Aggression, Social Interactions, and Reproduction in Orphaned (*Bombus impatiens*) Workers: Defining Dominance

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"Phenomena like worker oviposition…..are traditionally glossed over in studies of highly integrated colonies. But they are worthy of renewed attention because they are symptomatic of intragroup competition likely to be fundamental to larger patterns of behavior and social organization."

Mary-Jane West-Eberhard (p. 15, 1981)

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Abstract

At certain stages of a bumblebee colony life cycle workers lay eggs. Not all workers reproduce, however, since many continue to forage and care for the nest. This leads to questions regarding what differentiates a reproductive worker from a non-reproductive one. It is hypothesized that a form of reproductive competition takes place, where the most behaviourally dominant worker becomes reproductively dominant.

The behaviour of orphaned *Bombus impatiens* pairs was recorded and aggression, social interactions, egg-laying, and ovarian development were identified. Experiment 1 examined the association between aggression and egg-laying. Contrary to the hypothesis, the most aggressive worker did not lay more eggs. When the ovarian development of workers was manipulated and two workers with developed ovaries were paired (Experiment 3), they were more aggressive than pairs with discouraged ovarian development. This provides support for the supposition that aggression and reproduction are related, however, it is only partial support as worker pairs with encouraged ovarian development did not lay more eggs. Since aggression is believed to be only one part of behavioural dominance, Experiment 2 studied the association between social interactions and aggression and reproduction. Results showed that when two socially active bees were paired they were more aggressive than pairs including one or two socially inactive bumblebees. No significant difference in ovarian development between socially active pairs and socially inactive pairs was found.

Brood presence was also predicted to affect reproductive control. Experiment 1 found egg-laying and aggression were more likely to co-occur in the absence of brood. Results from Experiment 2 supplemented the first experiment since the absence of brood increased rates of aggression and ovarian development in pairs.

Whereas the results confirm aggression has a role in worker reproduction the findings also reveal that behavioural dominance does not equate to reproductive dominance under all conditions. The primary contributions of this thesis were the development of a method to distinguish behavioural dominance from reproductive dominance and determining their relationship under different environments (brood presence) and experimental manipulations (ovarian development). These contributions further define dominance in *Bombus impatiens.*

Introduction

Historically, the social organization of bumblebees (*Bombus* spp.) and other eusocial insects has been viewed as an oddity within the animal kingdom due to its high degree of co-operation and functional cohesion. These social insects have commonly been typified as harmonious entities that behave as a ‘superorganism’, paralleling the way each member works in cohesion similar to the components of a multi-cellular organism (Free & Butler, 1959; Moritz & Southwick, 1992; Wilson & Sober, 1989). This apparent cohesion extends to reproduction because although the workers of a bumblebee colony can produce eggs (specifically unfertilized eggs which give rise to males), for most of the colony’s life cycle the worker bees do not reproduce but rather assist the queen in rearing her offspring. The examination and understanding of the social dynamics of eusocial insects is essential to our understanding of the evolution of social behaviour (Hölldobler & Wilson, 2008). Indeed, the question of how altruism evolved has received significant attention and has been called the “central theoretical problem of sociobiology” (Wilson, 1975, p. 3). Altruism has been one aspect through which the study of social behaviour in eusocial insects has assisted in the understanding of this trait. In many eusocial insects, individuals in the group appear to be unaffected by the rules of natural selection since they are observed to perform altruistic acts that can assist the growth and development of the group at the cost of their own. For example, due to the physiology of the honeybee, she dies after she uses her stinger (Dizaji, Moeni, Sis, Shaddel, & Shaddel, 2008). On the surface, this may appear in contradiction to the theory of natural selection because the worker honeybee is producing an evolutionary ‘dead end’ through disemboweling herself to save her colony because her genes will not be passed to the next generation. Nevertheless, the study of these species has assisted in the development of models of altruism as well as models that are applied to explain human altruistic behaviour (Wilson, 1975).

The historical view of the ‘harmonious’ eusocial insect colony is unidimensional, however, since there are periods in a bumblebee colony’s life cycle, for example, where conflict does occur (defined as the competition phase, Bloch, 1999). Aggression may escalate, causing the queen to be expelled from the nest or even killed by her young (Bourke & Ratnieks, 2001; Foster, Brunskill, Verdirame, & O’Donnell, 2004). Worker aggression and oviposition (egg-laying) can also be observed if the colony is queen-less or “orphaned” (Owen, Rodd, & Plowright, 1980). Since intraspecies (i.e. worker-worker or queen-worker) aggression occurs within the context of worker oviposition (Bourke, 1988a; *Bombus bifarius*, Foster et al., 2004; *B. terrestris*: van Honk & Hogeweg, 1981; van Honk, Röseler, Velthuis, & Hoogeveen, 1981; van Doorn & Heringa, 1986), it is hypothesized that aggression has a role in the competition for reproductive rights (Bourke, 1988a; Johnstone, 2000; Penick, Trobaugh, Brent, & Liebig, 2013).

Theoretical explanations of reproductive altruism in eusocial insects

What leads to the shift from reproductive ‘altruism’ (refraining from oviposition) to ‘selfish’ reproduction in a worker bumblebee? To answer this question, one must understand why workers of a colony refrain from reproducing in the first place. Kin selection theory explains this reproductive altruism using the principles of natural selection and relatedness (Hamilton, 1963). Keller and Chapuisat (1999) provide a simple explanation of the rule. According to Hamilton, an act of altruism will depend on the costs incurred to the altruistic individual (c), the benefits received by the recipient (b), and the degree of (genetic) relatedness between the members (r). Altruism will be favoured when:

$$\mathrm{br} - \mathrm{c} > 0$$

Individuals cannot pass on their genes if they die before they procreate, but if through dying altruistic individuals save the lives of others who are likely to also carry their genes, the altruistic behaviour will be promoted. In humans, if an individual dies to save the lives of more than two siblings ($r = 0.5$), more than four half-siblings ($r = 0.25$), or eight cousins ($r = 0.125$), for example, the individual 'saved' a large enough portion of his genes to justify the sacrifice (Keller & Chapuisat, 1999). This theory can also be used to explain reproductive altruism in the social groups including bumblebees. In a way similar to saving the life of a relative at the cost of your own, individuals may benefit from assisting a relative to reproduce at their expense if the benefits (weighted by relatedness) exceed the costs. According to kin selection theory, bumblebee workers should favour the reproduction of their queen if the gains are greater than if they reproduced on their own. Following a diploid genetic system for procreation, the degree of relatedness would be the same whether an individual assisted a parent to successfully reproduce or reproduced themselves (e.g. an individual shares 50% of their genes with their offspring and 50% with their full siblings). However, this is not the case with bumblebees as a worker is more related to her sister than to her own offspring. This is because bumblebees follow a haplodiploid sex determination system. Only the females (workers and queens) develop from fertilized eggs and receive genes from both their mother and father. Males develop from unfertilized eggs and contain only the genetic material from their mother. All females receive the same genetic material from their father. If a queen mates only once, as is typical in most species of bumblebee such as *Bombus terrestris* and *B. bifarius* (Alaux, Jaisson, & Hefetz, 2006; Foster et al., 2004; Schmid-Hempel & Schmid-Hempel, 2000), the degree of relatedness between a worker and her sister is 75% ($r = 0.75$) whereas she is only 50% related to her son (Appendix A). Therefore, with regards to genetic relatedness, it would be in a worker's 'best interest' to assist the queen in producing reproductive females (i.e. sister queens).

There are certain situations when it is no longer genetically advantageous for a worker to refrain from reproducing, specifically when the queen starts producing male offspring. According to the haplodiploid system, the degree of relatedness between a bumblebee worker and her brother is only 25%. Therefore, a worker is more related to her own sons (50%) or the sons of her sister (37.5%) than to her brothers (Bourke, 1988a). This leads to a situation where under certain conditions, a divergence between the queen's reproductive interests and the reproductive interests of the workers will occur. According to kin selection, if a queen stopped producing daughters and started to produce males, the workers should lay their own eggs or encourage the reproduction of their sisters over the production of brothers. However, the queen's genes would be best promoted if she inhibited the reproduction of her workers because she is more related to her own sons (50%) than to her grandsons (25%). Therefore, kin selection theory predicts during periods of male production by the queen there would be increased attempts by workers to usurp the queen in producing males.

Mechanisms of reproductive control

Although Hamilton's rule of kin selection theory provides scenarios when reproductive altruism would be discouraged, it does not define the dynamics of the conflict between individuals nor how the interactions between members can affect reproduction. Even though it may be in a worker's best interest to reproduce directly, reproductive workers do not perform colony maintenance tasks and, as such, they reduce the overall productiveness of their colony (Bourke, 1988a; Endler, Hölldobler, & Liebig, 2007). Hence, not all bumblebee workers in a group reproduce with the rest performing activities related to colony care and maintenance (*B. terrestris*: Bloch, Borst, Huang, Robinson, & Hefetz, 1996; Bloch & Hefetz, 1999; *B. bifarius*, Foster et al., 2004; *B. terrestris*: van Doorn, 1987; van Doorn 1989; van Honk & Hogeweg, 1981). This is consistent with kin selection: if through competition, the genetic interests of a member cannot be met through reproducing directly, the member would collaborate through indirect reproduction to enhance her genetic 'interests'. Those who are defeated in their attempts to reproduce directly shall be more likely to perform tasks that enhance their indirect reproductive benefits (West-Eberhard, 1981). This also suggests that there is a form of reproductive competition among workers and the development of tools to enhance their reproductive chances may have evolved. Certain behaviours and environmental conditions are predicted to play a role in reproductive competition in eusocial insects such as the bumblebee, specifically; (1) aggressive behaviour, (2) social interactions, and (3) brood presence.

(1) Aggression and reproductive control

Aggression is associated with worker reproduction in a variety of different species of ants (e.g. *Harpegnathos saltator*, Liebig, Peeters, & Hölldobler, 1999; *Harpagoxenus sublaevis*, Bourke, 1988b) and wasps (e.g. *Parischnogaster mellyi*, Fanelli, Boomsma, & Turillazzi, 2008; Foster & Ratnieks, 2001; *Ropalidia marginata*, Premnath, Sinha, & Gadagkar, 1996). In the wasp *Ropalidia marginata*, for example, queens are commonly succeeded by nest-mates (Premnath et al., 1996). Immediately prior to succession, the colony becomes considerably more aggressive, with the most aggressive nest-mate becoming the new sole egg-layer (Premnath et al., 1996).

Similar associations between aggression and reproduction are observed in bumblebees. In *B. atratus*, for example, multiple queens compete in a fatal battle to become the sole egg-layer of the colony, with the most aggressive queens being the most likely to survive (Cameron & Jost, 1998). In *B. terrestris*, there is a positive association between aggressive behaviours and ovarian development (Duchateau, 1989; Free, 1955; Pomeroy, 1981; van Doorn, 1989) and aggressive behaviours are more likely to be performed by bees with developed ovaries and most often directed towards other bees with developed ovaries (Duchateau, 1989). Indeed, the aggressive behaviour oophagy (egg cannibalism) is used by both bumblebee queens and workers and is suggested to be used as a tool to control the amount of eggs that are successfully reared (*B. terrestris*, Alaux, Jaisson, & Hefetz, 2004; *B. impatiens*, Cnaani, Schmid-Hempel, & Schmidt, 2002; *B. melanopygus*, Owen & Plowright, 1982; *B. terrestris*, Ratnieks, 1988). Similar findings are also found in other eusocial insects (*Lasioglossum zephyrum*, Brothers & Michener, 1974; Fletcher & Ross, 1985; *Dinoponera quadriceps*, Monnin & Peeters, 1997).

Although there is an association between aggression and ovarian development in the bumblebee, it is not as clear whether this aggression is used to assert reproductive control. Ovariectomized bumblebee workers continue to display aggression (*B. terrestris*: van Doorn, 1987; van Doorn, 1989) and aggression serves other purposes in other eusocial insects such as to encourage foraging (*Ropalidia marginata*, Lamba, Chandrasekhar, & Gadagkar, 2008; *Polybia occidentalis*, O'Donnell, 2001) or other nest tasks (*Polistes fuscatus*, Sumana & Starks, 2004), or as a necessary behaviour to encourage the ovarian development in the worker herself (*R. marginata*, Lambda, Kazi, Deshpande, Natesh, Bhadra, & Gadagkar, 2007). Additionally, even if aggression is used in reproductive control it may be ineffective under certain conditions. Indeed, although the *Bombus* queen is aggressive towards her egg-laying workers, a proportion of males in a colony are worker derived (Alaux, Savarit, Jaisson, & Hefetz, 2004; Brown, Schmid-Hempel, & Schmid-Hempel, 2003; Owen &

Plowright, 1982; Paxton, Thoren, Estoups, & Tengo, 2001).

(2) Social interactions and reproductive control

Worker aggression is often viewed as an expression of social dominance, with the most socially dominant bee suppressing reproduction in another (Bourke, 1988a; Heinze, 2008). Similarly, non-aggressive social interactions are associated with aggression (*B. terrestris*, Bloch et al., 1996; van Honk & Hogeweg, 1981; van Honk et al., 1981) as well as ovarian development (*B. terrestris*, Alaux, Jaisson, et al., 2004; Bloch et al., 1996). Additionally, social interactions are often used to develop a reproductive dominance hierarchy in other eusocial insects. In the ponerine ant (*Pachycondyla villosa*), for example, social dominance is used to form a hierarchy among workers, of which the top-ranking individuals are the predominant egg-layers (Heinze, Trunzer, Oliveira, & Hölldobler, 1996). This close association between social dominance and reproductive dominance may account for why the term 'dominance' is often used interchangeably to represent both reproductive control and social control, which can cause confusion and accrue methodological problems (Bernstein, 1981). For example, the dominant bumblebee establishes her position through overt aggression and is often the one that lays the most eggs (*B. terrestris*, Larrere & Couillaud, 1993; van Doorn, 1989). However, this produces circular logic since a worker is defined as dominant because she is aggressive and lays eggs and, conversely, she is aggressive and lays eggs because she is dominant. Recent work has differentiated reproductive from social dominance giving empirical support to the distinction (Amsalem & Hefetz, 2010). Nevertheless, it is unclear what function social behaviours have on oviposition in worker bumblebees.

(3) Brood presence and reproductive control

If intraspecies aggression is used to compete for reproduction one may expect it to occur in nest areas where egg-laying normally takes place (Cole, 1988). In the ant (*Leptothorax*), for example, aggressive interactions are more likely to occur in nest areas where brood are present (Cole, 1988). The probability of escalated aggression is also more likely to occur around eggs compared to other areas of the nest (Cole, 1988). The presence of brood could also affect worker oviposition since egg cells are typically constructed on the cocoons of brood and as such may be a necessary stimulus to trigger egg-laying (*B. terrestris*, Duchateau & Velthuis, 1989). Indeed, common definitions of bumblebee egg-laying include brood interaction behaviour, specifically the opening or ‘inspecting’ (antennal touching of partly closed egg cells) of pre-existing egg cells (Bloch & Hefetz, 1999), suggesting brood rearing behaviours could be associated with reproduction and may have an important role in reproductive competition. Indeed, there is a significant correlation between ovarian development and task specialization in the bumblebee, with house-bees (those performing in-nest tasks such as brood feeding) having more developed ovaries (*B. bifarius*, Foster et al., 2004; *B. impatiens*, Pomeroy, 1981; *B. terrestris*, van Doorn, 1987) and more likely to become egg-layers compared to forager-bees (*B. terrestris*, Duchateau & Velthuis, 1989).

Reproductive conflict under orphaned conditions

Bumblebee colonies are generally founded by a single queen without the assistance of workers (Cnaani et al., 2002). Typically, queens emerge from hibernation in the spring and search for a nesting location. When a queen initially founds a colony she must continue to forage until there are enough workers to ensure that the colony receives sufficient resources to survive. This can be a particularly dangerous time for the queen since she may die while foraging or is otherwise unable to return to her nest and her brood. This results in an interesting situation where only a few workers are left to care for the nest. It is during queen-less conditions such as these that workers start egg-laying and aggressive behaviours are observed (*B. melanopygus*, Owen & Plowright, 1980). Considering that workers are observed to continue to lay eggs in the queen's absence it appears that they can continue to function in the absence of the queen (Owen et al., 1980). Although orphaned bumblebee colonies occur in nature (Owen et al., 1980) and are a common occurrence in the order of Hymenoptera (e.g. in the slave-making ant, *Harpagoxenus sublaevis*, Bourke, van der Have, & Franks, 1988; the Japanese ant, *Myrmica kotokui*, Kikuchi, Tomizuka, & Higashi, 2000; and the Japanese paper wasp *Polistes chinesis antennalis,* Miyano, 1986) relatively little is known about the functioning of queen-less groups. This may be because orphaned groups are associated with colony decline and death (Bourke, 1988a). In addition, considering that queen-right bumblebee colonies are of value for pollination purposes in commercial greenhouses (Veltuis & van Doorn, 2006), perhaps the study of queen-less colonies is limited because they are of little economic use. It has also been suggested that the use of the term 'orphaned' colony should also refer to the natural stage in the bumblebee colony life-cycle when the queen ceases production of workers and starts to lay male eggs, specifically the competition phase (Owen et al., 1980). As such, not only do queen-less colonies occur in nature and likely have had a significant role in the evolution of bumblebee social dynamics but similar behaviour patterns likely occur during the competition phase in the colony life-cycle. Nevertheless, the study of worker behaviour in the absence of the queen allows for the examination of the more subtle interactions between workers, those which are often masked or overlooked by the dominating presence of the queen.

Ovarian development and egg-laying

There are two common ways worker reproduction is measured; through physiological correlates (i.e. measures of ovarian development) and behavioural correlates (egg-laying). Although ovarian development is often used as an indicator of reproduction (*B. terrestris*, Amsalem & Hefetz, 2010; Amsalem & Hefetz, 2011; van Doorn, 1987) as it is associated with oviposition (Bloch & Hefetz, 1999), it is not necessarily an indicator of reproduction since a significant portion of workers in a colony have developed ovaries but do not lay eggs (*B. terrestris*: Bloch & Hefetz, 1999; Duchateau & Velthuis, 1989; *B. bifarius*, Foster et al., 2004; *B. terrestris*, van Doorn, 1989). Also, as previously indicated, oophagy can negatively affect the proportion of eggs that are successfully reared (*B. terrestris*, Alaux, Jaisson, et al., 2004; *B. impatiens*, Cnaani et al., 2002; *B. melanopygus*, Owen & Plowright, 1982; *Bombus*, Ratnieks, 1988). As such, even though a worker may have the ability to lay eggs, ovarian development does not indicate whether she is successful at reproducing. This leads to lingering questions regarding the association between reproductive 'potential' (i.e. ovarian development) and successful reproduction, which will be clarified here.

