

The Pennsylvania State University

The Graduate School

REGULATION OF BUMBLE BEE (*Bombus impatiens*) WORKER REPRODUCTION

BY BROOD

A Thesis in

Entomology

by

Jesse Starkey

© 2019 Jesse Starkey

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

August 2019

The thesis of Jesse Starkey was reviewed and approved* by the following:

Etya Amsalem

Assistant Professor of Entomology

Thesis Advisor

Christina Grozinger

Distinguished Professor of Entomology

Tom Baker

Distinguished Professor of Entomology

Gary Felton

Professor and Department Head of Entomology

*Signatures are on file in the Graduate School

ABSTRACT

Social insect species where egg laying is monopolized by a single or a few females have evolved multiple times in insects. These societies were always rooted in a simple family structure where adults care for the brood that is produce. Female reproduction in such families are often characterized by a tradeoff between reproduction and brood care. Yet, most work on the regulation of reproduction in social insects have focused on signals and traits exhibited by adults such as the queen or nestmate workers.

Reproductive division of labor, whether it is controlled by the brood or adults, is often regulated by a combination of behavioral and chemical means. However, it is hypothesized that behavioral interactions play a more important role in regulating reproduction of primitive eusocial species, while pheromones are typically used by large size, advanced eusocial species.

In this thesis, I examined the role of brood in regulating worker reproduction in *Bombus impatiens*, a primitively eusocial species where reproduction is dominated by the queen via an unknown mechanism. I further examined whether brood effect on worker reproduction is mediated via a pheromone produced by the offspring. I found that young larvae reduced worker egg laying, while the presence of pupae stimulated egg laying. These effects were quantity-dependent, with nearly complete suppression of egg laying in cages containing pair of workers and more than ten young larvae, and replicable regardless of worker age, relatedness to brood, or brood parentage/sex. I further showed that workers preferred pupae over larvae or control in a choice experiment, in line with their reproductive interests. However, odors from larvae or pupae were insufficient to inhibit worker reproduction. The youngest larvae were found to be particularly vulnerable to starvation, raising the hypothesis they produce a hunger signal.

However, despite a slight attraction and fewer eggs laid by workers in the presence of starved vs. fed larvae, these effects were insignificant.

The findings that any larvae can regulate worker reproduction in this simple eusocial species highlight the role of brood in the evolution of sociality and reproductive division of labor. Bumble bee workers may use a combination of cues or rely solely on behavioral interactions with brood and other females to make decisions about reproduction, in line with their small colony size and simple social organization. These findings also provide the first holistic explanation for the regulation of worker reproduction in *B. impatiens*, suggesting that either the queen inhibits worker reproduction through her brood, or workers forgo their own reproduction based on honest signals of queen fertility given off by the brood.

TABLE OF CONTENTS

LIST OF FIGURES	vi
LIST OF TABLES	vii
ACKNOWLEDGEMENTS.....	viii
Chapter 1: Introduction	1
Regulation of Reproductive Division of Labor by adults	3
Regulating of Reproductive Division of Labor by the brood	7
Why use bumble bees as a system?.....	10
Aim of the study	13
References	15
Chapter 2: The road to sociality: brood regulation of worker reproduction in the simple eusocial bee <i>Bombus impatiens</i>	27
Introduction	27
Methods.....	31
Results	35
Discussion	37
References	49
Chapter 3: Do bumblebees produce brood pheromones?	56
Introduction	56
Methods.....	60
Results	66
Discussion	70
References	83
Chapter 4: Discussion and Future Directions	90
References	96
Appendix	111

LIST OF FIGURES

Figure 1: An image of the custom plastic cages used in experiments.	43
Figure 2: The effect of related brood and wax on the cumulative number of eggs laid by <i>B. impatiens</i> workers.	44
Figure 3: The effect of relatedness, parentage/sex and amount of brood on the cumulative number of eggs laid by <i>B. impatiens</i> workers.	46
Figure 4: An image of the vacuum air-flow system that was used in volatile experiment.	75
Figure 5: The percent of choices made by <i>B. impatiens</i> workers in a choice bioassay between (A) young larvae and control, (B) pupae and control, and (C) young larvae, pupae, and control.	76
Figure 6: The effect of brood cuticular washes on the cumulative number of eggs laid by <i>B. impatiens</i> workers.	77
Figure 7: The effect of brood whole-body extracts on the cumulative number of eggs laid by <i>B. impatiens</i> workers.	78
Figure 8: The effect of brood volatiles on the cumulative number of eggs laid by <i>B. impatiens</i> workers.	79
Figure 9: Survival of <i>B. impatiens</i> larvae as a function of body mass in the absence of food.	80
Figure 10: The percent of choices made by <i>B. impatiens</i> workers in a choice bioassay between fed and starved larvae.	81
Figure 11: The effect of starved and fed larvae whole-body extracts on the cumulative number of eggs laid by <i>B. impatiens</i> workers.	82

