis using this study, because observations were not made from day five onwards.

As was found in other studies, the aggressive behaviors of the most dominant worker represented a high proportion (~70-76%) of the total group aggression in all treatments, indicating the formation of a dominance hierarchy, though these workers did not always have the highest ovarian activation (Amsalem and Hefetz 2011). The most aggressive worker had the largest oocytes in 46.15% (6 out of 13 groups) of the cases, though overall low levels of aggression necessitated removal of 5 groups because two or more workers had equivalent aggressive behaviors over the two-day observational period. Aggressive behaviors in our study are also extremely reduced compared to *B. terrestris* workers. Worker aggression was significantly higher in queenless, three worker *B. terrestris* groups compared to the same groups in *B. impatiens*. This substantiates the hypothesis that aggression is much less pronounced in *B. impatiens* (Cnaani et al. 2002), and calls into question whether aggression and ovarian activation are as tightly linked in this species compared to *B. terrestris*.

In this study, caged queens were unable to inhibit ovarian activation, suggesting that queen volatile cues, queen visual cues and limited, worker-initiated queen contact are not sufficient to inhibit worker ovary activation. However, it is important to note that ovary activation level may have been delayed; our 10-day time point may not have allowed us to precisely observe the effect of a delay. Similar results were obtained in *B. terrestris*: workers who can escape the influence of the queen into a chamber from which the queens are excluded still contact the queen but will activate their ovaries, although ovary activation is delayed compared to queenless workers (Alaux et al. 2004a). In our studies, it is unclear of ovary activation was delayed, but the reduced aggression observed in caged queen groups suggests this is possible. Worker-initiated contact may not be effective to inhibit reproduction in workers, though queen-initiated contact with workers may signal reproductive dominance to the workers. Workers can then “weigh” their option to reproduce based on the information they obtained from the queen. If so, this suggests that ovarian activation is self-controlled, not queen controlled. In this model, workers encountering an ineffective queen for a certain period of time (like our caged queens) will choose to reproduce. Thus, worker ovarian activation is only fully inhibited in situations where the queen can initiate contact with the workers (our data and Chapter 2). Her
contact with the workers may involve specific behavioral interaction, though we have not observed any signs of direct aggression between queens and workers in our studies. There was higher contact between workers and uncaged queens than workers and caged queens, and thus it is possible that a contact chemical cue could have been transferred in larger quantities to workers with uncaged queens, though it seems unlikely that the affect of a contact pheromone would be so precisely concentration-dependent. Alternatively, it is possible that caging stresses the queen so that she does not produce an adequate pheromonal bouquet.

Expression of *Kr-h1* was significantly lower in α-workers from the uncaged queen treatment, which matches expression patterns found in honey bees exposed to QMP and *B. terrestris* in the presence of a queen or dominant worker (Grozinger et al. 2003, Shpigler et al. 2010). Expression was high in α-workers from both caged queen treatments and queenless treatments, indicating that volatile cues, queen visual cues and limited, worker-initiated queen contact from caged queens had no effect on *Kr-h1* expression. Though, we may have also missed a window during our 10 day trials, when the formation of dominance hierarchy was delayed, *Kr-h1* expression in caged queen treatments may have been similar to uncaged queen treatments. Uncaged queen signals may lower *Kr-h1* expression, though we cannot rule out the possibility that there is an effect of worker ovarian activation on expression of *Kr-h1*, and because worker ovarian activation was inhibited in the uncaged queen groups, they may not display the same expression patterns. Interestingly, *vg* expression closely tracked aggression in our study, which indicates that that *vg* expression and aggression are linked in *B. impatiens*, which has also recently been found in *B. terrestris* (Amsalem et al. 2014). Both aggression and *vg* expression were lower in the presence of caged queen and uncaged queens indicating that signals produced by the queen do modulate aggression, which may explain the lack of the aggressive competition phase (Cnaani et al. 2002, 2007), despite the fact that workers activate their ovaries near the end of the colony cycle (Padilla et al. in preparation). This may indicate that *Kr-h1* expression/ovarian activation and *vg* expression/aggression are uncoupled in *B. impatiens* and are not regulated by the same behavioral or chemical signals. Expression of *vg* and aggression seem to be modulated by queen signaling, even if the queen is ineffective, the signal is robust and remains effective. For expression of *Kr-h1* and ovarian activation, queens may produce dominance signals, though workers may “choose” to reproduce if they sense an ineffective queen.
If workers do auto-regulate reproduction, queens must employ cues to signal their dominance to workers, though whether these cues are behavioral, chemical or a combination are still unknown. Our studies suggest that if these cues are chemical in nature, contact through a wire cage only acts to lower aggression and expression of *vg*, and has no effect on *Kr-h1* expression or reproduction. In future studies, aggression, ovarian activation and gene expression analyses can be conducted on workers exposed to queen cuticular extracts to see if differences are caused by pheromones or behavior. Egg laying and expression of *Kr-h1* at earlier time points can also be determined to resolve the question of delayed reproductive capabilities and interrupted formation of dominance hierarchies.