Significance

The study of insect behaviour provides important insights not only into the evolution and dynamics of social behaviour (Hölldobler & Wilson, 2008) but also to increase the understanding of colony functioning under conditions of queen death or absence. Bumblebee species have declined dramatically, with an astonishing number of species going extinct over the last century in the United Kingdom as well as worldwide (Goulson, 2010). Although the specific causes for species decline are not known, it is likely a combination of loss of habitat, pesticides, non-native bee introductions, and habitat fragmentation (Goulson, 2010). As competition for valuable nesting sites has likely increased, this may lead one to wonder the effect it would have on intracolony aggression and queen attempts at usurpation of valuable nest sites. Indeed, this has been observed to be a common occurrence in many species of *Bombus* found in Canada (Richards, 1978). This increased competition also places increased risk of further queen-less nesting situations from failed usurpation attempts or abandoned nests.

Reproductive conflict between a queen and her workers has been studied to some extent in the bumblebee (*B. terrestris*: Alaux, Savarit, et al., 2004; Alaux, Jaisson, & Hefetz, 2005; Amsalem, Twele, Francke, & Hefetz, 2009; Bloch, 1999; Bourke, 1988a; *B. hypnorum*, Brown et al., 2003; *Bombus*, Bulmer, 1981; *B. terrestris*, Cnaani, Robinson, Bloch, Borst, & Hefetz, 2000; *Bombus*, Keller & Nonacs, 1993; *B. melanopygus*, Owen & Plowright, 1982) but the research on reproductive conflict among workers is limited (*B. terrestris*: Amsalem & Hefetz, 2010; Amsalem & Hefetz, 2011; Bloch & Hefetz, 1999; *B. impatiens*, Cnaani et al., 2002; *B. terrestris*: Duchateau, 1989; Duchateau & Velthuis, 1989; *Bombus*: Free, 1955; Pomeroy, 1981; Röseler & Röseler, 1977; *B. terrestris*: van der Blom, 1986; van Doorn, 1987; van Doorn, 1989; van Honk et al., 1981). This is surprising considering that worker reproduction is suggested to have had significant consequences on queen behaviour, specifically with regards to the development of mechanisms used by the queen to control worker reproduction (Bourke, 1988a; Keller & Chapuisat, 1999). In addition, workers can contribute significantly to the number of males produced in a colony, which suggests that it has evolutionary significance (e.g. up to 90% of males produced in the colony are worker-derived in *B. atratus*, as cited in Bourke, 1988a; Owen & Plowright, 1982, although there is species variation, Alaux, Savarit, et al., 2004; Brown et al., 2003; Owen &

Plowright, 1982; Paxton et al., 2001). It is also hypothesized that worker reproduction has assisted in the development of characteristics such as aggression, matricidal behaviours, and temporal division of labour (Bourke, 1988a).

The predominant bumblebee species studied is the European *Bombus terrestris*. Considering bumblebees are prevalent in North America (Goulson, 2003; Kearns & Thomson, 2001) the study of a common local species can provide a unique perspective on worker bumblebee aggression. Additionally, although there are similarities between *B. impatiens* and *B. terrestris* (e.g. workers ovaries develop and they can lay eggs under queen-less conditions, Cnaani et al., 2002), there are differences (e.g. a proportionally smaller number of reproductive workers in a colony, Cnaani et al., 2002).

This thesis aims to study reproductive conflict in orphaned worker bumblebees (*B. impatiens*). These experiments not only provide the necessary bridge between the physiological and behavioural correlates of the phenomenon of bumblebee reproduction but they also test hypotheses regarding the relationship between aggression, social interactions, and reproduction in orphaned workers.

The objectives of the first experiment were to understand the relationship between aggression and oviposition in orphaned *B. impatiens* pairs as well as the role brood presence has on the two behaviours. The objectives of the second experiment were two-fold: (1) to examine the relationship between social interactions and aggression and reproduction, and (2) to experimentally manipulate brood presence to measure its effect on these dependent variables. Finally, the objectives of the third experiment were to manipulate the reproductive capabilities of a worker (i.e. ovarian development) to determine their effect on aggression and oviposition.

Experiment #1

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On the relationship between aggression and reproduction in pairs of orphaned worker bumblebees *(Bombus impatiens)*

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Preface

The first experiment is a correlation-based study measuring the relationship between two important variables that are present throughout the thesis; aggression and oviposition. The presence or absence of brood was also manipulated. This experiment examined the dependent variables under the condition of two orphaned bumblebees.

Abstract

This study characterized aggression and reproduction within pairs of orphaned bumblebee sisters (*Bombus impatiens*). Twenty-one pairs were filmed in the laboratory over 5–10 days. Frequencies of aggression and egg-laying were obtained for each bee, and the presence or absence of brood was manipulated. Aggression and egg-laying were more likely to co-occur in pairs placed without brood compared to pairs placed with brood. A significant positive correlation was found between members of a pair in the rates of aggression. In addition, a strong positive correlation was found in their rates of egg-laying: bees that had more sons also tended to have more nephews. The results show that under conditions of unrestricted food availability, behavioural interactions are compatible with continued reproduction by both orphaned workers. Though aggression may limit reproduction, it seems either to be an ineffective means of obtaining a reproductive monopoly in some situations and/or to be a set of behaviours invested with other possible functions.

Keywords: *Bombus impatiens*, orphaning, reproduction, aggression, dominance, inclusive fitness

Introduction

In bumblebees (*Bombus* spp.), workers retain their reproductive capabilities; although they do not mate, they can lay unfertilized eggs that result in male offspring (Sladen, 1989). Not all the workers lay eggs [by one estimate, 38 % of workers (*Bombus terrestris*) had laid eggs by the end of the colony cycle (Alaux, Savarit, Jaisson, & Hefetz, 2004)], and those that do, carry out little work while the rest perform the regular activities of colony maintenance (Ratnieks, Foster, & Wenseleers, 2006). This leads to the question of what differentiates the reproductive from the non-reproductive workers. The common assumption is that a form of reproductive competition takes place (Johnstone, 2000). Specifically, an agonistic encounter determines the egg-laying ‘winner’, while the loser must resort to caring for her sister’s offspring. In the slave-making ant (*Harpagoxenus sublaevis*), aggressive workers have increased ovarian development (Bourke, 1988). Similar associations are also observed in other eusocial insects such as the wasps, *Polistes chinensis antennalis* (Miyano, 1986), *Parachartergus colobopterus* (Platt, Queller, & Strassmann, 2004), and *Polistes instabilis* (Molina & O’Donnell, 2009). In worker bumblebees, aggressive workers are also more likely to have developed ovaries (*Bombus bifarius*) (Free, 1955; Foster, Brunskill, Verdirame, & O`Donnell, 2004); *Bombus bimaculatus* (Pomeroy, 1981); *B. terrestris* (Duchateau, 1989; van Doorn, 1989; Alaux, Jaisson, & Hefetz, 2004)) and are more likely to be egg-layers (van Doorn & Heringa, 1986; Alaux, Jaisson, et al., 2004), though there are interspecies differences within the genus *Bombus* with respect to the rates of worker aggression and worker reproduction (Cnaani, Schmid-Hempel, & Schmidt, 2002). The proportion of queen-less *Bombus impatiens* workers that have developed ovaries, e.g. is smaller (~11 %, Jandt & Dornhaus, 2011) than in *B. terrestris* (~50 %, Duchateau & Velthuis, 1989). In the present study, we investigate aggression and egg-laying in a North American species of bumblebee, *Bombus impatiens*.

Although there is a positive association between aggression and reproduction within individuals in *Bombus*, it is not so clear whether aggression by one bee directly influences the rates of egg-laying in another. Egg-laying workers, e.g. are not only more likely to initiate aggression, but they are also more likely to be the recipients of aggression (van Doorn & Heringa, 1986), and they continue to lay eggs in spite of aggression directed towards them. Additionally, ovariectomized workers display aggression (van Doorn, 1987, 1989). These findings suggest that ovarian development and aggression may not have a direct causal link, but rather that they may have a common antecedent—a third variable that affects both (Pomeroy, 1981).

While there may be conditions under which aggression may deter reproduction by workers, there may also be conditions where deterrence is unnecessary or even counterproductive. Although workers are potentially in conflict (Bourke & Ratnieks, 2001), since they are more highly related to their own sons (0.5) than to the sons of their sisters (0.375), they may nonetheless enhance their inclusive fitness by working to increase the number of males produced in the colony (Ratnieks & Reeve, 1992). One worker gains in inclusive fitness if she encourages the production of nephews, so long as the cost to her, in terms of producing her own sons, is less than her sister's reproductive benefits once they are weighted by the relatedness of the sisters (for a recent review on inclusive fitness theory, see Bourke, 2011). Even though one worker favours the production of her own sons over the production of her sister's sons, there may well be some advantage to her in making some allowances to her sister and her sister may in turn have little incentive to surrender her own reproductive rights.

If aggression were used to suppress reproduction, worker aggression directed at the queen also ought to have a significant impact on her ability to produce daughter queens, which it does not (Lopez-Vaamonde, Koning, Jordan, & Bourke, 2003). Queen aggression directed towards her workers also does not put a stop to worker oviposition (Alaux, Jaisson, et al., 2004) as a portion of males within a colony are worker-derived (Owen & Plowright, 1982; Alaux, Savarit, et al., 2004; Lopez-Vaamonde et al., 2003). Perhaps aggression in some contexts, such as two workers with abundant resources, is not so much for asserting reproductive control as for some other end. For example, a novel function for dominance behaviour in queen-less colonies of the wasp *Ropalidia marginata* has recently been proposed: the aggressive behaviour in an individual may be required for her own ovarian development (Lamba, Kazi, Deshpande, Natesh, Bhadra, & Gadagkar, 2007).

The association between aggression and egg-laying was tested here within pairs of orphaned (i.e. queen-less) workers. The study of pairs of workers has the advantage of reducing the phenomenon to its fundamental components. Small groups have been used in the past to study reproduction and aggression (Pomeroy, 1981; Bloch, Borst, Huang, Robinson, & Hefetz, 1996; Bloch & Hefetz, 1999b; Amsalem & Hefetz, 2011). Recent work on pairs of *B. terrestris* workers (monitored for 5 days after onset of pairing) has shown not only that one worker is usually more aggressive than the other, but also her behavioural dominance translates into reproductive dominance, as measured by terminal oocyte length (Amsalem & Hefetz, 2010). In addition, it has been reported that the submissive bee sends a ‘‘sterility signal’’ (ester compounds in the Dufour’s gland) that announces her non-reproductive status (Amsalem, Twele, Francke, & Hefetz, 2009) and has an appeasement effect (Amsalem & Hefetz, 2010). The previous work focused on the behaviour and physiology of workers in the first few hours and days after orphaning and made little attempt at extrapolation beyond that time. If oviposition behaviour were subsequently examined, and reproductive output were measured directly instead of indirectly, one possible scenario would be that the more aggressive bees within pairs would show higher rates of egg-laying. Though the focus of our study is on the behaviours of aggression and reproduction, it has also been suggested that chemical ‘‘signals’’ (i.e. pheromones) from *B. terrestris* queens and egg-laying workers affect the suppression of ovarian development in non-reproductive workers (Bloch & Hefetz, 1999a; Sramkova, Schulz, Twele, Francke, & Ayasse, 2008) though they may often be used in conjunction with behavioural means (e.g. in *Diacamma ceylonense* (Cuvillier-Hot, Gadagkar, Peeters, & Cobb, 2002).

Here, we also re-examined the very notion of reproductive competition (Amsalem et al., 2009). Perhaps ‘reproductive competition’ means only that in a pair one bee lays more eggs than the other, but perhaps it means more: a convincing ‘win’ by one bee may entail a convincing ‘lose’ by the other, in which case a negative correlation between two members of pairs in their rates of egg-laying ought to be observed. In other words, even if aggression plays a role in reproductive contests, there is some room for debate as to the extent of the conflict. The conflict may be such that there is a clear loser that bows out of the reproductive game, or it may be such that there is an ongoing ‘jostling for position’ where neither bee emerges as the victor and both persist in displaying aggression. The suggestion that egg-laying by one worker might, however, be positively rather than negatively associated with egg-laying in the other is not without precedent. Indeed, there is social facilitation of the ovarian development of bumblebee workers (Free, 1957; Cnaani et al., 2002).

In the present study, pairs of orphaned *B. impatiens* workers were given easily available and unlimited access to pollen and honey–water solution. The principal objectives were: (1) to determine whether egg-laying in one bee is associated with reproduction in her sister, and if so, whether the relationship is positive or negative, (2) to examine pairs for which we did and did not provide brood, in keeping with several studies that have considered a possible role of the presence or absence of brood both on measures of aggression (Foster et al., 2004) and reproduction (van Doorn & Heringa, 1986; Bloch, 1999; Lopez-Vaamonde et al., 2004; Geva et al., 2005) and (3) to also determine whether the bee with the higher rate of aggression in a pair tends to lay more eggs than her sister, as would be expected if aggression in one bee suppressed or inhibited reproduction in the other.

Methods

Orphaned pairs

Orphaned pairs of *B. impatiens* were collected from colonies (described below) between January and December, 2007. All pairs were maintained under the same laboratory conditions. Two sister callow workers of comparable size (individuals that were less than 12-h old, without full colouration and with curved wings) were selected randomly. While workers within *Bombus* colonies can vary in body mass by eight-to tenfold (Goulson, 2010) and worker size may affect dominance in queen-less colonies (Free, 1955; Röseler, 1977), it is not an important factor in pairs of workers that have been matched in size (Amsalem & Hefetz, 2010). Worker size was estimated at the end of the experiment by measuring the intertegular span [the distance between the tegulae, which is a strong indicator of body size (Cane, 1987)] in the video images. Age differences in orphaned pairs can have an effect on ovarian development (Pomeroy, 1981). For callow workers, however, any differences in reproduction or aggression are unlikely to be attributable to age differences in physiology (Bloch & Hefetz, 1999b).

All bees were marked by gluing coloured, numbered tags onto the thorax (Opalit Plättchen supplied by The Bee Works, Orillia, ON). The groups were provided with *ad* *libitum* 50 % (by volume) honey–water solution and fresh pollen mixed with honey–water solution to form uniform dough (Plowright & Jay, 1966). Three commercially supplied *B. impatiens* queens from Biobest Biological Systems were used solely to produce larvae to provide to the orphaned workers in the Pairs With Brood condition. These queens were unrelated to the orphaned workers. Although queen bumblebees (*Bombus occidentalis*) have been shown to discriminate between their own and foreign brood, they do not reject the foreign brood but rear them (Gamboa, Foster, & Richards, 1987).

Colonies

The orphaned workers in this study were pulled out of seven colonies. The Pairs With Brood condition consisted of a total of 11 orphaned pairs gathered from 2 commercial colonies between January and May, 2007. In an effort to increase levels of aggression that might be aimed at preventing oviposition, the Pairs Without Brood condition was subsequently implemented, using ten pairs from four laboratory-reared colonies and one commercial colony between May and December, 2007.

Apparatus

Each pair was housed in a wooden nest box (5.1 cm high x 10.2 cm x 10.2 cm), contained within an incubator in which temperature was maintained at 30^{o}C. A fan circulating air over a basin of water acted as a humidifier. The box was lined with honeybee wax to allow for a surface for egg-cup building. In the Pairs With Brood condition, the nest boxes contained queen-laid eggs and young larvae that were 5–7 days old.

The nest box was connected to a second same-sized box where a glass feeder tube was located. Glass plates covered the tops of the boxes to allow for easy observation. The laboratory was illuminated by a combination of fluorescent light fixtures and natural light. Digital video recordings were made on a JVC Hard Disk Camcorder attached to a tripod and positioned directly over the nest box.

Behavioural recording

The recording for each pair (duration approximately 8.5 h) on any one day began in the morning (starting time between 0730 and 1030 hours), afternoon (starting 1230–1530 hours) or evening (starting 1730–2030 hours), with the start times being chosen randomly. Recordings of the orphaned pairs commenced once worker oviposition was observed (5–11 days after placement, 8.4 ± 1.78). For the Pairs Without Brood condition, recordings concluded ten days after the first egg was laid. For the Pairs With Brood condition, recordings concluded when the first queen-laid offspring emerged as adults (5–10 days, 7.36 ± 1.75).

The description of worker egg-laying (oviposition) as characterized by Bloch and Hefetz (1999b) was used: ‘‘…a worker seen inserting her abdomen into an open egg cup for a few minutes while moving it up and down, along with typical kicking movement of the hind legs. Oviposition behaviour was often preceded by a period of new egg-cup construction or by opening and preparing an already existing one.’’ (p. 127). Using this definition, Bloch and Hefetz (1999b) found that for 90.5 % of observations of oviposition, at least one egg was seen in the egg-cups. The media file in Appendix B (B3) gives an example of oviposition.

Based on Duchateau’s (1989) definition, aggression was characterized as two behaviours. The first aggressive behaviour is defined as ‘‘butting’’, which consists of an accelerated movement of one animal towards the other resulting in contact and a change in direction if there was no previous interaction immediately preceding the event. The butting behaviour may or may not include agape mandibles (the mouthparts of the bee are open), or the raising of the hind legs. The second aggressive behaviour is defined as ‘‘grappling’’, which is a somersault motion between the two bees which may involve stinging positions in which the aggressor pulls the other by its hind legs (Gadagkar & Joshi, 1983). The end of the aggressive act was defined as breaking contact for more than 3 s. Further encounters were recorded as new acts. Examples of aggression in pairs of *B. impatiens* workers are presented in Appendix B (B1 and B2). To determine the reliability of the definition of egg-laying and aggression, randomly selected videos were rated by another trained observer.

Inter-observer reliability

Observer reliability was measured in six pairs of the Pairs With Brood condition. One trained observer re-analysed 435 h of video. There was a significant correlation between the first and second observers for both the total number of ovipositions (Pearson product-moment correlation coefficient $r = 0.97$, $df = 4$, $p = 0.001$) and the total number of aggressive acts per group ($r = 0.99$, $df = 4$, $p = 0.001$).