LIST OF TABLES

Table 1: The effect of related brood and wax on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	43
Table 2: The effect of related brood and wax on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	74
Table 3: The effect of related brood and wax on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	111
Table 4: The effect of related brood and wax on the cumulative number of eggs laid by callow <i>B. impatiens</i> workers.....	112
Table 5: The effect of unrelated brood and wax on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	112
Table 6: The effect of queen- and worker-laid larvae and wax on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	113
Table 7: The quantitative effect of larvae on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	114
Table 8: The effect of brood cuticular washes (1.4 brood equivalent/day) on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	115
Table 9: The effect of brood whole-body extracts (1.4 brood equivalent/day) on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	115
Table 10: The effect of brood whole-body extracts (7 brood equivalent/day) on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	116
Table 11: The effect of brood volatiles on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	116
Table 12: The effect of fed and starved larvae whole-body extracts (7 equivalent/day) on the cumulative number of eggs laid by random-age <i>B. impatiens</i> workers.....	117

ACKNOWLEDGEMENTS

I would like to first acknowledge and thank my principal investigator Dr. Etya Amsalem, Dr. Gary Felton, my committee members Dr. Tom Baker and Dr. Christina Grozinger, as well as the Pennsylvania State University Entomology Department for the funding provided for my Masters degree. Etya is one of the best PIs I could have ever hoped for, pushing me harder to achieve a greater project and a thesis that I am very proud to call my own. I owe much of my success to her continued confidence in my abilities and her support both within and outside the lab. I would also like to thank the College of Agricultural Sciences for their grant which aided in the funding for part of my thesis research.

I'm very thankful for the support given to me by my lab mates Erin Treanore, Gabriel Villar, Nathan Derstine, and Margarita Orlova. I was always able to turn to these people for help and support when I needed it the most. I would also like to thank all of the current graduate students and professors in the department of entomology for their additional support throughout my time here at Pennsylvania State University.

Finally, I'm grateful for the emotional guidance and continued support of my parents Eve Adams and Ron Starkey, my brother Dakota Starkey, as well as my partner Sierra Pregosin and my friend Brianna Flonc. Without everyone, I doubt I would've made it this far. I would like to thank my grandfather, James Starkey, who pushed me to strive to be better in life and in my academics and pushed me to obtain a higher education. Even though he passed on during my time here, his words always echoed in my head whenever I faltered.

Chapter 1

Introduction

Reproduction is a key aspect of nature that organisms pursue in order to maximize their fitness (Darwin 1859). However, social insects may seemingly present an exception to this. Social insect species exhibit varying levels of reproductive division of labor where one or a few females (usually a queen) dominate reproduction while other females (workers) delay or cease their own reproduction and function as helpers (Wilson 1971). As a result, workers of social insect species invest in the rearing of another female's offspring rather than their own. How such a phenomenon evolved and how it is regulated have been longstanding enigmas in the study of social evolution. These questions have been extensively studied in the last century (Beshers and Fewell 2001; Bonabeau et al. 1996; Robinson 1992; Schultner et al. 2017), yet, the proximate mechanisms regulating worker sterility are partially understood only in a handful of species (Bourke 2014; Ratnieks et al. 2006). Traditionally, these studies have focused on behavioral and chemical traits of adults (queen or other workers) and their potential effect on worker reproduction. For example, ants use both aggression (Liebig et al. 1999b) and chemical signaling to regulate worker sterility (Monnin et al. 2002; van Zweden et al. 2009), the honey bee queen produces a pheromone that inhibits worker reproduction (Hoover et al.

2003), and nestmates in multiple species may police other females who attempt to reproduce (Endler et al. 2004; Ratnieks and Visscher 1989; Wenseleers 2004).