In social insects, more advanced societies are hypothesized to use pheromone communication and more primitive species are thought to rely on aggression to regulate this division. Bumble bees are prime candidate organisms to explore this transition because both aggression and chemical communication are used by workers and queens to regulate this competition, but the relative contributions of each mechanisms seem to vary between species. In *B. terrestris*, aggression has a larger role in the reproductive division of labor than in *B. impatiens*, suggesting a higher reliance on pheromone communication. However, disentangling cues queens use to signal dominance to workers have proven to be difficult. In this study we have shown that caged queens can slightly lessen the aggression observed after 3-4 days in queenless workers, but workers in these groups activate their ovaries to the same degree after ten days. We have also shown that expression of *vg* is modulated by aggression while expression of *Kr-h1* is modulated by ovarian activation. Comparisons of *B. impatiens* and *B. terrestris* can provide valuable information about the genes, neurophysiological, and physiological mechanisms that underpin the hypothesized transition from aggression to pheromones to regulate the reproductive division of labor.
Figure 9: Experimental design. A laying queen from a queenright colony was caged on developing brood and three callow workers were then placed into the chamber. Queens and workers were provided access to a 50% sucrose solution via a modified feeder, fitted with a filter in the side to deliver the sucrose.
Figure 10. Worker contact with queens and cages. Worker contact with uncaged queens was significantly higher than worker contact with caged queens (Wilcoxon multiple comparisons, z=3.37, p=0.0007) and empty cages (Wilcoxon multiple comparisons, z=4.671, p<0.0001). Worker contact with caged queens was significantly higher than worker contact with empty cages (Wilcoxon multiple comparisons, z=-3.895, p<0.0001). Each group was observed every 5 minutes for 45 minutes on days 2, 3 and 4 at 12:00pm. Average contact events over the 3-day period are reported here. Data are presented as mean +/-SE with the number of replicates presented at the bottom of the bar. Different letters denote statistical differences using a Wilcoxon multiple comparisons test.
**Figure 11: Aggression levels observed in worker groups.** Aggressive behaviors are significantly higher in queenless worker groups than uncaged queen groups (Wilcoxon multiple comparisons, $z=1.70$, $p=0.013$). There were no significant differences between queenless worker groups and caged queen groups (Wilcoxon multiple comparisons, $z=-0.955$, $p=0.089$) or between caged queen groups and uncaged queen groups (Wilcoxon multiple comparisons, $z=-2.48$, $p=0.339$) though there is a trend of reduced aggression in caged queen groups compared to queenless worker groups. Each group was observed for a total of 60 min over a two-day period. Additive total of attacking, humming and darting are presented here. Data are presented as mean +/-SE with the number of replicates presented at the bottom of the bar. Different letters denote statistical differences using a Wilcoxon multiple comparisons test.
**Figure 12: Levels of aggression displayed by most aggressive worker.