Data analysis

If aggression is an expression of conflict over reproduction, then it ought to be observed in close temporal association with oviposition, i.e. at least within the same observation period. We examined whether aggression was more likely to occur in observation periods during which oviposition was also observed, using a logistic model fit to the data using generalized linear interactive modelling (GLIM) (Francis, Green, & Payne, 1993).

The remainder of the statistical analyses was performed using PASW Statistics 18. The frequencies of the aggressive and egg-laying behaviours over the recording periods were summed for each individual bee and divided by the total observation time. Because the data were positively skewed, they were square-root transformed.

To understand the behavioural interactions within pairs of orphaned workers, two data sets were created. Prior to computing correlation coefficients, it was necessary to assign identity to each member of a pair in a consistent and non-arbitrary way. First, we examined pairs in which there was any difference, statistically significant or not, in rate of aggression (*n* = 13, seven pairs with and six pairs without brood). One bee in the pair could then be identified as the more aggressive bee and the other as the less aggressive bee. Secondly, we examined pairs in which there was any difference, statistically significant or not, in rate of egg-laying (*n* = 19, eleven pairs with and eight pairs without brood). One bee could then be identified as the one with the higher rate of egg-laying and the other with the lower rate.

Results

We analysed 1780 hours of video recordings. The mean difference in intertegular span between workers in a pair, together with the standard deviation, was 7.7 ± 4.5 % (range 0.08–16 %).

Comparisons between pairs with and without brood

In the Pairs With Brood condition, all pairs were observed to lay eggs. Aggression occurred in seven of the eleven pairs. In the Pairs Without Brood condition, there was egg-laying in all pairs but one. Aggression occurred in six of the ten pairs. The difference in rate of aggression between the two members of a pair was compared between groups. The mean difference score for Pairs With Brood (mean and standard deviation 0.32 ± 0.29) was not significantly different (independent samples *t* test, $t_{11} = 0.20$, $p = 0.85$) from that for Pairs Without Brood (0.35 ± 0.22). Including the pairs for which the frequency of aggression was zero for both members of the pair (three pairs With Brood and five pairs Without Brood) left the outcome unchanged ($t_{19} = .29$, $p = 0.77$).

The difference in rate of egg-laying between members of a pair was also compared between groups. The mean difference score for the Pairs With Brood (mean and standard deviation 0.09 ± 0.05) was no different (independent samples *t* test $t_{17} = 0.58$, $p = 0.57$) from that for the Pairs Without Brood (0.13 ± 0.07).

Timing of aggression

Although the analysis above revealed no differences between the Pairs With and Without Brood, Figure 1 shows differences between the two groups in the timing of aggression with respect to oviposition. Thirteen pairs displayed both aggression and oviposition during the observation period. Observation periods during which aggression was seen seemed no more likely to have begun at one of the three times of day, which were counterbalanced, than the others: 14 in the morning, 14 in the afternoon and 12 in the evening. For the seven Pairs With Brood, aggression occurred during approximately one-third of the observation periods, whether oviposition also occurred or not. For the six Pairs Without Brood, however, aggression was much more likely to occur during observation periods in which oviposition was also observed. A logistic model (which specifies a binomial error term) was fit to the data. The interaction between group (With or Without Brood) and presence or absence of oviposition was significant ($\chi^2_1 = 12.21$, $p = 0.0005$). A term for time of year was then entered into the linear model, in 2-month blocks, within each group, to determine whether there were any time-related fluctuations in the laboratory aside from the experimental introduction of larvae. No effect of time of year was detected ($\chi^2_4 = 7.46$, $p = 0.11$).

The relationship between behaviours within pairs

For the two following correlational analyses, the groups of pairs with and without brood were combined since there was no effect of group on the difference scores, either in aggression or egg-laying (see above). Figure 2 shows that the more one bee in a pair initiated aggression, the more the other initiated aggression ($r = 0.59$, df = 11, $p = 0.03$). Figure 3 shows that bees that had more sons also had more nephews. A strong positive correlation in the rates of egg-laying between members of a pair was found ($r = 0.77$, df = 17, $p = 0.0001$).

A paired samples t test was performed to determine whether the more aggressive bees of the pair laid more eggs than the less aggressive bees, as would be expected if aggression by one bee served to inhibit reproduction in her sister. Though the mean was higher for the more aggressive bees (mean and standard deviation 0.20 ± 0.07) than for the less aggressive ones (0.15 ± 0.10), the difference was not significant ($t_{12} = 1.90$, $p = 0.08$). Conversely, the bees that laid more eggs in a pair did not show more aggression than the bees that laid fewer eggs ($t_{18} = 0.84$, $p = 0.41$).

Discussion

The correlational analyses of this study were aimed at characterizing the extent of the conflict between pairs of queen-less, worker bumblebees. In one sense, our results are perfectly compatible with the notion that there is reproductive suppression of one bee by her sister, because there was always one bee in a pair that laid more eggs than the other did. Our results, however, are incompatible with a more restrictive meaning of ‘suppression’, in which one worker’s gain in egg-laying amounts to a loss in her sister’s. This is the scenario that has been disconfirmed by our results: there was a positive correlation in egg-laying within pairs of orphaned workers (Figure 3): bees that had more sons also had more nephews. Our results circumscribe the level of competition: the positive correlation is not consistent with any level of suppression. In other words, one worker does no more than ‘keep a lid’ on reproduction by her sister. The positive correlation is in line with the suggestion that there is social facilitation of ovarian development in bumblebee workers (Free, 1957; Cnaani et al., 2002). It is difficult to reconcile with the view that the ‘subordinate’ bee signals her functional sterility (Amsalem et al., 2009; Amsalem & Hefetz, 2010)—at the very least it shows that such a signal is deceptive, temporary or both. Indeed van Doorn (1989) has pointed out that ‘dominance hierarchies’ are fluid.

There was no evidence that aggression by one bee was met with surrender by her sister: a positive correlation was also found in levels of aggression within pairs (Figure 2). Under this study’s conditions of unrestricted food availability, there were virtually no foraging costs, and neither worker had an incentive to become a functionally sterile forager (and by the same reasoning, queens should also not interfere with worker oviposition when resources are plentiful). On the contrary, both workers likely stood to gain the most by having both sons and some nephews. Although orphaning of bumblebee colonies does occur in nature (Owen, Rodd, & Plowright, 1980), it is likely that orphaning of only a pair of workers in nature is rare. Future research investigating worker aggression and reproduction in larger groups will likely place more foraging costs and potentially increase the incentive for a portion of *B. impatiens* workers to become functionally sterile.

While the presence or absence of brood had no discernible effect on differences in the rates of oviposition or aggression within a pair, a pronounced effect was found in the timing of these two behaviours (Figure 1): in Pairs Without Brood, aggression was more likely to occur at times when there was also oviposition, i.e. at times when it could serve either to prevent oviposition or to guard against oophagy. No such context dependence was found for the Pairs With Brood. One explanation for this pattern of results would be that the presence of larvae and subsequent pupae interferes with the aggression that may accompany oviposition. Egg-laying often occurs on top of or in close proximity to larvae. Aggressive events within the context of oviposition may become interrupted by activities such as inspecting larval cups, feeding larvae (Pendrel & Plowright, 1981; Ribeiro, Velthuis, Duchateau, & van der Tweel, 1999) and incubating pupae.

The results on the co-occurrence of aggression and egg-laying are compatible with the understanding that aggression is ‘about’ reproduction, even though there is some debate as to whether the competition is for a place with thermal insulation (Pomeroy, 1981) and/or should be viewed as a race to lay eggs in time for them to be reared to reproductive age (Amsalem & Hefetz, 2011). In this context, it seems curious that while the more aggressive worker laid more eggs compared to the less aggressive one, as would have been expected if aggression by one bee functioned to suppress oviposition by the other, the difference was not significant. Moreover, the bees that laid more eggs were not more aggressive overall than the bees that laid fewer. The failure to find significant differences is likely not due to insufficient observations of behaviour. We logged 1780 hours of digital recordings, when compared to about 5 h by Foster et al. (2004) or 320 h by Cameron (1989). Moreover, we used daily observation periods of approximately 8.5 h, in comparison to 30 min (3 x 10 min) in Amsalem and Hefetz (2010). Even if pairs of workers of *B. impatiens* are like *B. terrestris* in that individuals that are ‘‘behaviourally dominant’’ within the first five days of placement together tend to have the most developed ovaries (Amsalem & Hefetz, 2010), this ovarian development seems not to translate very well into oviposition. Physiological measures are not a substitute for behavioural measures of reproduction, and indeed not all workers with developed ovaries lay eggs (van Doorn, 1989; Bloch & Hefetz, 1999b; Foster et al., 2004).

Our study highlights the multidimensional nature of aggression in *B. impatiens* workers. Aggression is often viewed as an expression of conflict over reproduction in honeybees (Malka, Shnieor, Katzav-Gozansky, & Hefetz, 2008), bumblebees (van Doorn, 1987, 1989; Duchateau, 1989; Bloch & Hefetz, 1999b; Foster et al., 2004) and other insect groups (Bourke, 1988b; Cole, 1988; Liebig, Peeters, & Hölldobler, 1999). This study raises the possibility that there are boundary conditions on the view that aggression is the means towards egg-laying monopolization and suggests aggressive and reproductive behaviours might activate rather than suppress egg-laying in other individuals. In this vein, the term dominance, whether behavioural or reproductive, might possibly lead to oversimplifications of ongoing interactions between two workers.

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Figure 1. The relative frequencies (expressed as proportions from 0 to 1), with standard error bars, of daily observation periods during which aggression was observed are given as a function of two variables: whether oviposition was also seen during that same daily observation period or not and whether the pairs of orphaned workers were placed with or without brood.

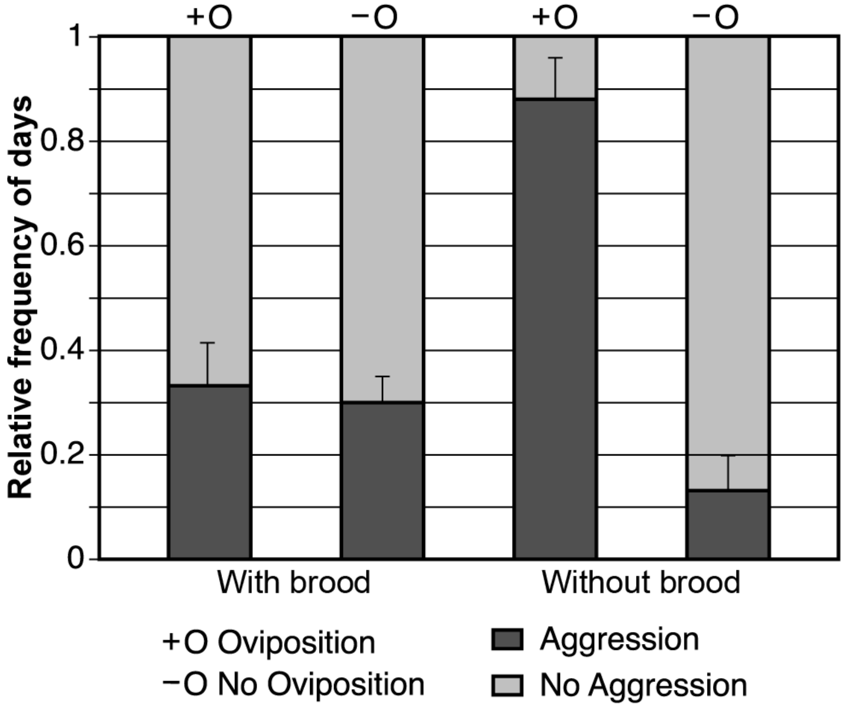


Figure 2. Rates of aggression per hour (square-root transformed) by bees showing the higher and lower rates of aggression within each pair.

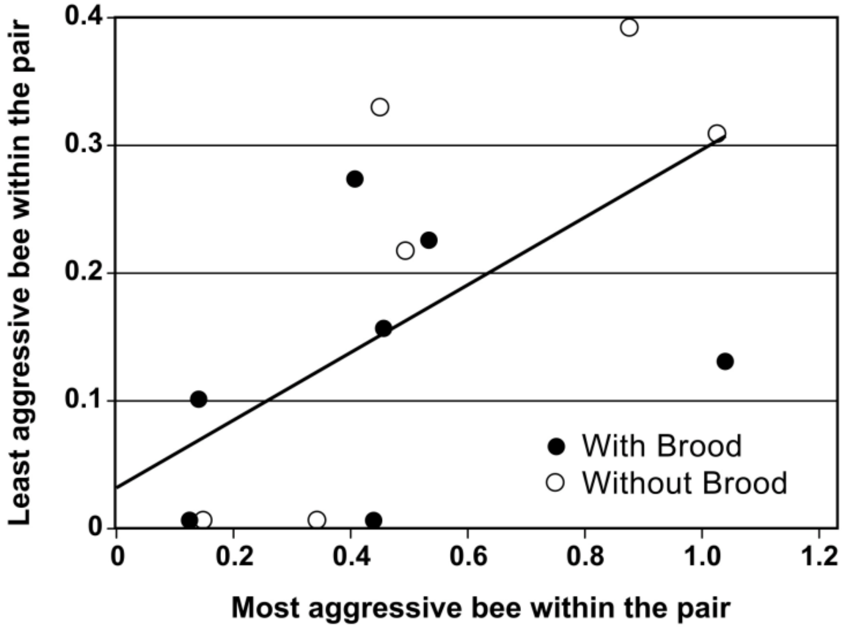
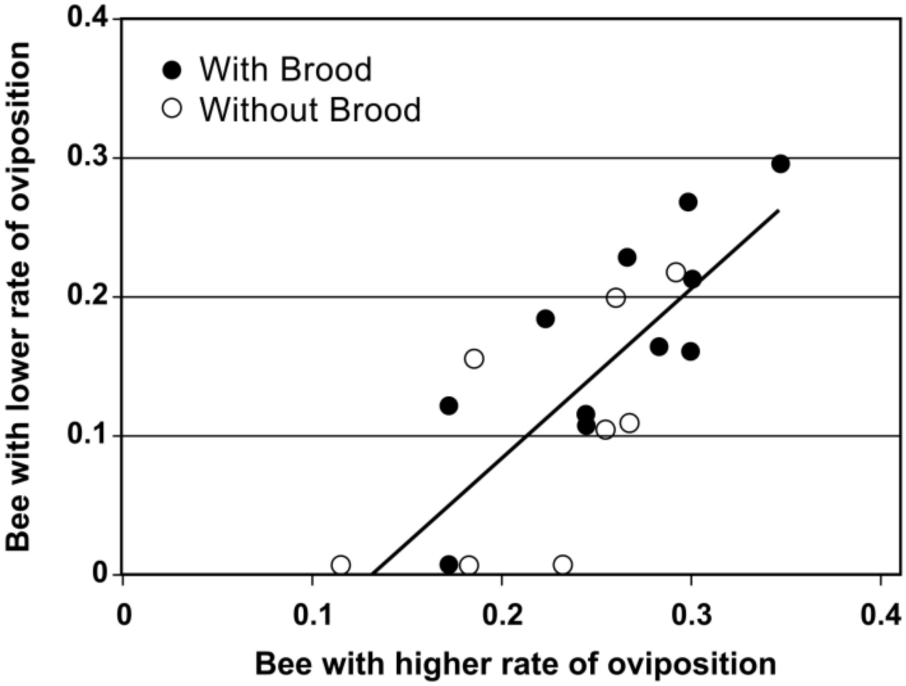


Figure 3. Rates of egg-laying per hour (square-root transformed) by bees showing the higher and lower rates of egg-laying within each pair.



Experiment #1 Addendum

Introduction

Two additional experiments were done as part of Experiment 1 that were not included in the published manuscript. The first experiment was performed to determine if isolated workers could successfully oviposit (1A) and the second was performed to validate the presence of egg-cups as an index of the presence of eggs and determine the average number of eggs laid per cup (1B). Both of these variables are important as they are part of later experiment protocols.

General Methods

Colonies and workers

Bombus impatiens workers were collected from the colonies described in Experiment 1 between May and November, 2007. All bumblebees were marked by gluing coloured, numbered tags onto the thorax (Opalit Plättchen, The Bee Works, Orillia, ON). Workers were provided with unlimited 50 % (by volume) honey–water solution and fresh pollen mixed with honey–water to form uniform dough.

Apparatus

Workers were housed in the same manner as described in Experiment 1 (e.g. a wooden nest box contained within an incubator). All nest boxes were connected to a second same-sized box where a glass feeder tube was located. Glass plates covered the tops of the boxes to allow for easy observation. The laboratory was illuminated by a combination of fluorescent light fixtures and natural light.

Data analysis

The statistical analyses were performed using PASW Statistics 18.

Experiment 1A: Can isolated workers reproduce?

It was important to determine if isolated workers could successfully oviposit under these conditions as this was to be part of later experiment protocols (e.g. part of the procedure to create 'behaviour testers' in Experiment 2). Isolated workers have been shown to have reduced ovarian development (*Bombus impatiens*, Cnaani, Schmid-Hempel, & Schmidt, 2002) even when compared to a worker placed with a queen (*Bombus terrestris*, Duchateau & Velthuis, 1989). This reduced ovarian development may represent delayed development rather than an inability to develop ovaries (*B. impatiens*, Cnaani, Wong, & Thomson, 2007). This experiment was performed to determine if single worker *B. impatiens* could indeed successfully oviposit and rear their offspring under our nesting conditions.

Methods

The methods were as described under the General Methods section. Eleven callow workers of various sizes were gathered from three colonies between May and July 2007. One callow was placed per nest box, without brood. Each nest box was observed daily and the dates of the first oviposition, when the offspring pupated, and worker mortality rates were recorded.

Results and Discussion

Ten of the eleven isolated workers laid eggs. Four out of the ten workers died before their offspring hatched. The average number of days between placement and oviposition was 11.8 (*SD* = 3.26). The number of days between placement and oviposition was compared with data collected from Experiment 1 Pairs Without Brood (*M* = 9.2, *SD* = 2.57) condition. An independent samples t-test was performed to determine whether the isolated workers were significantly delayed in the date of their first oviposition. The difference between the two groups was not significant, t (18) = 1.98, p = 0.06.

Experiment 1B: Presence of egg-cups as an index of the presence of eggs.