Social insect colonies may contain a few to millions of individuals. They also contain varying level of brood at different developmental stages that is produced by the dominant female/s. Thus, worker reproduction may be regulated not only by behavioral or chemical means exhibited by adults, but also by the presence of the brood. Brood care behavior was suggested to play a dominant role in the social evolution of insects (Kronauer and Libbrecht 2018). Maternal behavior in solitary insects, for example, has evolved to regulate sibling care in eusocial species (West-Eberhard 1996), and most eusocial societies are rooted in a simple family structure where there is a tradeoff between brood care and future reproduction (Boomsma 2009; Linksvayer and Wade 2005; Toth et al. 2007). However, the role of brood in regulating female reproduction was mostly examined in solitary insects (Hunt and Simmons 2004; Kolliker 2007; Zink 2003) or in species exhibiting advanced social behavior such as the honey bee (Le Conte et al. 2001; Maisonnasse et al. 2010) and fire ants (Bigley and Vinson 1975). Deciphering the regulators of reproduction by multiple players in the social unit and in simple-eusocial compared to solitary and advanced social species can provide valuable insights into one of the major transitions in social evolution (Amsalem et al. 2015b; Kronauer and Libbrecht 2018).

The simple eusocial *B. impatiens* is an excellent system to study the role of brood because unlike other eusocial species, bumble bees form relatively small size colonies where reproduction is first monopolized by the queen, but later in the season is performed

by both queen and workers (Duchateau and Velthuis 1988). Additionally, the regulation of bumble bee worker reproduction has been a source of debate with conflicting results and, despite extensive research, remains unclear. In my thesis, I will describe our current knowledge on the regulation of worker reproduction in social insects with a focus on the simple eusocial bumble bee *Bombus impatiens*, delving into the mechanisms used by both adults and brood to regulate worker sterility.

Regulation of Reproductive Division of Labor by Adults

Adults (i.e, queens and workers) in social insect societies regulate reproductive division of labor through behavioral means directed towards individuals attempting to reproduce, chemical signals that inhibit reproduction in workers through advertising the female reproductive status, or a combination of both (Kocher and Grozinger 2011; Ratnieks et al. 2006).

Regulating reproduction through aggression is widespread in social species lacking morphological differences between female castes like the simple eusocial wasps *Ropalidia marginata* (Premnath 1996) and *Polistes fuscatus* (Reeve 1983) as well as queenless ants like *Dinoponera quadriceps* (Monnin and Peeters 1999). In different species, aggression may be exhibited by individuals in three different scenarios: (1) reproductive individuals competing against one another (reproductive competition); (2) reproductive individuals controlling subordinates' reproduction or behavior (reproductive

inhibition), or (3) sterile workers aggressing females who are reproductively active (policing behavior).

Reproductive competition is common in primitively eusocial insects where several morphologically identical females competing for reproduction. Reproductive inhibition using aggression occurs across many social insects, but is less common among advanced eusocial insect species that show little to no overt aggression (Leonhardt et al. 2016). For example, in the wasp *Polistes carolina*, the first arriving foundresses are more aggressive and have reproductive superiority over other females in nests (Seppa et al. 2002). In the ant *Dinoponera quadriceps*, there is no queen present in the colony, but rather 5-10 morphologically identical females who dominate reproduction (Monnin and Peeters 1999). In this species, higher ranked females form behavioral and reproductive hierarchies by physically blocking other reproductive females from walking away (Monnin and Peeters 1999). In the wasp species *Ropalidia marginata* and *Polistes fuscatus*, dominant, reproductive females aggress subordinate females after the establishment of a new nest, forcing subordinate sterile females to perform colony maintenance (brood care, cleaning, etc.) and foraging, to support the rearing of offspring laid by the dominants, resulting in reproductive inhibition in the aggressed individuals (Premnath 1996; Reeve 1983).

Aggression may be also exhibited by sterile females towards individuals attempting to reproduce (“policing behavior”). Worker policing is predicted to occur in social insects where workers are more related to their brothers than nephews, and thus prefer the queens’ sons and prevent their sisters from producing males by directly

attacking the reproducing females, or by devouring their eggs (Wenseleers 2004). For example, *Apis mellifera* workers identify (through antennation) and attack reproductive workers (Ratnieks 1988; Ratnieks and Visscher 1989), and reproductively active workers of the queenless ant *Harpegnathos saltator* are aggressed by sterile workers resulting in inhibition of ovarian activation in the aggressed female (Liebig et al. 1999b). Furthermore, in the ant *Camponotus floridanus*, workers are able to differentiate between worker- and queen- laid eggs and devour eggs laid by workers (Endler et al. 2004).