** The most aggressive female from the queenless worker group had significantly higher aggressive behaviors compared to the most aggressive female from the uncaged queen groups (Wilcoxon multiple comparisons, $z=2.328$, $p=0.019$). There were no significant differences between the most aggressive females from queenless worker groups compared to the caged queen groups (Wilcoxon multiple comparisons, $z=1.59$, $p=0.1127$) or between the most aggressive female from caged queen groups compared to the uncaged queen groups (Wilcoxon multiple comparisons, $z=-0.782$, $p=0.434$) though there is a trend of reduced aggression in the most aggressive female from caged queen groups compared to the queenless worker groups. Each group was observed for a total of 60 min over a two-day period. Additive total of attacking, humming and darting are presented here. Data are presented as mean +/- SE with the number of replicates presented at the bottom of the bar. Different letters denote statistical differences using a Wilcoxon multiple comparisons test.
Figure 13. Queenless worker aggression comparison. Worker aggression data for *B. terrestris* was obtained from Amsalem & Hefetz (2011) and was compared to worker aggression data from this study. Aggressive behaviors of *B. terrestris* groups were significantly higher than *B. impatiens* groups (Wilcoxon rank sums; $z=2.69$, $p=0.007$). Data are presented as mean +/- SE with the number of replicates presented at the bottom of the bar.
Figure 14. Worker ovarian activation. There was a significant effect of treatment on worker ovary activation (one-way ANOVA; $f_{2,15}=4.468$, $p=0.030$). Worker ovary activation was higher in the queenless and caged queen groups relative to the uncaged queen groups ($p<0.025$) and there was no significant different between queenless workers and workers housed with the caged queen ($p=0.769$). Data are presented as mean +/- SE with the number of replicates presented at the bottom of the bar. Different letters denote statistical differences using a Student's t-test.
Figure 15. Relative vitellogenin expression. There was a significant effect of treatment on vitellogenin expression. Expression was significantly higher in α female heads from the queenless treatments compared to the caged and uncaged treatments (Wilcoxon multiple comparisons: $z=2.482$, $p=0.013$; $z=-2.082$, $p=0.005$). Expression was also significantly higher in workers from the caged treatment compared to the uncaged treatment (Wilcoxon multiple comparisons: $z=-2.162$, $p=0.031$). Data are presented as mean +/- SE with the number of replicates presented at the bottom of the bar. Different letters denote statistical differences using a Wilcoxon multiple comparisons test.
Figure 16. Relative $Kr-h1$ expression. There was a significant effect of treatment on $Kr-h1$ expression. Expression was significantly higher in $\alpha$ female heads from the queenless and caged treatments compared to the uncaged treatments (Wilcoxon multiple comparisons: $z=-2.482$, $p=0.013$; $z=-2.162$, $p=0.031$). No significant differences were found between females from the queenless and caged treatments. (Wilcoxon multiple comparisons $z=1.201$, $p=0.229$). Data are presented as mean +/- SE with the number of replicates presented at the bottom of the bar. Different letters denote statistical differences using a Wilcoxon multiple comparisons test.
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Table 1. Gene and primer information
Chapter 4.
Conclusion and Future Directions