It was important to validate the presence of eggs within the egg-cups as this was used as an indicator of reproductive capability in later experiments (e.g. Experiment 2). Information regarding the average number of eggs laid per cup also provides information regarding the reproductive abilities of *B. impatiens* workers, something which has not been reported to our knowledge.

Methods

The methods were explicated in the General Methods section. Twelve pairs of *B. impatiens* workers of unknown age and various sizes were gathered from a commercially supplied colony and tested over 18 days in November, 2007. Pairs were placed in a nest box and observed daily. When at least one egg-cup was observed, the workers were removed from the nesting box and the cup was dissected, the presence of eggs was recorded as well as the number of eggs per cup. The eggs were then removed but the cup was left undisturbed. The workers were then reintroduced to the box. The procedure was continued if new cups were observed over the testing period.

Results and Discussion

Eleven of the twelve pairs laid eggs during the testing period. The date of first oviposition varied from day 4 to day 11 (*M* = 7.09, *SD* = 2.43). All cups contained eggs. The number of egg-cups produced in 24 hours varied from one to four (*M* = 1.48, *SD* = 0.70) and the number of eggs per cup varied from one to seven (*M* = 3.05, *SD* = 1.31).

General Discussion

This research was performed to determine if isolated workers can reproduce and to know the number of eggs *B. impatiens* worker pairs lay per egg-cup. Isolated workers are successful at laying eggs under these conditions as only one of the eleven single workers died before she oviposited. There was no significant mean difference in the date of first oviposition between the isolated workers and the Pairs Without Brood.

The proper identification of an egg-cup is a necessary component of this thesis. Using this validation task, it is possible to make a correct identification of an egg-cup within 24 hours of an oviposition. An increased understanding of worker reproduction was also developed through answering this question. The total number of worker-produced eggs can vary significantly over a 24 hour period, from one to 28, with an average of three cups and three eggs per cup. Not surprisingly, the queen may produce more eggs per cup as she generally lays between eight and 16 eggs (Free & Butler, 1959; Goulson, 2003). Of note, we did not identify the egg-layer. It is possible both workers could have contributed to the number of eggs laid in a single egg-cup, although this is not typically observed. Nevertheless, the findings show workers can reliably lay eggs in easily observed egg-cups and can successfully produce multiple egg-cups in a two-week period.

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Experiment #2

Social interactions and their connection to aggression and ovarian development in orphaned worker bumblebees (*Bombus impatiens*)

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Preface

The second study expanded on the first study by using an experimental design rather than a correlational one. The social activity of the bumblebees was assessed and workers were defined as either socially active or socially inactive. This was based on the rationale that there may be other variables that may have affected the patterns of aggression and reproduction in the first experiment that were not captured in the experiment, mainly the social activity of the workers. The presence and absence of brood was also manipulated to further explicate the relationhip between brood and aggression and reproduction. A second measure of reproduction, specifically ovarian mass, was included as a physiological measure of worker reproduction.

Abstract

This study examines the effect of pairing together orphaned worker bumblebees (*Bombus impatiens*) with comparatively high or low levels of social activity ('social dominance') on their ovarian development and aggression. The workers were paired according to their levels of social activity (a socially active + another socially active worker, socially active + socially inactive, and two socially inactive workers). The presence or absence of brood was also manipulated. The absence of brood increased both aggression and ovarian development, suggesting that aggression and reproduction are associated or that there is a third variable that affects both. Socially active pairs were significantly more aggressive: here, social activity can be taken as an early indicator of aggression. No such effect, however, was obtained on ovarian development as the socially active pairs did not have more developed ovaries compared to the others. Within the socially active + socially inactive pairs, the socially dominant worker did not have more developed ovaries and was not more aggressive than her socially submissive partner. Results highlight environmental conditions (the absence of brood) can predict ovarian development and although social activity can be a precursor to aggression, it does not translate to increased ovarian development under these conditions.

Keywords: bumblebee, *Bombus impatiens,* queen-less workers, reproduction, aggression, dominance, social interactions

Introduction

Bumblebee workers retain their reproductive capabilities and have the potential to produce male offspring. Nevertheless, through most of the colony life cycle they forego reproduction in favour of rearing the queen's offspring (Sladen, 1989). When worker oviposition (egg-laying) does occur, only a subset of workers become reproductive, becoming 'false' or pseudo queens (van Doorn & Heringa 1986; van Doorn, 1987; van Honk & Hogeweg, 1981). They are given this label because they cease foraging and only lay eggs in a way reminiscent of the behaviour of queens. A form of reproductive competition is hypothesized to occur that will distinguish the reproductive workers from the non-reproductive ones and that this competition is expressed through social dominance interactions and aggression (Bourke, 1988a; Heinze, 2008). In the wasp for example, rigid dominance hierarchies are created with a single, reproductive queen. If the queen is removed from the nest, aggression is observed between the highest-ranked socially dominant workers to establish their status as a reproductive (*Harpagoxenus sublaevis*, Bourke, 1988b; *Ropalidia marginata*, Premnath, Sinha, & Gadagkar, 1995). Similar suggestions are made with respect to bumblebees, specifically that aggression and characteristic social interactions differentiate a reproductive worker from a non-reproductive worker. This is based on the finding that social interactions and aggression coincide with ovarian development (*Bombus terrestris*, Alaux, Jaisson, & Hefetz, 2004; Bloch, Borst, Huang, Robinson, & Hefetz, 1996; *B. bifarius*, Free, 1955; *B. bimaculatus*, Pomeroy, 1981; *B. terrestris*, Duchateau, 1989; van Doorn, 1989; *B. bifarius*, Foster, Brunskill, Verdirame, & O`Donnell, 2004).

The relationship between ovarian development and aggression may not be as clear in *B. impatiens* compared to other species of *Bombus*. Although *B. impatiens* are similar to other species of bumblebee, e.g. similar patterns of worker ovarian development compared to

B. terrestris workers (Cnaani, Schmid-Hempel, & Schmidt, 2002), there are differences, e.g. a proportionally smaller number of reproductive workers in a colony (Cnaani et al., 2002). Additionally, when the behaviour of pairs of orphaned workers was monitored 5-11 days after the bees were placed together, the more aggressive bee in a pair did not lay significantly more eggs than the less aggressive one. Moreover, the bee that laid more eggs in a pair did not show more aggression than the bee that laid fewer eggs (Sibbald & Plowright, 2013). This may suggest that under conditions of unlimited access to food, aggression may be ineffective at suppressing reproduction in another or it serves another purpose in worker pairs. In other eusocial species, for example, aggression is used to encourage foraging and other nest duties (Lamba, Chandrasekhar, & Gadagkar, 2008; Clarke & Faulkes, 2001), is a necessary behaviour to encourage the ovarian development in itself (Lamba, Kazi, Deshpande, Natesh, Bhadra, & Gadagkar, 2007) or is a combination of functions (Clarke & Faulkes, 2001; Premnath et al., 1995). Conversely, the lack of an association between aggression and reproductive suppression in *B. impatiens* pairs may be indicative of other variables that have a more predominant role in bumblebee pairs.

Non-aggressive social interactions were not measured in our previous study (Sibbald & Plowright, 2013) on aggression and reproduction in pairs of orphaned bumblebees. Since workers were randomly paired, it is likely that bees of differing degrees of social dominance were paired together. Social interactions are likely a component of reproductive competition in that social interactions are signals of social dominance (van Honk & Hogeweg, 1981). In the present study, in addition to obtaining measures of aggression and reproduction, we recorded social interactions as soon as workers were paired to determine whether these interactions could serve as an early indicator of reproductive conflict. Moreover, we harnessed this variable by experimentally assigning workers to groups depending on their levels of social activity. If social interactions serve to mitigate future aggression, i.e. to resolve dominance contests, one possible outcome of this study would be that when workers are unequal in their levels of socially activity (as opposed to both being socially active or inactive) then future aggression would be reduced.

The presence of brood is predicted to play a significant role in reproductive competition. Based on the research, however, its relationship is mixed. Brood interactions and feeding, for example, are positively associated with oviposition (Foster et al., 2004) and ovarian development (Duchateau & Velthuis, 1989). Aggression is also more likely to occur in areas of the nest where the brood are located (such as in *Leptothorax allardycei*, Cole, 1988). Nevertheless, these studies were correlational and therefore specific conclusions regarding causation cannot be made. In an experimental manipulation of the presence versus absence of brood, in contrast, aggression and oviposition were more likely to co-occur in pairs placed without brood compared to pairs with brood (Sibbald & Plowright, 2013). This suggests that brood presence inhibits aggression that occurs during egg-laying. Conditions that further induce aggression (i.e. pairing two socially active bees together) may accentuate the effect of brood.

This study was performed on a common North American species of bumblebee, *B. impatiens*, to address three main questions: (1) Does pairing orphaned worker bumblebees according to their levels of non-aggressive social activity affect their levels of aggression? (2) Does it also affect their ovarian development? (3) Does the presence of brood, when experimentally manipulated, promote or inhibit aggression and ovarian development?

Methods

Colonies

Two commercial *B. impatiens* colonies supplied by BioBest, Biological Systems and three *B. impatiens* colonies derived from wild-caught queens and reared according to the procedure of Plowright and Jay (1966) were used. All colonies were maintained in the laboratory under identical conditions. Orphaned workers were collected from the colonies between July and December, 2008.

All bees were marked by gluing coloured, numbered tags (Opalit Plättchen, The Bee Works, Orillia, ON) onto the thorax. The groups were supplied with food *ad libitum*: 50% honey-water solution and pollen collected by honeybees that is mixed with honey-water solution to form an uniform dough.

Target workers

Target workers (the objects of study) were 118 *B. impatiens* orphaned workers from the five queen-right colonies. Workers that were within 12 hours of emergence from their cocoon (defined as 'callow workers'; bees not having full colouration and having curved wings) were randomly selected. Callow workers were chosen to ensure that workers were of comparable age. The callow workers were kept in isolation for 12 days to allow for ovarian development (as previous research has found it takes an average of 11.8 days for single *B. impatiens* workers to lay eggs (Experiment 1 Addendum –1A)).

Behaviour testers

To manipulate levels of social activity across pairs of target workers, it was necessary to assess these levels first. To this end, nine *B. impatiens* adult workers of unknown age from three queen-right colonies (the two BioBest colonies, and one of the colonies derived from a wild-caught queen) were randomly selected as 'testers' of social interactions in the target workers. These testers were placed singly in nesting boxes. To ensure these workers had developed ovaries the testers were not used for behaviour testing until eggs were observed in their nest boxes.

Apparatus

Once the bees were removed from their colonies they were housed in wooden nest boxes (10.2 cm x 10.2 cm x 5.1 cm) under humid conditions at 30^{o}C. The box was lined with honeybee wax to allow for a surface for egg-cup building. For the With Brood group, eggs were gathered from queen-right colonies and placed in the nesting box. The box was connected to a second nest box where a glass feeder tube was located. Glass plates covered the top of the boxes to allow for easy observation. The laboratory was illuminated by a combination of fluorescent light fixtures and natural light. Digital video recordings were made on a JVC Hard Disk Camcorder positioned directly over the nest box.

Assessment period

After their 12 days of isolation, the target workers' 'baseline' social activity was assessed. The target workers were placed in a new nesting box with one of three behaviour tester bees. Their behaviour was observed for five minutes and all social interactions and aggression were coded. A score of the degree with which the target workers initiated interactions versus retreated from them was calculated and workers were categorized as either socially active (SA) or socially inactive (SI). This was based on van Doorn and

Heringa's (1986) as well as van Honk and Hogeweg's (1981) definition of dominant social interactions of the bumblebee. Aggressive interactions were rare, occurring in only 6% of all assessments and were not included in the calculation. In other words, the definition of social activity does not include the behaviours that it is being used to predict. The pair was then separated and the same protocol was performed with two other behaviour testers. An average of the target workers' levels of interaction was determined. The following formula was used to calculate the target workers' level of social activity for each behaviour tester.

$$\frac{\text{No. of Initiations – Retreats}}{\text{Total No. of Interactions}}$$

An interaction is defined as antennal contact where the initiating bee is different from the bee that ends the interaction (see the non-aggressive social interactions definition below for more detailed information). Using the above calculation, for example, if a target worker initiates 8 interactions and retreats from 0 interactions with a behaviour tester its interaction score with that behaviour tester would be (8 - 0) / 8 = + 1.00. If a target worker initiates 0 interactions and retreats from 8 interactions, its interaction score would be – 1.00. An average over all three behaviour testers was calculated and workers were categorized as either SA or SI. An SA worker is one who scored above 0, meaning she initiated more interactions than she retreated from them. If a worker had a score of 0 or below, meaning she retreated from social interactions more than she initiated them, she was categorized as a socially inactive (SI) worker.

Testing Conditions

On the same day as the assessment the target workers were paired in a new nesting box under one of six conditions; three social groups and two brood groups. For the social group, each worker was paired in one of three ways; (1) a socially active worker + another socially active worker (SA+SA), (2) a socially active worker + a socially inactive worker (SA+SI), (3) a socially inactive + another socially inactive worker (SI+SI). For the brood group, each social group was also paired either (A) with brood or (B) without brood. There were 10 pairs per condition (except for the SA+SI, pairs with brood condition where there were 9 pairs as one worker in a pair died) and a total of six conditions (3 social groups x 2 brood groups), for a total of 59 pairs of orphaned workers. Each target worker was paired with a sister worker.

Procedure

The recordings were started approximately 24 hours after pairing. The recordings were randomly started either in the morning (defined as anytime between 7.30 h -10.30 h), afternoon (12.30 h -15.30 h) or evening (17.30 h – 20.30 h) and continued for 3 hours a day for 6 consecutive days. Following a previously developed, statistically reliable training method (Sibbald & Plowright, 2013), five behaviour coders were trained to identify aggression, social interactions, and oviposition. Four of the five were blind to the conditions and all coders analyzed a similar number of pairs per condition. The frequency and duration of aggression was coded in each pair. Oviposition was also recorded.

Measures

Aggression

We used Sibbald & Plowright's (2013) definition of aggression, which was adapted from that of Duchateau (1989). This consists of two behaviours; butting and grappling. Butting is defined an accelerated movement of one bee towards the other resulting in contact.

Grappling consists of a somersault motion between the two individuals which may involve stinging positions in which the aggressor is on the back of the other, dragging the other by her hind legs. Both the frequency and duration of each aggressive act was measured. This definition of aggression was found to be reliable (Sibbald & Plowright, 2013). Appendix B1 and B2 contain examples of butting and grappling behaviours.

Non-Aggressive Social Interactions

The definition from van Doorn and Heringa (1986) and van Honk and Hogeweg (1981) was used. An interaction is defined as any non-aggressive encounter where antenna contact is made between two bees and the initiating bee is different from the bee that ends the interaction. This definition was based on the observation that the queen rarely retreats from an interaction she initiates (van Honk & Hogeweg, 1981). The frequency and duration of all social acts were measured. Appendix B4 shows an example of a social interaction.

Oviposition and Ovarian Development

Oviposition was identified using Bloch and Hefetz's (1999) definition. This has shown significant inter-observer reliability (Sibbald & Plowright, 2013). To measure ovarian development, the ovaries of the target workers were dissected within 24 hours of the final day of testing and weighed on a Mettler-Toledo balance (PB 303). Ovary mass was divided by body mass.

Data analysis

The statistical analyses were performed using SPSS Statistics 20.

Results

There were 1047 hours of video recordings analyzed. Although new eggs were present within the nest of 40 of the 59 pairs, oviposition was not regularly captured on video (52.5% of pairs that had laid eggs) and therefore could not be included in the analysis. Of the 19 pairs that did not lay eggs, most were in the SA+SI social group (With Brood = 6, Without Brood = 4), compared to the SA+SA group (With Brood = 3, Without Brood = 0), and the SI+SI group (With Brood = 4, Without Brood = 2).

As not all testers were from the same colony as the target workers, a logistic regression was performed to determine whether the classification of a target worker as socially active or inactive depends on how many of the three behaviour testers were from the same colony as the target worker (in which case target and tester workers were relatives) as opposed to another colony (i.e. target and testers were unrelated). The four possibilities were: all three of the testers were from the same colony as the target ($n = 18$), only two were ($n = 6$), only one was ($n = 8$), or none were ($n = 86$). How many of the testers were related to the target was not an important variable, $\chi^2(1, N = 118) = 0.97, p = 0.33$. This is consistent with other research which has found that related worker pairs did not significantly differ from unrelated pairs in levels of aggression or ovarian development (Amsalem & Hefetz, 2010).

Due to the substantial positive skew of the frequency distribution for aggression frequency and duration, results are reported with a logarithmic transformation.

To determine the effect of social pairing and brood presence on levels of aggression and ovarian mass, a 3x2 multivariate analysis of variance was performed. The results are presented below. Additionally, a second 3x2 multivariate analysis of variance was performed to determine if socially active workers differ from socially inactive workers on levels of aggression and ovarian development. Therefore, a total of two tests were performed which typically warrants the Bonferroni adjustment to correct for the increased chance of significance when conducting multiple tests. Due to increased criticism of the adjustment, however, (Moran, 2003; Nakagawa, 2004; Perneger, 1998) the *p*-values are presented without a Bonferroni correction.

Differences between Pairs

Figures 1 and 2 show an effect of social pairing on aggression duration (F (2, 116) = 10.87, $p < 0.0001$, partial $\eta^2 = 0.17$) and aggression frequency (F (2, 116) = 13.48, $p < 0.0001$, partial $\eta^2 = 0.20$), respectively, Wilks' λ = 0.79. On both aggression duration and frequency, post-hoc comparisons using a Tukey's HSD test revealed the SA+SA groups were significantly more aggressive ($M_{duration}= 0.62$, $SE_{duration} = 0.07$; $M_{frequency}= 0.57$, $SE_{frequency} = 0.06$) compared to the SA+SI ($M_{duration} = 0.19$, $SE_{duration} = 0.07$; $M_{frequency}= 0.16$, $SE_{frequency} = 0.06$) and the SI+SI groups ($M_{duration} = 0.20$, $SE_{duration} = 0.07$; $M_{frequency}= 0.17$, $SE_{frequency} = 0.06$), $p < 0.05$. There was no significant difference between the SA+SI and SI+SI groups, which may reflect a floor effect. The groups without brood were significantly more aggressive as measured by aggression duration (F (1, 116) = 3.99, $p = 0.048$) but not aggression frequency (F (1, 116) = 1.64, $p = 0.20$), Wilks' λ = 0.85. The interaction between brood presence and social pairing was not significant for aggressive duration or frequency, Wilks' λ = 0.93 (F (2, 116) = 1.70, $p = 0.19$ and F (2, 116) = 1.76, $p = 0.18$, respectively).