Reproductive division of labor is further regulated by chemical communication using chemical compounds (pheromones) that are produced by individuals and induce behavioral and/or physiological changes in the receiver (Karlson and Luscher 1959). This mode of communication is typically found in larger social insect species where the dominant female cannot behaviorally interact with all workers in a colony (Leonhardt et al. 2016). Pheromones may induce either a primer or releaser effect. Releaser pheromones cause short-term behavioral responses in recipients of the signal, such as attraction. For example, in *Dinoponera quadriceps* and *Pachycondyla inversa* (among other ants), reproductive females mark competing reproductive females with a hydrocarbon secretion which elicits worker aggression (Monnin et al. 2002; van Zweden et al. 2009). The queen of the wasp *Vespula vulgaris* marks her eggs with queen characteristic cuticular hydrocarbons, resulting in reduced policing behavior by workers to marked vs. unmarked eggs (Oi et al. 2016). Additionally, the honey bee queen

mandibular pheromone induces retinue and suppress swarming behavior in workers (Keeling et al. 2003; Winston et al. 1989).

Primer pheromones trigger long-term physiological changes in the recipients. One of the most well-known examples of a primer pheromone is the queen mandibular pheromone of the honey bee *Apis mellifera*, which has been shown to inhibit ovarian activation (Grozinger et al. 2007; Keeling et al. 2003; Winston et al. 1989) and delay behavioral maturation in workers (Robinson and Vargo 1998). Other pheromones produced by reproductives can also act like a fertility signal, an honest signal providing information about the mating and/or reproductive status of females (Smith 1994). Fertility signals correlating with reproductive status (but not necessarily capable of inhibiting worker reproduction) have been found to be produced by many species, including ants (Abril et al. 2018; Cuvillier-Hot et al. 2004; Leibig et al. 2000), honey bees (Keller and Nonacs 1993), bumble bees (Amsalem et al. 2017; Sramkova et al. 2008), and wasps (Sledge et al. 2001; Vargo 1999). Workers are predicted to ‘listen’ to these signals and forgo reproduction in order to increase their fitness through the rearing of related kin. In non-hymenopteran eusocial insects like termites, pheromones regulating reproduction have been discovered and identified. Polyunsaturated alkenes indicate reproductive status of the species *Zootermopsis nevadensis* and n-butyl-n-butyrate and 2-methyl-1-butanol inhibit the development of new reproductive females in the species *Reticulitermes speratus* (Liebig et al. 2009; Matsuura et al. 2010a).

Kin selection theory (Smith 1964) has been used as a framework to explain conflicts over reproduction in social insects, particularly, in hymenopteran species where

the haplo-diploid mechanism for sex determination creates an asymmetry in the relatedness between colony members (Hamilton 1964b). How relatedness between colony members affects worker reproduction in a social insect colony depends on whether the queen is singly or multiply mated and whether the colony is monogyne or polygyne. For example, multiple mating by the queen in species with a single queen results in reduced relatedness among female sisters compared to species with a single, singly mated queen. As a result, in species with a monogyne multiply mated queen workers will be more related to brothers compared to nephews and are predicted to police their sisters' eggs. In contrast, in species with a monogyne singly mated queen, workers are more related to nephews over brother, and are predicted to unite in competition against the queen when she transition to lay haploid male eggs (Trivers and Hare 1976). Thus, relatedness plays a large role in how workers interact with brood produced by the queen and other workers.

Regulating Reproductive Division of Labor by the Brood

Social behavior originates from solitary ancestors where adults cared for their young (Queller 1994; Tallamy 1984). Brood is known to regulate female reproduction in many insect species, even outside of social insects (Engel et al. 2016; Hunt and Simmons 2004; Kolliker 2007; Schultner et al. 2017; Zink 2003). In several highly eusocial insect species, young offspring were shown to produce pheromones that alter worker foraging

behavior, increase brood care and reduce workers' reproduction (Bigley and Vinson 1975; Le Conte et al. 2001; Maisonnasse et al. 2010; Morel and Meer 1988).