There has long been interest in understanding the proximate mechanisms underlying the evolution of social behavior in social insects. It has been hypothesized that pheromonal regulation of reproductive division of labor evolved from aggressive regulation of reproductive of division of labor, and thus these two modes of communication should use the same suite of genes and neurophysiological pathways. However, thus far, this theory has not been comprehensively examined using closely related species that employ aggression or pheromones. Bumble bees represent a unique, transitional species where the reproductive division of labor is regulated both by aggression and chemical communication, although the relative contributions of these mechanisms is hypothesized to differ among species. A comparative approach using two different bumble species allows us to directly investigate the mechanisms regulating reproductive division of labor.

Differences in the mechanisms that regulate the reproductive division of labor in B. terrestris and B. impatiens may make these two species an appropriate comparative system, though this has not been tested. In this thesis I investigated if B. impatiens queens use volatile pheromones, contact pheromones, aggression or visual cues to inhibit ovarian activation leading to egg laying in workers. I then used candidate gene expression analysis to determine how genes in signal receivers are modulated by reproductive dominance (aggression and pheromones). Finally, I compared this data to the known mechanisms controlling reproductive dominance in B. terrestris and found significant differences between the two species.

My results demonstrate that B. impatiens queens do not use volatile pheromones to inhibit worker reproduction. Workers that received volatiles from both reduced colonies and full colonies displayed activated ovaries consistent with unexposed control workers. Workers in direct contact with queens in reduced colonies were reproductively inhibited, evidenced by significantly smaller oocytes than unexposed and workers exposed to volatiles. Thus, direct contact with the queen is required to inhibit worker ovary activation.
Interestingly, my results suggest that a queen-produced contact pheromone modulates worker-worker aggression, while behavioral interactions between queens and workers appear to be necessary to inhibit worker ovary activation. Workers exposed to cues from caged queens displayed reduced aggression compared to control workers exposed to an empty cage. Although behavior was modulated, ovarian activation of these workers was unaffected by cues from a caged queen; both workers exposed to a caged queen and control workers exposed to an empty cage activated their ovaries. Ovarian activation and aggression was inhibited in workers exposed to uncaged queens.

The model we present above, queen contact pheromone mediates aggression and queen behavior mediates ovary activation, is also supported by gene expression. Expression of vitellogenin (vg), which is associated with aggression in B. terrestris, was significantly downregulated in workers exposed to caged and uncaged queens. Expression of Krüppel homolog 1 (Kr-h1), which is associated with reproductive dominance in B. terrestris, was not significantly different in workers exposed to cues from a caged queen and control workers exposed to an empty cage, although expression was significantly downregulated in workers exposed to an uncaged queen. These results also confirm that gene expression associated with reproductive dominance and aggression is conserved in B. terrestris and B. impatiens.

Taken together, my results indicate that contact pheromones produced by B. impatiens queens modulate worker aggression and gene expression associated with aggression, while queen behavior inhibits worker ovarian activation and modulates gene expression associated with reproductive dominance. This model also provides an explanatory framework in which to interpret the results from the full colony analyses in Chapter 1. Workers in full colonies activated their ovaries during the colony cycle, although aggression and egg laying was never observed. Thus, we postulate that near the end of the colony cycle when worker population peaks, queens cannot behaviorally interact with every worker, resulting in an increase of worker ovarian activation. However, we hypothesize that queen pheromone is still adequately spread throughout the colony, thereby inhibiting worker-worker aggression. However, we have not determined which cue regulates worker egg laying. Volatile cues from queens do not effect worker egg laying, as workers exposed to reduced colony volatiles and full colony volatiles laid eggs consistent with unexposed
controls. Worker egg laying was not analyzed when exposed to caged queens, however, worker egg laying was not witnessed during the cull colony cycle analysis, suggesting that queen pheromone also regulates worker egg laying.

These studies lay the groundwork for future investigations into the cues that regulate queen dominance in bumble bees and how behavior and pheromones function to inhibit aggression and ovarian activation. Definitively separating behavioral cues from chemical cues is the next step to determine the origin and effects of these various cues. To achieve this; workers can be exposed to a variety of chemical extracts (glandular, cuticular and whole bodies) from queens in similar assays to those used in our studies. Ovarian activation, aggression, worker-egg-laying, and gene expression analyses can be performed which will yield concrete conclusions regarding the effects of queen chemicals on workers. Isolating queen behavior will prove to be much more difficult, as methods used to eliminate queen pheromones will undoubtedly modify queen behavior, though non-living queen bodies can be washed free of chemicals to determine if visual cues are necessary for inhibition of aggression or reproduction. Though disentangling the mechanisms underlying the reproductive division of labor in bumble bees will be challenging, these studies will provide us with new insights into the mechanisms mediating the evolution and maintenance of eusocial behavior.

The origin and evolution of queen dominance, the conversion from aggression to pheromone control of worker reproduction, and the underlying gene expression changes in signal producers and receivers are still mostly unknown. However, bumble bees use both behavior and pheromones to regulate the reproductive division of labor; making bumble bees an exceptional biological model to investigate the molecular and neural mechanisms mediating the evolution of complex social behavior.
References