Figure 3 shows that the results on ovarian mass did not parallel those on aggression duration (Fig. 1) and aggression frequency (Fig. 2); no effect of social group pairing was detected, *F* (2, 116) = 0.46, $p = 0.63$. In all three social groups, however, ovarian mass was larger when the pairs were placed without brood (*M* = 0.11, *SE* = 0.006) compared to when they were placed with brood (*M* = 0.08, *SE* = 0.004), *F* (1, 116) = 13.03, $p < 0.0001$, partial $\eta^2 = 0.11$. No interaction between social grouping and brood presence or absence was detected (*F* (2, 116) = 1.98, $p = 0.14$).

Differences within Pairs

As the SA+SI pairs are heterogeneous with regards to social activity prior to pairing, we performed an analysis to determine if aggression and ovarian development differed within the pair (i.e. is the SA bee more aggressive than the SI bee?). As such, a 3x2 multivariate analysis of variance was performed. The overall *F* test was not significant, showing the variance in aggression duration, aggression frequency, and ovarian mass is not explained by differences in social activity (Wilks' $\lambda = 0.93$, *F* (3, 32) = 0.76, $p = 0.53$). The SA bees were not more aggressive than the SI bees. The overall *F* test was also not significant for the presence of brood, Wilks' $\lambda = 0.90$, *F* (3, 32) = 1.17, $p = 0.34$.

Discussion

Through controlling social activity pairing we were able to isolate its separate effects on aggression and ovarian development. In *B. terrestris*, characteristic social interactions in a worker are associated with her aggressive behaviour (Bloch et al., 1996; van Honk & Hogeweg, 1981; van Honk et al., 1981). Similarly, in this study, pairs of socially active bees were significantly more aggressive than pairs with one or two socially inactive bees. In *B. terrestris*, the most socially dominant bee has more developed ovaries (Alaux et al., 2004) and aggression is believed to be used in this 'battle' over reproduction, with the most successful aggressor laying the eggs. In the socially active (SA+SA) pairs, aggression was the highest, suggesting increased competition between these bees. This aggression, however, did not translate into heightened reproduction since socially active pairs did not have significantly larger ovaries than the other groups. However, these results do not conclusively suggest that aggression has no role in reproduction. If there are two socially dominant workers who are taking part in a reproductive competition, for example, they both may be effective at suppressing reproduction in the other which translates to reduced ovarian development at the pair level. A slightly different view is that the aggression may represent a competition to determine who is the first to lay eggs rather than who monopolizes egg-laying. The production of male eggs typically occurs during the end of the bumblebee colony life cycle (Bloch & Hefetz, 1999) and as such the earlier laid male eggs would have a better chance of being successfully reared before the death of the colony and the end of the season (Amsalem & Hefetz, 2011). Either way, our results show that social dominance is affected by the characteristics of a bee's nestmate.

It is curious that there was not a significant difference between socially active workers and their socially inactive partners, either in their ovarian development or in their levels aggression. Here, we suggest possible explanations in addition to the usual caution regarding interpretation of null results. If social activity is a signal of a bee's potential to 'win' the reproductive battle, SI bees are at a disadvantage compared to the SA bees and therefore are predicted to remove themselves from the competition. The socially active bees however did not have more developed ovaries. The lack of a significant difference in ovarian development within the SA+SI pairs may be due to the fact that there are only two bees in the group. Although orphaned pairs have been shown to maintain behavioural interactions consistent with larger orphaned groups (Amsalem & Hefetz, 2010), larger groups of queen-less *B. impatiens* have bigger differences in ovarian development compared to pairs, who are more evenly matched (Cnaani, Wong, & Thomson, 2007). Additionally, there is a positive correlation in egg-laying within pairs of workers; the more one worker lays eggs, the more her partner lays eggs (Sibbald & Plowright, 2013). This may mean that, at least under paired conditions, both workers can successfully rear eggs. It also suggests that having a partner may serve an activating role in another worker's ovaries rather than a suppressive one (as has been suggested in other insects, Lamba et al., 2007). Indeed, single workers have significantly reduced ovarian development compared to workers placed in groups and even a worker placed with a queen (Cnaani et al., 2002).

Ovarian development was greater in the pairs placed in nesting boxes without brood. The presence of brood therefore may have an inhibitory effect on reproduction. A similar association is also found in honeybees (Jay, 1972) as well as in ponerine ants (Heinze, Trunzer, Oliveira, & Hölldobler, 1996). Such an inhibiting effect may be due to a type of pheromone release (Jay, 1972) or alternatively, brood presence may cue rearing behaviours which interrupt reproduction. Indeed, when workers are removed from a colony the remaining workers take on more feeding responsibilities (Pendrel & Plowright, 1981). Since there are only two workers placed with brood there would be increased feeding responsibilities compared to larger groups. Future research with larger queen-less groups would help to clarify this association between brood presence and reproduction as it may reduce the increased feeding responsibilities placed on the individual workers. Nevertheless, since brood absence increases both aggression and ovarian development, these findings suggest these two variables are associated in some way or there is a third variable that affects both (e.g. hormonal activity, Pomeroy, 1981).

This study is distinctive from other research as the social conditions were experimentally manipulated (through pairing workers with sisters of varying degrees of social activity). Environmental conditions were also manipulated through placing pairs either with or without brood. Experimental manipulation of reproductive competition within bumblebee research is the exception rather than the rule, with most research focusing on correlational data only (e.g. Bloch et al., 1996; Foster et al., 2004; van Doorn & Heringa, 1986). Additionally, exceptionally long periods of observation (~1000 hours) ensured that the behaviours observed were likely representative of the interactions that occurred within each pair. The findings of this study emphasize the complex nature of aggression and reproduction within worker *B. impatiens*. The absence of brood increased both aggression and ovarian development in pairs, which suggests the latter two variables are associated in some way. The findings however cannot make specific conclusions about the characteristics of the relationship and cannot exclude the possibility that a there is a third variable that may affect both. Socially active pairs were more aggressive but did not have larger ovaries and socially active workers were not more aggressive nor had increased ovarian development than their socially inactive counterparts. As such, aggression is not necessary for ovarian development as has been suggested in other insects (Lamba et al., 2007). Further research with larger groups examining the relationship between social dominance and reproductive dominance in worker *B. impatiens* can help clarify the relationship in this species of bumblebee. Nevertheless, the findings of the present study underscore that ovarian development can be predicted by certain environmental conditions (e.g. the absence of larvae) and although social interactions can be a precursor to aggression it does not translate to increased ovarian development under all conditions.

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Figure 1. Mean aggression duration (log transformed) of orphaned *B. impatiens* workers for each social group with standard error bars. SA: Socially Active SI: Socially Inactive.

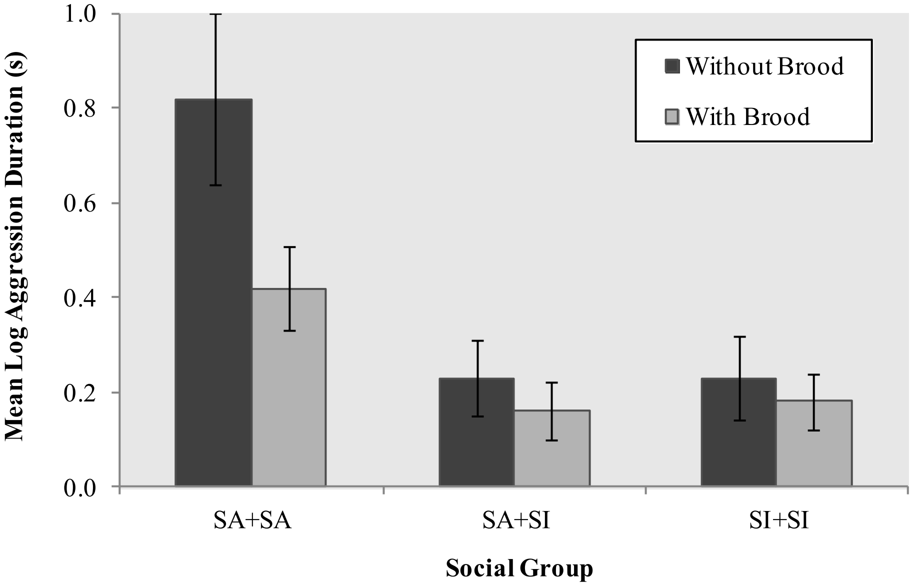


Figure 2. Mean aggression frequency (log transformed) of orphaned *B. impatiens* workers for each social group with standard error bars. SA: Socially Active   SI: Socially Inactive.

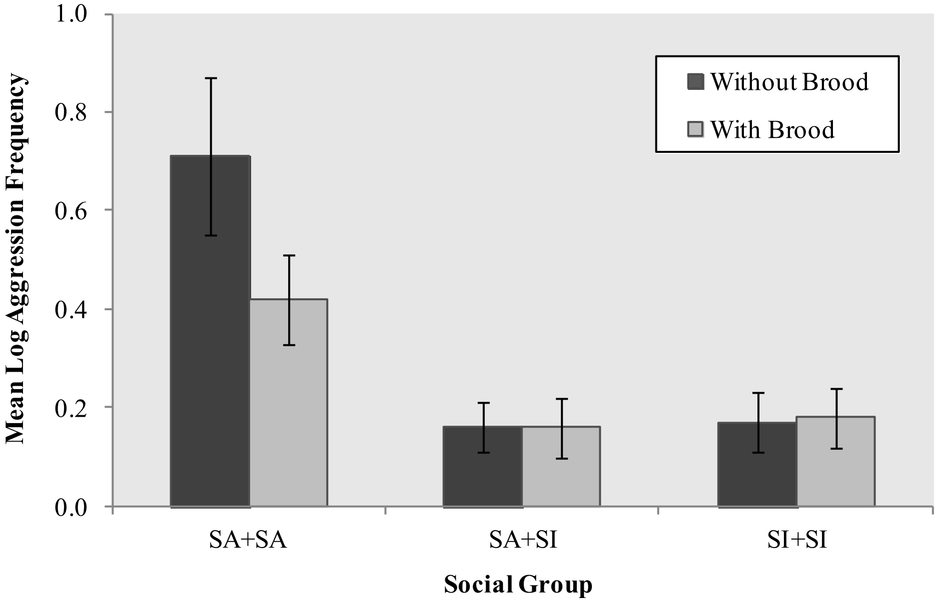
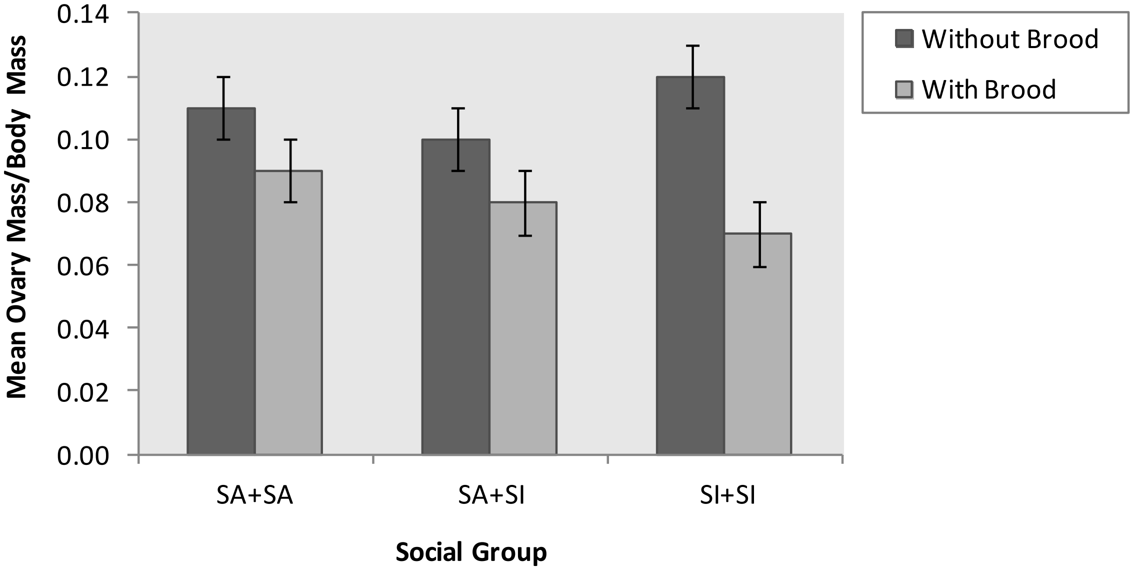


Figure 3. Mean ovarian mass (divided by body mass) of orphaned *B. impatiens* workers for each social group with standard error bars. SA: Socially Active SI: Socially Inactive.



Experiment #2 Addendum

Introduction

An extra condition was done as part of Experiment #2 that was not included in the manuscript. The condition was included to determine if aggression is observed when a worker is paired with a male. If aggression is indeed associated with reproductive control, pairing a worker with a male is predicted to reduce the degree of reproductive competition and as such, the frequency of aggression.

Methods

Workers and males

Bombus impatiens workers were collected from the colonies described in Experiment 2 between July and December, 2008. Ten *B. impatiens* males of unknown age produced from a pair of orphaned workers were also used. Workers and males were marked by gluing coloured, numbered tags onto the thorax (Opalit Plättchen, The Bee Works, Orillia, ON). They were supplied *ad libitum* 50% honey-water solution and fresh pollen collected by honeybees and mixed with honey-water solution to form uniform dough.

Apparatus

Workers were housed in the same manner as described in Experiment 2 (e.g. a wooden nest box contained within an incubator). All nest boxes were connected to a second same-sized box where a glass feeder tube was located. Glass plates covered the tops of the boxes to allow for easy observation. The laboratory was illuminated by a combination of fluorescent light fixtures and natural light.

Procedure

A socially inactive worker was placed with a male (*SI+Male*). Aggression and ovarian mass were measured.

Data analysis

The statistical analyses were performed using SPSS Statistics 20.

Results

A one-way analysis of variance was performed to compare the mean ovary mass between conditions (all without brood): SA+SI, SI+SI, and SI+Male pairs. Ovarian mass did not significantly differ by social group, F (2, 49) = 1.77, $p = 0.18$.

The dependent variables; aggressive frequency and duration, were highly positively skewed and their modes were at the extreme left of the distribution. As such, both variables were represented with a negative binomial distribution and a Generalized Linear Model (GLM) was performed. The main effect for social group was significant for both aggressive frequency (Wald χ^2 (2) = 7.23, $p < 0.05$) and aggressive duration (Wald χ^2 (2) = 12.53, $p < 0.05$). A simple contrast analysis showed the SI+Male group had a significantly lower mean frequency of aggression (Table 1) and aggression duration (Table 2) compared to the other two groups.

Discussion

If intra-species aggression is associated with worker reproduction, one would predict pairing a worker with a male would significantly reduce the aggression initiated by a worker. The results of this study support this hypothesis. One possible explanation for the lack of worker-male aggression was that the male was paired with a socially inactive worker. Nevertheless, the SI+SI group was significantly more aggressive (in both frequency and duration) compared to the SI+Male group.

Table 1. Simple Contrast Analysis of Mean Aggression Frequency

	$M_{SA+SI} = 0.70$	$M_{SI+SI} = 0.90$	$M_{SI+Male} = 0.05$
$M_{SA+SI} = 0.70$	0	-0.20	0.65 *
$M_{SI+SI} = 0.90$		0	0.85 *
$M_{SI+Male} = 0.05$			0

Note. * $p < 0.05$, ** $p < 0.001$

Table 2. Simple Contrast Analysis of Mean Aggression Duration

	$M_{SA+SI} = 1.85$	$M_{SI+SI} = 2.10$	$M_{SI+Male} = 0.05$
$M_{SA+SI} = 1.85$	0	-0.77	1.80 **
$M_{SI+SI} = 2.10$		0	2.05 **
$M_{SI+Male} = 0.05$			0

Note. * $p < 0.05$, ** $p < 0.001$

Experiment #3

Reproductive potential and its effect on aggression and egg-laying in worker bumblebee (*Bombus impatiens*) pairs

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Preface

The first two experiments manipulated characteristics thought to be associated with reproduction (e.g. aggression and brood presence). The final experiment was designed to manipulate reproduction to measure its effect on aggression. In this experiment, two variables were chosen to measure reproduction; oviposition and ovarian development.

Abstract

In eusocial insects the competition for reproductive rights has been hypothesized to be manifested through physical acts of aggression. This has not, however, been measured in the common North American species of bumblebee, *Bombus impatiens.* To measure the effect of reproductive potential (ovarian development) on aggression and egg-laying, the ovarian size of worker *B. impatiens* was manipulated. A paired system was used whereby a worker with 'encouraged' ovarian development (an 'E' worker) was paired either with another encouraged ovarian development worker (E+E pairs) or with a bee whose ovarian development was discouraged (a 'D' worker; E+D pairs). The E+E pairs were more aggressive suggesting there is an association between aggression and ovarian development in this species of bumblebee. The E+E pairs, however, did not have significantly larger ovaries or lay more eggs compared to the E+D pairs. Additionally, although the E workers of the E+D pairs had larger ovaries, they did not lay significantly more eggs or were more aggressive then their D worker partners. The results show that under these conditions, although ovarian development and aggression are associated, aggression is not necessary for egg-laying. Results also underscore that ovarian development is not necessarily an indicator of a worker's reproductive success.