Although producing offspring is the direct manifestation of reproduction, parent-offspring relationships are not conflict-free. Since parents are equally related to all the offspring they produce, conflict is predicted to arise in the allocation of resources by the parent between current offspring and future siblings (Gilbert and Manica 2010). This conflict is apparent in holometabolous insects where young are completely reliant on parental care in order to survive, resulting in young developing more ways to elicit parental care while adults try to maximize their reproduction (Kilner and Johnstone 1997; Mas et al. 2009; Noonan 1986; Rauter and Moore 1999; Trivers 1972).

Parent-offspring conflicts are even more apparent in social insect colonies where the offspring are often not produced by their caregivers (Trivers and Hare 1976). Offspring in a social environment can vary in age, quality, and relatedness to the caregiver, thus requiring an accurate assessment of the brood's value to the caregiver. In social insects workers are predicted to gain a higher inclusive fitness by rearing offspring that they are more related to (Hamilton 1964b). Because of this, it is expected that workers have developed ways to identify kin vs. non-kin, as well as the sex of brood. Chemical signals are well suited to convey such information in a crowded colony and may serve the interests of the brood, the caregiver, and the colony. For example, workers of the honey bee can identify and distinguish between worker-produced and queen-produced brood as well as brood that is more related (Free and Winder 1983; Page et al. 1989; Pirk et al. 2004; Visscher 1996).

Signals produced by the brood vary greatly between insect species and convey information such as need and quality. This can take form as either physical behaviors or chemical signaling. Behavioral signals produced by larvae have been documented in insects from many social settings. For example, burying beetle (*N. vespilloides*) larvae use a specialized begging behavior to touch their parents' mouthparts in order to convey their hunger (Engel et al. 2016). *Gnamptogenys striatula* ant larvae also perform a begging behavior, swaying side to side in order to elicit more feedings by adults (Kaptein et al. 2005). Larvae may play an integral role in the production of offspring like in ant colonies of *Monomorium pharaonis*, where late instar larvae are known to process solid protein for the colony, which then affects the number of reproductive offspring that can be produced (Warner et al. 2016). Other brood mechanisms regulating brood care and reproductive division of labor such as auditory signals and visual cues are less common but were described in several species. For example, *Vespa orientalis* larvae produce acoustic signals to communicate their presence by scraping their mandibles against the cell walls (Ishay and Landau 1972).

Chemically, larvae have only been shown to produce signals regulating worker reproduction in two species; fire ants (Bigley and Vinson 1975) and honey bees (Le Conte et al. 1990; Maisonnasse et al. 2010). Yet, in these few cases, brood produced pheromones have been shown to cause similar effects to that of adults (specifically the queen) in regard to long- and short-term effects on worker behavior and physiology. Larvae of *A. mellifera* produce the brood pheromone *E-β-Ocimene* which alters worker foraging behavior (He et al. 2016; Maisonnasse et al. 2010). Similar releaser pheromones

have been identified in the fire ant *Solenopsis invicta* as well, where exposure to this non-volatile pheromone induced brood tending behavior in workers (Bigley and Vinson 1975). These pheromones were shown to have primer effects in workers. *E-β-Ocimene* reduces worker ovarian activation in *A. mellifera*, and is produced in greater amounts by starved larvae (He et al. 2016; Maisonnasse et al. 2010). Furthermore, ethyl palmitate and methyl linolenate, two brood ester pheromones, have both been shown to partially inhibit ovarian activation of queenless workers (Arnold 1994; Mohammedi et al. 1998). When these brood esters were synthesized and applied to workers, they were also shown to reduce worker and colony lifespan (Smedal et al. 2009). Unfortunately, primer effects of brood pheromones in other species remain relatively unexplored.

Bumble bees as a model organism

The regulation of worker reproduction in bumble bees is not fully understood and much of the previous work done on this has focused on queen superiority in *B. terrestris* and *B. impatiens*. While studies have shown that direct queen presence reduces worker reproduction (Alaux et al. 2004; Amsalem et al. 2017; Melgarejo et al. 2018; Padilla et al. 2016), the mechanisms used by the queen to regulate reproduction remain unclear. It was first suggested that worker reproduction is inhibited in response to pheromones produced in the queen's mandibular glands (Honk van et al. 1980; Roseler et al. 1981), to which no supporting evidence was found (Bloch and Hefetz 1999). Further studies have focused on