Keywords: *Bombus impatiens,* bumblebee, queen-less workers, reproduction, aggression, egg-laying, ovarian development

Introduction

There are periods in the development of a bumblebee colony where intraspecies aggression occurs (*Bombus terrestris*, Bloch, 1999; *Bombus bifarius*, Foster, Brunskill, Verdirame, O'Donnell, 2004; *B. terrestris*, van der Blom, 1986). This worker-worker and queen-worker aggression occurs only within the context of worker oviposition (egg-laying) (Bourke, 1988a; Foster et al., 2004; van Honk & Hogeweg, 1981; van Honk, Röseler, Velthuis, & Hoogeveen, 1981; van Doorn & Heringa, 1986). As such it is hypothesized that aggression has a role in the competition for reproductive rights (Bourke, 1988a; Johnstone, 2000; Penick, Trobaugh, Brent, & Liebig, 2013). Indeed, bumblebee workers with more developed ovaries are more aggressive (*B. terrestris*, Alaux, Jaisson, & Hefetz, 2004a; Bloch, Borst, Huang, Robinson, & Hefetz, 1996; Duchateau, 1989; *B. bifarius*, Foster et al., 2004; *Bombus*, Pomeroy, 1981) and aggressive bees are more likely to lay eggs compared to non-aggressive workers (*B. terrestris*, Alaux et al., 2004a; van Doorn & Heringa 1986). A similar pattern is also observed in other eusocial insects. In the monogynous queen-less ant (*Dinoponera quadriceps*) for example, egg-laying is performed by the most behaviourally dominant ant who maintains her position through agonistic interactions. This behaviourally dominant ant is often overthrown whereby through intense aggressive interactions, a new worker becomes the main egg-layer (Monnin & Peeters, 1999). Similarly, if the main egg-layer of a queen-less group of the ant, *Harpagoxenus sublaevis*, is removed from the nest, another worker will establish herself as the reproductively dominant individual (Bourke, 1988b). The reintroduction of the reproductive worker induces worker aggression and an interesting relationship between aggression and reproduction is noted: if the reintroduced worker is the recipient of aggression upon reintroduction, she is more likely cease egg-laying. Conversely, if she initiates aggressive attacks upon reintroduction then she is more likely to remain egg-laying (Bourke, 1988b).

The role of aggression in reproduction in the common North American species of bumblebee, *Bombus impatiens*, may not be as straightforward as it is in other bumblebee species. In research involving pairs of orphaned *B. impatiens* workers, for example, the more aggressive bee of a pair did not lay significantly more eggs than the less aggressive bee of the pair (Sibbald & Plowright, 2013a). Additionally, although two socially dominant workers (defined as a worker that initiates more non-aggressive social interactions than she retreats from) displayed increased aggression compared to pairs consisting of at least one socially submissive worker, the socially dominant workers do not have more developed ovaries (Sibbald & Plowright, 2013b). These results may suggest aggression serves a different function in this context. Indeed, aggression does serve other purposes in other eusocial insect species (e.g. to encourage foraging (*Ropalidia marginata*, Lamba, Chandrasekhar, & Gadagkar, 2008; *Polybia occidentalis,* O'Donnell, 2001) or other nest duties (*Polistes fuscatus,* Sumana & Starks, 2004)), which may be due to the different evolutionary constraints in which the different species evolved (*Ropalidia*, Deshpande, Sumana, Surbeck, & Gadagkar, 2006; Kardile & Gadagkar, 2002). It may also suggest that the relationship between aggression and reproduction is more complex in the context of two *B. impatiens* workers compared to larger groups. There continues to be a division of labour in queen-less colonies, with some workers foregoing reproduction to ensure the maintenance of the colony since reproductive workers spend more time performing social dominance behaviours (Gobin, Heinze, Strätz, & Roces, 2003) and do little else for the care and maintenance of the colony (Bourke, 1988b; Endler, Holldobler, & Liebig, 2007). In pairs, however, there may be no need for a division of labour since these worker pairs have unlimited food resources. As such, both may be able to successfully reproduce. Aggression in *B. impatiens*, however, continues to be observed under paired conditions (Sibbald & Plowright, 2013a; Sibbald & Plowright, 2013b) and it is more likely to happen within the context of egg-laying (Sibbald & Plowright, 2013a). It may be that a worker attempts to assert her reproductive dominance but as the *B. impatiens* workers are more equally matched with regards to reproductive capabilities, the aggression is not effective. Indeed, aggression has been shown to be ineffective in some cases. Queen aggression directed at egg-laying *Bombus* workers, for example, is not effective at suppressing reproduction since a portion of males are produced by the workers (*B. terrestris*, Alaux, Savarit, Jaisson, & Hefetz, 2004b; *Bombus melanopygus*, Owen & Plowright, 1982). Additionally, the most aggressive worker is not necessarily the predominant egg-layer (*B. terrestris*, Duchateau, 1989). Regardless of whether ovarian development is significantly different in pairs, ovarian development is not a direct indicator of oviposition since a portion of workers in queen-right colonies have developed ovaries but do not lay eggs (*B. terrestris*, Duchateau & Velthuis, 1989). Therefore, although both workers could have developed ovaries there may still be a reproductive division of labour in groups of two.

One way to investigate the association between aggression and oviposition in *B. impatiens* is to encourage the ovarian development of a worker prior to pairing. As such, through manipulating a worker's ovarian development (i.e. reproductive capabilities) it is predicted that one can create a worker that is more reproductively 'advantaged' over her partner. Creating pairs that are more unbalanced with respect to reproductive potential may result in less aggression if it is an indicator of reproductive competition in this species of bumblebee. As a worker is more related to her sons than her nephews (Bourke, 1988b), it is hypothesized that a worker will continue to prioritize the production of sons versus nephews under these conditions (Foster & Ratnieks, 2001) and display aggression under conditions when both workers have developed ovaries.

The present study was designed to experimentally manipulate the ovarian development (i.e. the reproductive potential) of *Bombus impatiens* worker pairs to measure its effect on aggression and reproduction. This study was performed to address the following questions: (1) Does pairing orphaned worker bumblebees according to their levels of ovarian development affect their levels of aggression? and (2) Does it also affect their egg-laying?

Methods

Colonies and workers

Three commercial *Bombus impatiens* colonies were supplied by BioBest, Biological Systems and one *B. impatiens* colony derived from a wild-caught queen and reared in the laboratory according to the procedures developed by Plowright and Jay (1966) were used. One hundred and six workers that were within 12 hours of hatching from their cocoon (defined as 'callow' workers; bees with curved wings and without full colouration) were selected from the colonies and identified with coloured, numbered tags (Opalit Plättchen, The Bee Works, Orillia, ON) attached to the thorax. Workers were tested between June and November, 2010. All workers and colonies were fed 50% (by volume) honey-water solution as well as pollen lumps (pollen collected by honeybees mixed with honey-water to form a dough-like mixture).

Encouraged Ovarian Development Workers ('E Workers')

To encourage ovarian development, a modified protocol derived from Alaux, Boutot, Jaisson, & Hefetz (2007) was used. Specifically, the tagged callow workers were returned to their natal colonies for 48 hours to allow for maturation. After the 48 hours, the workers were removed from the colony and placed with a callow sister worker. The tagged worker was placed with the younger worker as older workers are more likely to be dominant egg-layers in queen-less groups (*B. terrestris*, Alaux et al., 2007). The pair remained together for nine days to allow for ovarian development as previous findings observed it takes an average of nine days for orphaned pairs to lay eggs (Sibbald & Plowright, 2007, unpublished results).

Discouraged Ovarian Development Workers ('D Workers')

To discourage ovarian development, after the 48 hours of maturation in their natal colony, the tagged workers remained in the colony for an additional nine days. Workers placed with a queen or in colonies have reduced ovarian development compared to queen-less groups (*B. terrestris*, Duchateau & Velthuis, 1989). Workers were kept in the colony for an additional nine days to ensure that the discouraged ovarian development workers were the same age as the encouraged ovarian development workers and therefore any differences in ovarian development were not due to differences in age.

Apparatus

Once removed from their colonies, the workers were housed in wooden nesting boxes (10.2 cm x 10.2 cm x 5.1 cm) that were located in an incubator that maintained warm (30^{o}C) and humid conditions (through a fan circulating air over a water basin). The nest box was lined with honeybee wax to produce a substrate for egg-cup building. The box was connected to another nesting box containing a feeder tube. Both boxes were covered with glass plates to allow for observation. The laboratory was illuminated by a combination of natural and fluorescent light. Digital video recordings were made on a Vivotek IP8161 fixed network camera positioned directly over the nest box.

Experimental Condition

After the period of ovarian development encouragement (E workers) or discouragement (D workers), the bees were paired in a new nest box under one of the following conditions: (1) An encouraged ovarian development worker paired with another encouraged ovarian development worker (E+E pairs), (2) an encouraged ovarian development worker and a discouraged ovarian development worker (E+D pairs). There were 10 pairs per condition for a total of 20 pairs. All workers in each pair were the same age (both hatched within 12 hours of each other) and from the same colony.

Control Condition

To determine if the conditions used to encourage ovarian development and egg-laying were effective, 12 E (encouraged ovarian development) workers and their callow partners were dissected after their nine days of pairing to determine their ovarian development. These pairs were also video recorded for seven hours a day for nine days. Ovipositions were coded in the videos to determine the identity of the egg-layer.

To determine the ovarian size of workers whose ovarian development was discouraged, 12 D workers were dissected after eleven days. At eleven days, D workers would be of comparable age to the E workers (i.e. 48 hours within the colony plus nine days of testing).

Procedure

The recordings were started immediately after pairing. Recordings were randomly started in the morning (defined as anytime between 7.30 h -10.30 h), afternoon (12.30 h - 15.30 h) and evening (17.30 h – 20.30 h) and continued for seven hours. The different recording start times were selected to ensure any group differences in aggression or reproduction were not due to circadian rhythm differences. The pairs were recorded for approximately seven hours a day for seven days. There were 977 hours of coded video. The frequency and duration of aggression as well as the frequency of oviposition were coded. Due to the considerable number of hours of videos to code (approximately 49 hours per pair), the computer program ObjectTracker was used to crop the video to short clips where two bees were within close proximity of one another. This was employed to increase the efficiency of the coding as the computer program created two different video files; one that contained all occurrences when two bees were in close proximity and a second video file that contained all times when the bees were in not close proximity (and therefore cannot be aggressive). Four coders were trained to identify aggression following a statistically reliable training method (Sibbald & Plowright, 2013a). All coders were blind to the experimental condition of the workers. As bees do not have to be within close proximity of one another to oviposit, the ObjectTracker program could not be used and egg-laying was coded through manually analyzing the video at 16x normal speed to identify potential oviposition events. When a potential oviposition was identified, it was watched at normal speed to confirm the event. The 16x normal speed is slow enough for the coder to identify egg-laying acts but would be too fast to identify aggression, hence the use of ObjectTracker in conjunction with fast-forward.

Measures and Behavioural Definitions

Oviposition was identified using Bloch and Hefetz's (1999) definition, which has shown a significant inter-observer correlation in *B. impatiens* (Sibbald & Plowright, 2013a). Appendix B3 contains an example of an oviposition. Aggression consists of butting and grappling behaviours (Sibbald & Plowright, 2013a). Oophagy was also measured, which is defined as the destruction of egg-cups and consumption of eggs (Alaux, et al., 2004a; Cnaani, Schmid-Hempel, & Schmidt, 2002; Owen & Plowright, 1982; Ratnieks, 1988). This behaviour has been found to be a reliable indicator of overt conflict (Bloch & Hefetz, 1999; van der Blom, 1986). The initiator of the behaviour was identified and the duration of each act was measured. There was a significant inter-observer correlation between coders using this definition (Sibbald & Plowright, 2013a). Examples of butting, grappling, and oophagy are found in Appendix B1, B2, and B5 respectively.

To measure ovarian development the ovaries were dissected within 24 hours of the final day of testing. They were weighed on a Mettler-Toledo balance (PB 303). Ovarian mass was divided by body mass.

Data analysis

The statistical analyses were performed using SPSS Statistics 20. A combination of parametric and nonparametric tests was used. Both types of statistical tests were selected because there was a combination of normally distributed and not normally distributed dependent variables. Most of the aggression variables, for example, were positively skewed. Nonparametric statistical tests were chosen for such variables as these tests provide powerful and accurate results for small datasets that are not normally distributed (Mehta & Patel, 2011). Multiple statistical tests were performed which can warrant a Bonferroni adjustment as it corrects for the increased chance of significance when conducting multiple tests. However, there is increased criticism of the adjustment (Moran 2003; Nakagawa 2004; Perneger 1998) and as such the following *p*-values are presented without a Bonferroni correction.

Results

Control Condition - Encouraged ovarian development validation

An Analysis of Variance was performed to determine if E workers have more developed ovaries than their callow partners and D workers. Figure 1 shows a significant group difference on ovary development, $F(2, 35) = 49.62$, $p < 0.0001$. Post-hoc comparisons using a Tukey's HSD test revealed the D workers had significantly smaller ovaries than E workers and callow workers, $p < 0.0001$. There was no significant difference, however, in ovary size between E workers and callow workers, $p = 0.11$. With respect to oviposition, a Mann-Whitney U test showed the E workers laid more eggs (mean rank = 15.88) than callow workers (mean rank = 9.13), $U = 31.5$, $p < 0.05$ (Figure 2).

Experimental Condition – Differences between E+E and E+D pairs

A Mann-Whitney U test shows E+E pairs were significantly more aggressive than E+D pairs as measured by oophagy (mean rank $_{\text{E+E}} = 23.43$, mean rank $_{\text{E+D}} = 17.58$), $U = 141.5$, $p < 0.05$ (Figure 3). There were no significant group differences in butting frequency, $U = 188.5$, $p = 0.73$ (Figure 4a), butting duration, $U = 188.00$, $p = 0.72$ (Figure 4b), grapple frequency, $U = 158.00$, $p = 0.22$ (Figure 4c), and grapple duration, $U = 145.50$, $p = 0.11$ (Figure 4d). Although there is not a significant group difference in these variables of aggression, the mean rank suggests there may be a similar trend as the E+E pairs had a higher mean rank (e.g. grapple frequency = 22.60; grapple duration = 23.23) than the E+D pairs (grapple frequency = 18.40; grapple duration = 17.77). Descriptively, E+E pairs also displayed very long grapples (up to 27 minutes in some cases) compared to E+D pairs (the longest being 1.28 minutes).

With respect to reproduction, independent samples t-tests displayed no significant group differences in ovary mass $t\ (38) = 1.24$, $p = 0.22$ or oviposition $t\ (38) = -0.32$, $p = 0.75$.

Experimental Condition – Differences within E+D pairs

To determine if there are differences within the pairs, it was determined if E workers were more aggressive and/or reproductive than D workers. The ten E+D pairs were used for these analyses. Wilcoxon tests indicated E workers were not significantly more aggressive than their D worker counterparts (oophagy $Z = -1.34$, $p = 0.18$, butting frequency $Z = -0.65$, $p = 0.52$, butting duration $\mathrm{Z} = -0.65$, $p = 0.52$, grapple frequency $Z = -0.21$, $p = 0.83$, or grapple duration $\mathrm{Z} = -0.52$, $p = 0.60$). A paired samples t-test showed, however, that the E workers had more developed ovaries ($M = 0.11$, $SE = 0.01$) than D workers ($M = 0.05$, $SE = 0.01$), $t\ (9) = 6.35$, $p < 0.0001$ (Figure 5) but E workers did not lay more eggs, $t\ (9) = 1.91$, $p = 0.09$.

Discussion

Workers with developed ovaries are predicted to be at a reproductive advantage compared to workers with undeveloped ovaries. If aggression is used in a ‘battle’ over reproductive dominance, pairs who are more matched with regards to reproductive ability would be expected to have increased conflict over reproductive rights and as such would differ on levels of aggression compared to pairs where the reproductive potential is more unmatched (i.e. an encouraged ovarian development worker + a discouraged ovarian development worker). The protocol to encourage ovarian development in a worker was effective as the encouraged ovarian development (E) workers had larger ovaries than the discouraged ovarian development (D) workers prior to pairing. This difference was maintained over the seven days of testing as the E workers continued to have significantly larger ovaries compared to their D worker partners.

Between pairs, the encouraged ovarian development (E+E) pairs were more aggressive than the E+D pairs. This suggests there is an association between ovarian development and aggression in *B. impatiens* worker pairs (as is observed in *B. terrestris* pairs (Amsalem & Hefetz, 2010)). Also, it could be consistent with the hypothesis of reproductive competition as the E+E pairs would be more competitive for reproductive rights as both are reproductively able compared to E+D pairs. Additionally, the E+E pairs did not lay more eggs or have larger ovaries. This may also represent reproductive competition as it can indicate that both workers are competing to suppress reproduction in the other, with one worker being no more effective than her partner.

With the E+D pairs, the E workers were not more aggressive than the D workers. This may be unsurprising as one worker is already at a reproductive advantage over her partner. In other eusocial insects, for example, once the main reproductive individual is established, aggression is no longer observed by the predominant egg-layer (*Streblognathus peetersi*, Cuvillier-Hot, Lenoir, Crewe, Malosse, & Peeters, 2004; *Ropalidia marginata*, Premnath, Sinha, & Gadagkar, 1996), which may be the result of a switch from behavioural enforcement to chemical signalling (Cuvillier-Hot, Gadagkar, Peeters, & Cobb, 2002;

Cuvillier-Hot, Lenoir, Crewe, Malosse, & Peeters, 2004). If one looks at the measures of ovarian development, the results could support this hypothesis as the E workers had bigger ovaries. When one looks at the rates of oviposition, however, the encouraged ovarian development workers did not lay more eggs and as such, were not more reproductively successful. In sum, even when one worker is at a reproductive advantage prior to pairing, there was no reproductive dominance. This may be because both workers can successfully rear offspring under conditions of food *ad libitum*. In larger queen-less groups of *B. impatiens*, for example, there is a variation in ovarian development as only a few workers have developed ovaries with the rest having immature ovaries (Cnaani, Wong, & Thomson, 2007). In pairs, however, there was less of a difference in ovary development between the bees as the worker with the smaller ovaries still has larger ovaries than the bees with the smaller ovaries in groups consisting of 3, 4, 6, or 12 workers (Cnaani et al., 2007). Of note, one worker can have significantly larger ovaries than her partner in *B. terrestris* pairs (Amsalem & Hefetz, 2010) suggesting there may be species differences. Although it may be at a worker's genetic advantage to produce sons rather than nephews (Bourke, 1988b), if both workers can produce offspring, it may be unnecessary to partake in behaviours (e.g. aggression) that reduce the success of the group or colony (Hammond & Keller, 2004; Monnin & Ratnieks, 1999). Therefore, if both can successfully reproduce, without costs to the success of rearing offspring, there is no need to partake in a reproductive competition. Still, as there are group differences in aggression, it may be that aggression between two reproductively able workers functions to determine who is the first to lay eggs rather than who asserts reproductive dominance (Amsalem & Hefetz, 2011). Indeed, as bumblebee males can reproduce multiple times, those males who are produced earlier will likely mate with more queens (Beekman & Oldroyd, 2008).