the queen's cuticular secretions or in the wax containing these compounds. Pentacosane (C25), one of the queen's cuticular compounds, caused increased ovarian resorption in *B. terrestris* workers (Van Oystaeyen et al. 2014). However, this compound is produced in large amounts by both workers and queens in both *B. impatiens* and *B. terrestris*, and ovary resorption was demonstrated in both laying queens and workers and also in response to other hydrocarbons (Amsalem et al. 2015c; Duchateau and Velthuis 1989). Additionally, an exposure to C25 in both *B. impatiens* and *B. terrestris* workers failed to decrease worker ovarian activation (Amsalem et al. 2015c; Van Oystaeyen et al. 2014). Wax from pre-competition colonies reduced aggression and ovary size in *B. terrestris* workers, but only in the presence of the queen (Rottler-Hoermann et al. 2016). Further studies focused in the production of gynes and males by queens and the effects of these events on worker reproduction. While male production by the queen did not precede the competition (Duchateau and Velthuis 1988), the production of new queens did (Alaux et al. 2006), suggesting that workers delay their reproduction until new queens are produced. Finally, several more studies supported the idea that the queen loses her superiority due to increase in worker population in the colony (Amsalem and Hefetz 2011; Bloch 1999). Overall, despite much effort to identify potential queen pheromones in bumble bees, their existence, identity, and whether they are combined with dominance behavior or depend on social context, remain elusive.

Bumble bee workers is regulated not only by the queen, but also by their nestmates. Dominant workers have been shown to reduce nestmates' ovarian activation and juvenile hormone biosynthesis in *B. terrestris* (Bloch and Hefetz 1999). *B. terrestris*

sterile workers were also shown to produce sterility signals, ie high amounts of octyl esters, compared to workers with active ovaries that possess almost none of these esters (Amsalem and Hefetz 2010; Amsalem et al. 2009). The presence of these esters is associated with inactive ovaries and was suggested to signal the sterility of these workers in an attempt to reduce aggressive behavior directed at them.

Some of the studies that examined the regulation of worker reproduction by the queen and nestmates in bumble bees included brood in their treatments. However, results only provided anecdotal observations about its role in regulating worker reproduction, and sometimes conflicting results regarding its impacts on workers. For example, Padilla et al. 2016 found no relationship between worker ovarian activation and direct contact and volatiles from pupae or from the entire colony (containing brood at different developmental stages). However, this study did not examine the effect of other life stages of bumble bee brood on worker behavior and reproduction (Padilla et al. 2016). Sibbald and Plowright in 2013 found that the presence of larvae increases aggression between queenless workers at the time of oviposition in *B. impatiens* (Sibbald and Plowright 2013). They further showed that the presence of larvae reduces worker aggression and ovarian development when compared to workers provided with no brood (Sibbald and Plowright 2014). Several other studies demonstrated a clear impact of brood on worker behavior but have not investigated how brood affects worker reproduction. Pereboom et al. 2003 showed that starved larvae of *B. terrestris* are fed significantly more often than fed larvae (Pereboom et al. 2003), and Boer & Duchateau 2006 supported this by showing that young larvae produce a hunger signal which has a releaser effect in *B.*

terrestris workers, inducing attraction and feeding behavior (Boer and Duchateau 2006). These observations do not give us a clear understanding on how brood of different developmental phases affects worker reproduction in bumble bee as well as the mechanism by which it is done.

Aim of the Study

Previous studies in bumble bees suggested that worker reproduction is unlikely to be regulated by queen produced-pheromones alone (Alaux et al. 2004; Amsalem et al. 2017; Bloch and Hefetz 1999; Honk van et al. 1980; Melgarejo et al. 2018; Padilla et al. 2016; Roseler et al. 1981). In this thesis, I examine the role of brood in regulating worker reproduction in the common Eastern bumble bee, *Bombus impatiens*. Workers of this species have inactive ovaries up until the death or removal of the queen (Cnaani et al. 2002), as well as having more workers activated ovaries the older the colony gets (Observations). Brood care has been identified as one of the paths by which eusociality has evolved in insects (Kronauer and Libbrecht 2018) due to a simple trade-off between brood care and future reproduction (Queller 1994; Tallamy 1984), suggesting that brood may play a role in the regulation of reproduction of simple eusocial species such as *B. impatiens*. Bumble bee sociality exhibit characteristics of both highly eusocial species such as reproductive dominance by a single queen (albeit temporary), along with extensive competition over reproduction in the second phase of the life cycle. Primitively eusocial insects have been previously suggested as great models for examining the

interplay between behavioral and chemical means in regulating reproduction of social species (Amsalem et al. 2015b; Jandt et al. 2014; Kronauer and Libbrecht 2018).

In Chapter 2, I examined the reproduction of workers following exposure to live brood. First, I tested how different developmental stages of brood affect egg laying and ovary size in workers. I then examined whether the observed effects depend on previous worker experience, worker relatedness to the brood, the number of larvae, or its parentage/sex. In Chapter 3, I examined whether *B. impatiens* brood produces a brood pheromone to communicate their presence or hunger. I examined the releaser effects of odors produced by live brood on worker attraction. Then, I examined the primer effects of these odors by testing how cuticular washes, whole-body extracts and volatiles drawn from the brood affect worker reproduction. Next, I examined the survival of larvae as function of their body mass, hypothesizing that young larvae are more vulnerable to starvation and are thus more dependent on worker care. I then examined the releaser effects of starved and fed larvae on worker and whether odors extracted from starved and fed larvae have a primer effect on worker reproduction.

References

- Abril S, Diaz M, Lenoir A, Ivon Paris C, Boulay R, Gomez C (2018) Cuticular hydrocarbons correlate with queen reproductive status in native and invasive Argentine ants (*Linepithema humile*, Mayr) PLoS One 13:e0193115
doi:10.1371/journal.pone.0193115
- Alaux C, Jaisson P, Hefetz A (2004) Queen influence on worker reproduction in bumblebees (*Bombus terrestris*) colonies Insectes Sociaux 51:287-293
doi:10.1007/s00040-004-0741-5
- Alaux C, Jaisson P, Hefetz A (2006) Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal Behavioral Ecology and Sociobiology 60:439-446 doi:10.1007/s00265-006-0184-2
- Amsalem E, Grozinger CM, Padilla M, Hefetz A (2015a) The physiological and genomic bases of bumble bee social behaviour. In: Amro Z, Clement FK (eds) Advances in Insect Physiology, vol 48. Academic Press, pp 37-93
- Amsalem E, Hefetz A (2010) The appeasement effect of sterility signaling in dominance contests among *Bombus terrestris* workers Behavioral Ecology and Sociobiology 64:1685-1694 doi:10.1007/s00265-010-0982-4
- Amsalem E, Hefetz A (2011) The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris* PLoS One 6:e18238
doi:10.1371/journal.pone.0018238

Amsalem E, Orlova M, Grozinger CM (2015b) A conserved class of queen pheromones?

Re-evaluating the evidence in bumblebees (*Bombus impatiens*) Proc Biol Sci

282:20151800 doi:10.1098/rspb.2015.1800

Amsalem E, Padilla M, Schreiber PM, Altman NS, Hefetz A, Grozinger CM (2017) Do

Bumble Bee, *Bombus impatiens*, Queens Signal their Reproductive and Mating

Status to their Workers? J Chem Ecol 43:563-572 doi:10.1007/s10886-017-0858-

4

Amsalem E, Twele R, Francke W, Hefetz A (2009) Reproductive competition in the

bumble-bee *Bombus terrestris*: do workers advertise sterility? Proc Biol Sci

276:1295-1304 doi:10.1098/rspb.2008.1688

Arnold G, Le Conte, Y., Trouiller, J., Herve, H., Chappe, B., Masson, C. (1994)

Inhibition of worker honeybee ovaries development by a mixture of fatty acid

esters from larvae Acad Sci

Beshers SN, Fewell JH (2001) Models of division of labor in social insects Annu Rev

Entomol 46:413-440 doi:10.1146/annurev.ento.46.1.413

Bigley WS, Vinson SB (1975) Characterization of a Brood Pheromone Isolated from the