The results of the present study highlight that ovarian development is not necessarily an indicator of egg-laying in *B. impatiens*. Although the encouraged ovarian development workers had significantly larger ovaries than their discouraged ovarian development partners in the E+D condition, they did not lay more eggs. Additionally, within the control condition, although the encouraged ovarian development workers laid more eggs than their callow partners, they did not have significantly larger ovaries. It may be that ovarian mass is not an effective indicator of ovarian development since the worker with the smaller ovaries can still lay eggs. Ovarian mass, however, has been used as a valid measure of ovarian development in the bumblebee since it can reliably differentiate groups (Fisher, 1983; Pomeroy, 1981; Sibbald & Plowright, 2013b). Therefore, if one bee does have more developed ovaries than her partner, the results of the present study may suggest that control over reproduction cannot be maintained in pairs. Indeed, Cnaani et al. (2007) did observe that the difference in ovarian development between pairs of *B. impatiens* was not as great as the differences between workers in larger queen-less groups. Even though the encouraged ovarian development workers from the control condition laid more eggs than their callow partners, the callow workers were only nine days old at the end of testing. Considering it takes a worker approximately seven days to start egg-laying, this does not conclusively suggest reproductive dominance can be maintained in these pairs, although age has been shown to be a strong indicator of reproductive dominance in the bumblebee (*B. terrestris*, Bloch, et al., 1996; Bloch & Hefetz, 1999; *Bombus,* Pomeroy, 1981) as well as in other insects (*Polistes instabilis*, Hughes & Strassmann, 1988). Nevertheless, the results of the present study indicate that although ovarian development is often used as a measure of reproductive dominance (Amsalem & Hefetz, 2011; Jandt & Dornhaus, 2011; Malka, Schnieor, Katzav-Gozansky & Hefetz, 2008) since it is associated with it (*B. terrestris*, Bloch & Hefetz 1999), most workers with developed ovaries do not lay eggs. It is clear that more than just developed ovaries are necessary for oviposition, rather there is a determination made, perhaps on the part of the worker, regarding when to reproduce or when not to reproduce (Alaux et al., 2007; Beekman & Oldroyd, 2008).

The present study is distinctive as it experimentally manipulated ovarian development to measure its effect on aggression and egg-laying in an eusocial insect. The results indicate the manipulation of reproductive capability (i.e. ovarian development) does affect aggression but does not guarantee a worker bumblebee will produce more sons than her partner. It may be that under conditions of unlimited food, both workers can successfully reproduce and hence reproductive competition is not necessary. Instead the competition lies in who is the first to lay eggs so that they can be successfully reared in time to mate with virgin queens (Amsalem & Hefetz, 2011). Further research to determine if the most aggressive worker produces offspring before the least aggressive bee can help elucidate this suggestion. Nevertheless, the present study highlights ovarian development is not necessarily an indicator of egg-laying in worker *B. impatiens* and that under these conditions, ovarian development is not necessarily a predictor of aggressiveness.

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Figure 1. Control condition: Comparison of mean ovarian development (ovarian mass divided by body mass, with standard error bars) by encouraged ovarian development workers, their callow partners, and discouraged ovarian development workers.

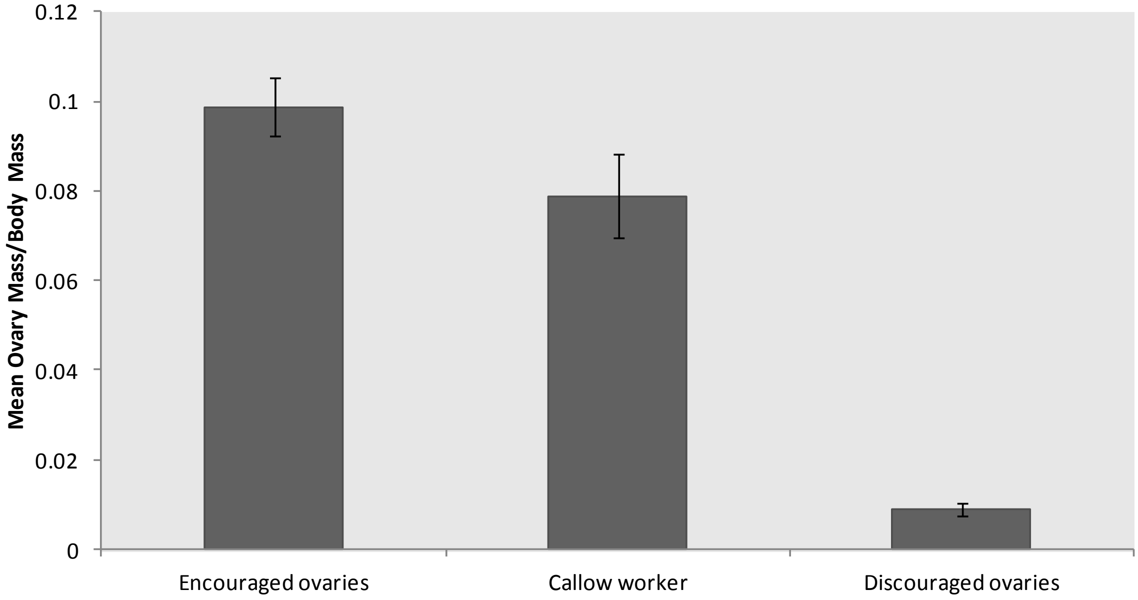


Figure 2. Control condition: Comparison of mean oviposition (with standard error bars) in encouraged ovarian development workers and their callow partners.

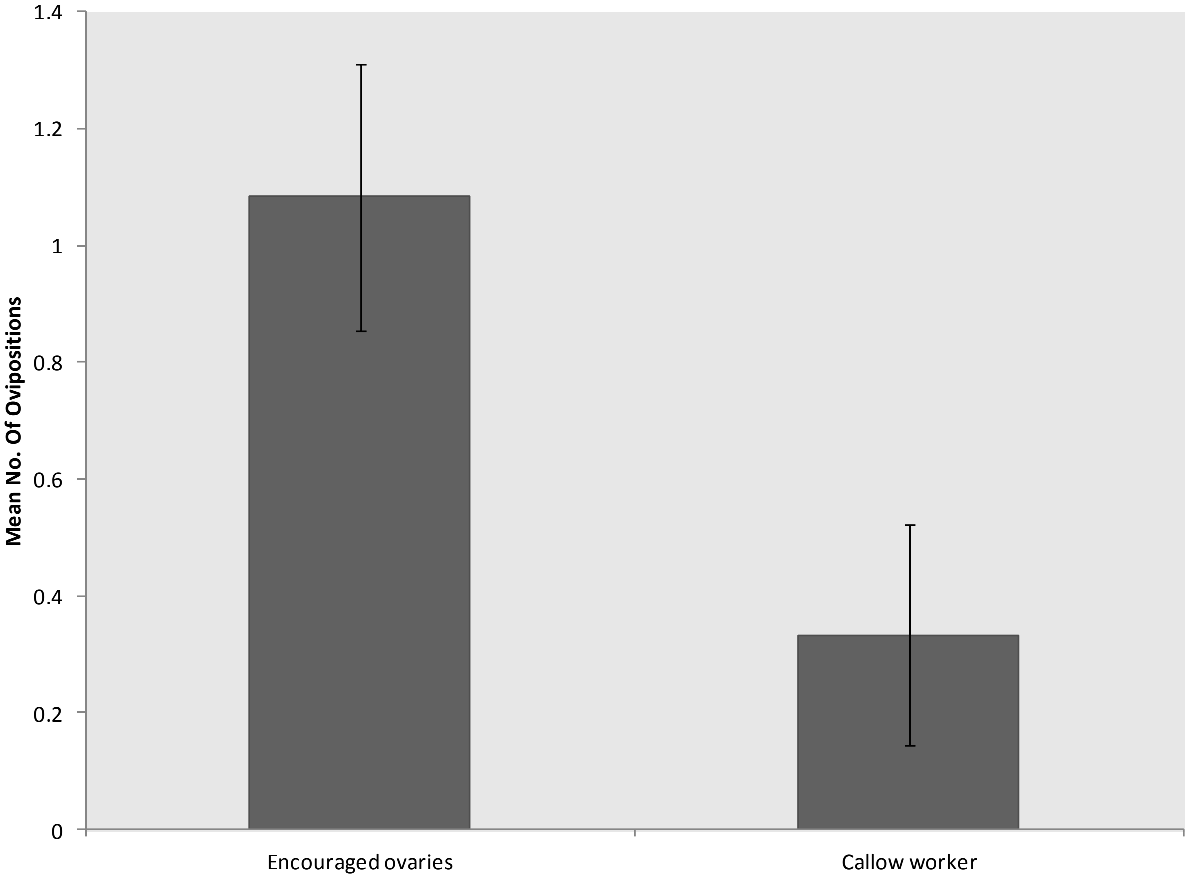


Figure 3. Experimental condition: The contrast of aggression (mean oophagy with standard error bars) between E+E worker bumblebee pairs and E+D worker pairs.

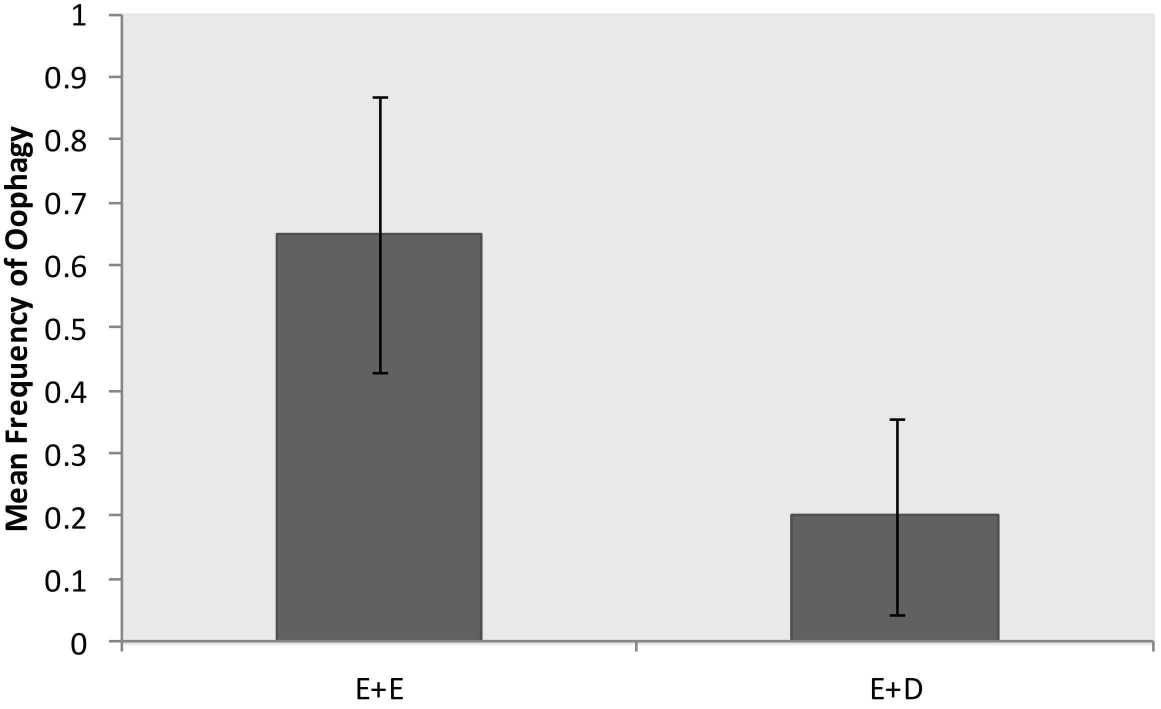
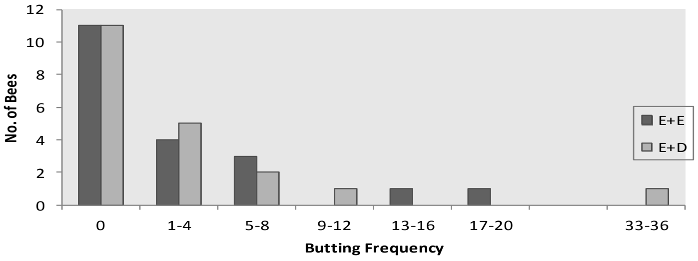
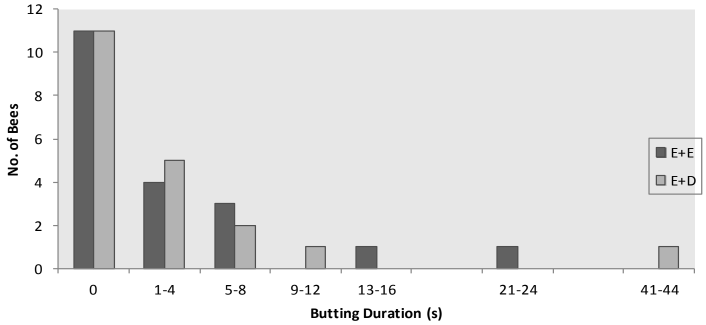


Figure 4. Experimental condition: The frequency distribution of aggression between E+E worker bumblebee pairs and E+D pairs.

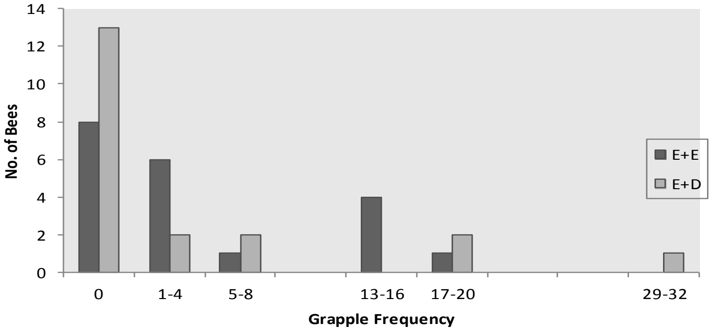
(a) Butting Frequency



(b) Butting Duration



(c) Grappling Frequency



(d) Grappling Duration

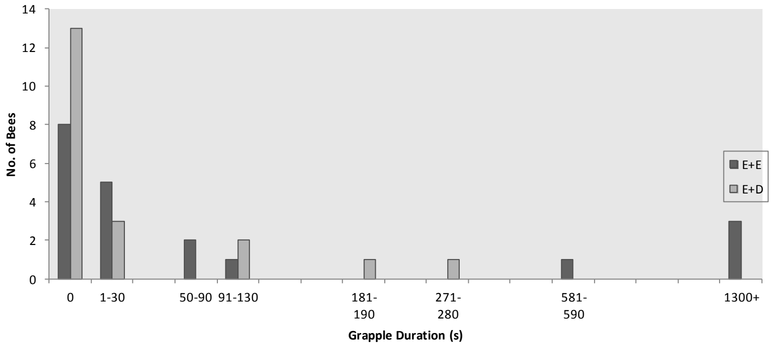
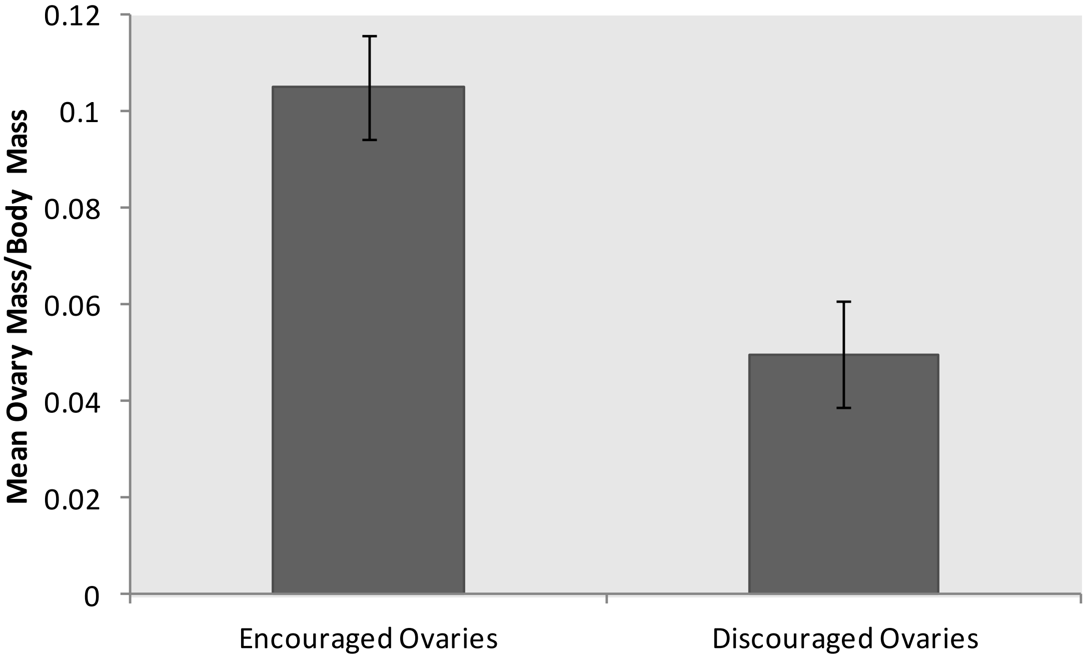


Figure 5. Experimental condition: Differences in ovarian development between E and D orphaned worker bumblebees in the E+D paired condition.



General Discussion

As concisely stated by Bourke (1988), "...worker reproduction is crucial to our understanding of hymenopteran eusociality" (p. 291) and understanding its relationship with aggression assists in defining dominance in the bumblebee. Results from the first experiment found a complex relationship between aggression and reproductive control in worker pairs, with a positive correlation in aggression between members of a pair as well as a positive correlation in rates of oviposition between members of a pair (i.e. the more one worker laid eggs, the more her partner laid eggs). Research with *Bombus terrestris* pairs found aggression and ovarian development were positively correlated (Amsalem & Hefetz, 2010); the most aggressive worker has more developed ovaries. This association was not found in our first experiment with *B. impatiens*. Results from the third experiment supplemented our findings from the first study through experimentally manipulating ovarian development in workers and measuring its effect on aggression and egg-laying. The supposition that aggression has a role in reproductive control was partially supported as pairs consisting of workers with developed ovaries upon pairing were more aggressive than pairs consisting of at least one worker with immature ovaries. Indeed, aggressive workers are more likely to have developed ovaries (Duchateau, 1989; Free, 1955; Pomeroy, 1981; van Doorn, 1989). However, *B. impatiens* workers whose ovarian development was encouraged prior to pairing did not lay more eggs than their partners whose ovarian development discouraged. Reasons for such discrepancies were discussed in the final experiment.

To further define dominance in the bumblebee, the second experiment examined the association between social interactions (believed to be a part of behavioural dominance, Bourke, 1988a; Gadagkar, 2009) and aggression and ovarian development. Results showed that when two socially active bees were paired they were more aggressive than pairs consisting of one or two socially inactive bumblebees. The results of the second experiment were consistent with other reports of the association between aggression and social interactions (in *B. terrestris*, Bloch, Borst, Huang, Robinson, & Hefetz, 1996; van Honk & Hogeweg, 1981; van Honk, Röseler, Velthuis, & Hoogeveen, 1981). The findings were only partially consistent, however, since the second experiment did not find a significant difference in ovarian development between socially active pairs and socially inactive pairs. Research involving larger groups of *B. terrestris* has suggested an association between social activity and ovarian development (Alaux, Jaisson, & Hefetz, 2004; Bloch et al., 1996). Reasons for the differences in results were discussed in the second experiment.

Brood presence was predicted to have a significant role in reproductive control. Results from the first experiment found the presence or absence of brood affected oviposition and aggression since the behaviours were more likely to co-occur in the absence of brood. Results from the second experiment found the absence of brood increased rates of aggression and ovarian development in pairs. This may be different from other species (such as the ant) where such behaviours are more likely to occur in nest areas where brood are present (*Leptothorax*, Cole, 1988). Reasons for these differences were discussed in the first and second experiments.

Kin selection theory (Hamilton, 1963) and the haplodiploid genetic system of sex determination may not be sufficient to explain the evolution of eusociality (Wilson & Wilson, 2007). Nonetheless, they provide an understanding of conditions when it may no longer be advantageous in terms of inclusive fitness for a worker to remain reproductively altruistic. Although this theory suggests when such situations may occur, it does not elucidate how it will occur or what differentiates a reproductive worker from her non-reproductive sister. The results of this thesis show aggression is ‘about’ reproduction in that two workers with developed ovaries were more aggressive (Experiment 3). The results also suggest aggression is 'about' reproductive competition as workers placed with a male were significantly less aggressive (Experiment 2-Addendum). This competition may even be successful to a limited extent since there was typically one predominant egg-layer per pair (Experiment 1). Nevertheless, the results do not suggest that the most aggressive bee of a pair lays more eggs (Experiment 1). The reasons for this are explored in the discussion sections of each experiment, however, there are a few hypotheses, specifically: (1) aggression is used to assist a worker in developing her ovaries (Lamba, Kazi, Deshpande, Natesh, Bhadra, & Gadagkar, 2007), (2) it represents the competition to determine which is the first to lay eggs (Amsalem & Hefetz, 2011), or (3) it is a 'last ditch' effort to obtain reproductive control. All three of these hypotheses pertain to reproduction and are not mutually exclusive. The data of this thesis cannot exclude these suppositions as potential explanations and as such, each hypothesis will be further explicated.

Aggression functions to encourage ovarian development

In the primitively eusocial wasp, *Ropalidia marginata*, worker aggression is hypothesized to be used to assist the worker in rapidly developing her ovaries (Gadagkar, 2009; Lamba et al., 2007). The evidence for this supposition is based on the observations that (1) isolated workers have significantly reduced ovarian development compared to those in groups (Lamba et al., 2007) and (2) the most aggressive worker is not the main egg-layer (Gadagkar, 2009). Similar findings are also observed in the *B. impatiens*; isolated workers have significantly reduced ovarian development (Cnaani et al., 2002) and the most aggressive worker in worker pairs is not always the predominant egg-layer (Experiment 1). However, it has been observed that *B. impatiens* workers placed with a male were also significantly less aggressive compared to worker-worker pairs (Experiment 2-Addendum). It stands to reason that a worker could continue to display aggression towards her male partner and hence encourage her ovarian development. This is not observed, however, and as such, may suggest aggression is not used to activate a *B. impatiens* worker's ovarian development under these conditions.

Aggression functions to determine which is the first to lay eggs

Since the bumblebee colony has an annual life cycle, it is hypothesized there would be competition between reproductive workers to produce males early so they would have the opportunity to mate with virgin queens before the end of the season (Alaux et al., 2006; Amsalem & Hefetz, 2011). This hypothesis could account for the lack of aggression observed in the encouraged ovarian development + discouraged ovarian development worker pairs (i.e. one worker has been identified as the reproductive 'loser'). Although temporal changes in aggression were not assessed in the experiments of this thesis, aggression was observed throughout the entire testing period in all experiments. Research measuring temporal changes in aggressive behaviour would be beneficial to further examine this hypothesis. Nevertheless, this hypothesis only serves as a partial explanation, since one may conclude that reproductive competition would also involve maximizing the number of eggs laid as well (Amsalem & Hefetz, 2011). This supposition highlights the possibility that reproductive timing may be as important as reproductive output, insofar as it affects the probability of reproduction by male progeny. Nonetheless, speed is not necessarily of the essence; producing males too early, before the emergence of queens from other colonies, may be just as costly as producing them too late.

Aggression functions as a 'last ditch' attempt to assert reproductive control

Aggression serves some role in reproductive control since oophagy reduces the number of eggs laid by another, aggression and oviposition are more likely occur on the same day in pairs without brood (Experiment 1), and *B. terrestris* research as well as research with other eusocial insects has found evidence supporting the supposition (Amsalem & Hefetz, 2011; Bourke, 1988a; Johnstone, 2000; Penick et al., 2013). Nevertheless, the findings of this thesis show that aggression is not only employed by the reproductive 'winner' but by the 'loser' as well. As such, a worker may use aggression as a form of a last ditch attempt to control the reproduction of another when other forms of reproductive control (e.g. social interactions or pheromones) are no longer effective. For most of the *Bombus* colony life cycle, workers do not reproduce and worker-worker and queen-worker aggression is not observed. However, when workers start reproducing, aggression can be observed and although the queen is aggressive towards her egg-laying workers, she is not effective at suppressing their reproduction as a proportion of males produced are worker derived (Alaux et al., 2004; Brown et al., 2003; Owen & Plowright, 1982; Paxton et al., 2001). The explanation for this phenomenon is that the queen is no longer able to control her worker's reproductive behaviour (Fletcher & Ross, 1985, although it has also been suggested that worker reproductive behaviour is due to decision-making on the part of the worker that reads signals from the queen rather than queen-controlled suppression, (Alaux et al., 2004)). As such, worker aggression may also be a final, often ineffective, attempt to gain reproductive control.

The methodological design of these experiments successfully captured the behaviours and physical attributes in question. Nonetheless, certain adjustments, which could have provided more information, are noted below. The improvements to the method include: (1) increasing the resolution of the digital recordings so the bees were better contrasted in the videos. Although the original resolution was sensitive enough for human trackers to code behaviours, changes in video resolution would have increased the sensitivity of the ObjectTracker software, making data collection more efficient and allowing for its use in earlier experiments. (2) Including the dependent variable of the amount of food consumed to better differentiate aggressive and/or reproductive bees from their counterparts. (3) Including the identity of the first egg-layer as an additional dependent variable. This could provide evidence regarding Amsalem and Hefetz's (2011) hypothesis that competition between two reproductively capable bumblebees functions to determine which is first to lay eggs rather than the quantity of eggs laid. (4) Including other measurements of ovarian development. As discussed in Experiment 3, although ovarian mass can reliably differentiate groups, other measures (such as the mean terminal oocyte length) have been regularly used to measure ovarian development in *Bombus* (Amsalem & Hefetz, 2010; Cnaani, Schmid-Hempel, & Schmidt, 2002) and may be more sensitive to quantifying ovarian development differences. (5) Finally, recording the animals under red light. Although bumblebees that perform mainly nest-tasks are not as influenced by diurnal rhythms as bees that perform mainly foraging tasks (*B. terrestris*, Yerushalmi, Bodenhaimer, & Bloch, 2006), nest conditions are typically underground and as such, in darkness.

Although using orphaned worker pairs was advantageous as it reduces the event of study to its fundamental components, the study of larger queen-less groups can provide more information regarding the relationship between reproductive dominance (i.e. ovarian development and egg-laying) and behavioural dominance (aggression and social interactions) in *B. impatiens*. A larger group size may increase competition for reproductive rights as more workers would be needed to perform other tasks such as foraging or caring for the brood. Indeed, results of this thesis show the presence of brood does reduce the frequency of aggressive interactions, suggesting care behaviours interrupt aggression. With respect to group size and ovarian development, a larger group (e.g. between 2 - 12 worker *B. impatiens*) can result in a greater dichotomy of ovarian development, with a smaller proportion of workers in the group having more developed ovaries (Cnaani, Wong, & Thomson, 2007). Cnaani, Wong, & Thomson (2007), however, could not capture enough aggressive or social interaction behaviours to include the data in their analysis. Research involving *B. terrestris* found the most aggressive workers of a group were more aggressive as group size increased (group sizes of 3, 5, and 10, Amsalem & Hefetz, 2011). Combined, these results suggest a reproductive division of labour increases as group size increases (Cnaani et al., 2007) with a corresponding escalation of aggression as well (Amsalem & Hefetz, 2011). This may suggest aggression is employed to maintain reproductive control, however, these findings cannot comment on causality. To gather more evidence regarding causality, a potential research design would involve both collecting more data of the test groups in order to capture aggressive behaviours and manipulating ovarian development (similar to the third experiment) and placing a larger number of encouraged ovarian development workers with a larger group of discouraged ovarian development workers. An experimental manipulation of the ratio of encouraged ovarian development workers to discouraged ovarian development workers in a larger group size may produce a greater incentive for a reproductive division of labour. This increased reproductive division of labour would also be predicted to coincide with an increase in behaviours hypothesized to be associated with reproductive competition; mainly aggression, if such behaviour is used to assert reproductive control.

Restricting resources or making foraging necessary for resource acquisition are other possibilities for future work. Orphaned pairs in this thesis were given unlimited access to food. As such, each worker could acquire her own resources without relying on her partner and without exerting much energy. This could suggest more time can be devoted to behaviours such as aggression and reproduction. Indeed, there is increased worker ovarian development and aggression in colonies supplemented with sugar solution. This is hypothesized to be due to reduced worker energy expenditure as foraging is not necessary (*B. terrestris*, Tod, 1986).

There are many avenues for further research with respect to reproductive control and the presence or absence of brood. For example, determining the causal nature of brood presence will help understand this relationship. Does brood presence affect aggression and reproduction because brood care activities merely interrupt other behaviours? Or, is it a pheromone or other compound release that affects worker behaviour and physiology? Using an apparatus such as a wire mesh separator to isolate workers from brood (e.g. design protocols similar to Dornhaus & Chittka, 2001; Lopez-Vaamonde, Brown, Lucas, Pereboom, Jordan & Bourke, 2007) could inhibit workers from performing brood care behaviours but not inhibit their exposure to volatile pheromones. There is little research involving this apparatus and measuring its effect on worker reproductive behaviour. There are experiments, however, examining the degree of the queen's control over worker reproduction using wire mesh separators (e.g. Alaux et al., 2004; Lopez-Vaamonde et al., 2007; Pomeroy, 1981). In these experiments, there was no evidence of volatile queen pheromones that affect queen-less workers' aggressive or egg-laying behaviour (Alaux et al., 2004). There was also no evidence that there is a non-volatile queen pheromone (Alaux et al., 2004), specifically that contact with other workers or brood that have had contact with the queen have any effect on worker behaviour (Lopez-Vaamonde et al., 2007). These findings may suggest brood do not emit pheromones that would affect worker oviposition or aggression. Nevertheless, there is evidence that workers can differentiate hungry brood from satiated brood (Pomeroy, 1981) suggesting workers can identify characteristics of the brood. This may suggest that employing wire mesh separator may, in the least, exclude the possibility that it is the contact with brood pheromones or pheromones left on the brood by the queen that suppresses worker reproduction.

The primary contribution of this thesis is the development of a method to define dominance in the bumblebee through distinguishing behavioural dominance (aggression and social interactions) from reproductive dominance (oviposition and ovarian development) and determining their relationship under different environments (e.g. with brood) and experimental manipulations (ovarian development). Coding approximately 3800 hours of video recordings ensured there were sufficient observations of behaviour and an accurate behavioural ethogram of *B. impatiens* interactions. The inclusion of multiple measures of reproduction (oviposition and ovarian mass) provided the necessary connection between the behavioural and physiological correlates of the phenomenon. This method revealed the complexities of reproductive conflict in the bumblebee and highlighted the difference between reproductive and behavioural dominance, of which these results may suggest bypassing the term all together (Bernstein, 1981). One premise of dominance involves the most reproductive animal maintaining their reproductive monopoly through 'winning' aggressive and other social interactions with other potentially reproductive individuals. This conceptualization of dominance is typically applied to many different animal species (e.g. primates, Klinkova, Hodges, Fuhrmann, de Jong, & Heistermann, 2005, although not without controversy, Cowlishaw & Dunbar, 1991). Although some vertebrate species have a similar social structure as eusocial insects (e.g. Clarke & Faulkes, 2001) it may be that this conceptualization of dominance is too unidimensional for eusocial insect species. Indeed, the haplodiploid genetic system and kin selection theory would predict eusocial insects like the bumblebee are under different selection pressures than diploid genetic systems where the original dominance models were derived.

As hypothesized in the general introduction of this thesis, there may not be a causal relationship between aggression and reproductive control. This thesis controlled for variables believed to be associated with aggression, specifically social activity, to measure its separate effects on aggression and ovarian development. Finally, we controlled for ovarian development to measure its effect on aggression. Applications of this method to larger orphaned or queen-right groups have promise in further expounding reproductive conflict in eusocial insects like *B. impatiens*, providing a greater understanding of the social organization and reproductive decision making in primitively eusocial insect species like this common and economically useful North American bumblebee.

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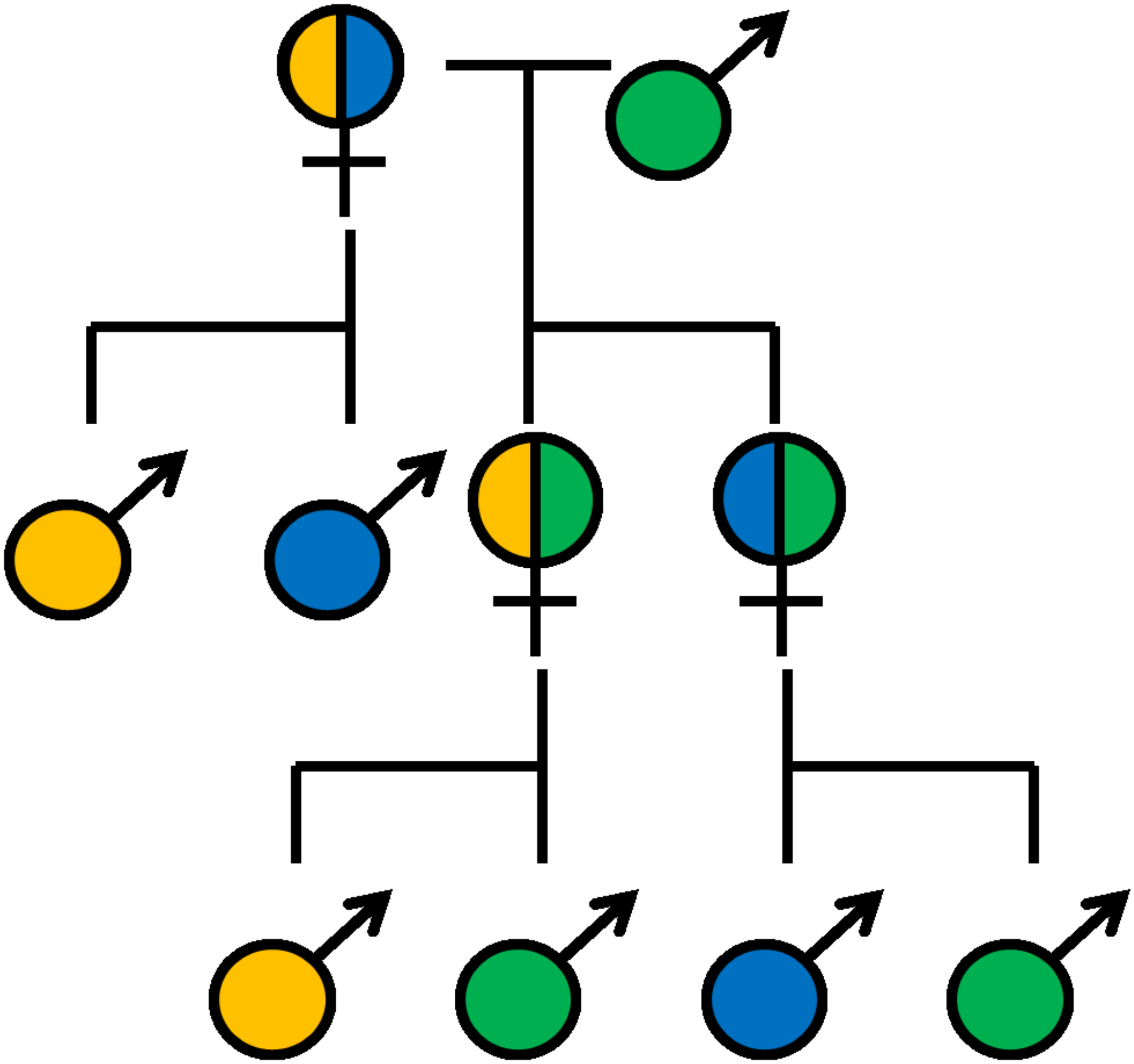
APPENDIX A

Haplodiploidy in the bumblebee



* Different colours represent different genes

APPENDIX B

Video Examples of *Bombus impatiens* Behaviour

Supplementary material B1: An example of butting behaviour in a pair of orphaned workers (*Bombus impatiens*).

Obtained from: Sibbald, E. D., & Plowright, C. M. S. (2013). On the relationship between aggression and reproduction in pairs of orphaned worker bumblebees *(Bombus impatiens)*. *Insectes Sociaux, 60,* 23-30. doi: 10.1007/s00040-012-0261-7

Headbutt.wmv

Supplementary material B2: An example of grappling behaviour between two orphaned workers (*B. impatiens*).

Grapple.wmv

Supplementary material B3: An example of worker *B. impatiens* oviposition.

Oviposition.wmv

Supplementary material B4: A social interaction in a pair of orphaned workers (*B. impatiens*).

Social Interaction.wmv

Supplementary material B5: An example of oophagy in *B. impatiens*.

Oophagy.wmv