Sexual Brood of the Imported Fire Ant, *Solenopsis invicta*1,2 Annals of the

Entomological Society of America 68:301-304 doi:10.1093/aesa/68.2.301

Bloch G (1999) Regulation of queen-worker conflict in bumble-bee (*Bombus terrestris*)

colonies Proc Biol Sci 266:2465-2469 doi:10.1098/rspb.1999.0947

Bloch G, Hefetz A (1999) Reevaluation of the role of mandibular glands in regulation of

reproduction in bumblebee colonies Journal of Chemical Ecology 25:881-896

doi:Doi 10.1023/A:1020805103379

- Boer SPAd, Duchateau MJHM (2006) A larval hunger signal in the bumblebee *Bombus terrestris* *Insectes Sociaux* 53:369-373 doi:10.1007/s00040-006-0883-8
- Bonabeau A, Theraulaz G, Deneubourg JL (1996) Mathematical model of self-organizing hierarchies in animal societies *Bulletin of Mathematical Biology* 58:661-717
- Boomsma JJ (2009) Lifetime monogamy and the evolution of eusociality *Philosophical Transactions of the Royal Society B: Biological Sciences* 364:3191-3207
doi:doi:10.1098/rstb.2009.0101
- Bourke AF (2014) Hamilton's rule and the causes of social evolution *Philos T R Soc B* 369:20130362 doi:10.1098/rstb.2013.0362
- Cnaani J, Schmid-Hempel R, Schmidt JO (2002) Colony development, larval development and worker reproduction in *Bombus impatiens* *Cresson Insectes Sociaux* 49:164-170 doi:10.1007/s00040-002-8297-8
- Cuvillier-Hot V, Lenoir A, Crewe R, Malosse C, Peeters C (2004) Fertility signalling and reproductive skew in queenless ants *Anim Behav* 68:1209-1219
doi:10.1016/j.anbehav.2003.11.026
- Darwin C (1859) *The origin of species.*
- Duchateau MJ, Velthuis HHW (1988) Development and reproductive strategies in *Bombus terrestris* colonies *Behavior* 107:186-207
- Duchateau MJ, Velthuis HHW (1989) Ovarian Development and Egg-Laying in Workers of *Bombus-Terrestris* *Entomologia Experimentalis Et Applicata* 51:199-213
doi:DOI 10.1111/j.1570-7458.1989.tb01231.x

- Endler A, Liebig J, Schmitt T, Parker JE, Jones GR, Schreier P, Holldobler B (2004) Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect Proc Natl Acad Sci U S A 101:2945-2950 doi:10.1073/pnas.0308447101
- Engel KC, Stokl J, Schweizer R, Vogel H, Ayasse M, Ruther J, Steiger S (2016) A hormone-related female anti-aphrodisiac signals temporary infertility and causes sexual abstinence to synchronize parental care Nat Commun 7:11035 doi:10.1038/ncomms11035
- Free JB, Winder ME (1983) Brood Recognition by Honeybee (*Apis-Mellifera*) Workers Anim Behav 31:539-545 doi:Doi 10.1016/S0003-3472(83)80077-3
- Gilbert JD, Manica A (2010) Parental care trade-offs and life-history relationships in insects Am Nat 176:212-226 doi:10.1086/653661
- Grozinger CM, Fan Y, Hoover SE, Winston ML (2007) Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*) Mol Ecol 16:4837-4848 doi:10.1111/j.1365-294X.2007.03545.x
- Hamilton WD (1964) The genetical evolution of social behaviour. II J Theor Biol 7:17-52
- He XJ, Zhang XC, Jiang WJ, Barron AB, Zhang JH, Zeng ZJ (2016) Starving honey bee (*Apis mellifera*) larvae signal pheromonally to worker bees Sci Rep 6:22359 doi:10.1038/srep22359
- Honk van CGJ, Velthuis HHW, Roseler PF, Malotiaux ME (1980) The mandibular glands of *Bombus terrestris* queens as a source of queen pheromone. Entomologia Experimentalis Et Applicata 28:191-198

- Hoover SE, Keeling CI, Winston ML, Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development *Naturwissenschaften* 90:477-480 doi:10.1007/s00114-003-0462-z
- Hunt J, Simmons LW (2004) Optimal maternal investment in the dung beetle *Onthophagus taurus*? *Behavioral ecology and sociobiology* 55:302-312
- Ishay J, Landau EM (1972) *Vespa* larvae send out rhythmic hunger signals *Nature* 237:286-287 doi:10.1038/237286a0
- Jandt JM, Tibbetts EA, Toth AL (2014) *Polistes* paper wasps: a model genus for the study of social dominance hierarchies *Insect Soc* 61:11-27 doi:10.1007/s00040-013-0328-0
- Kaptein N, Billen J, Gobin B (2005) Larval begging for food enhances reproductive options in the ponerine ant *Gnamptogenys striatula* *Animal Behavior* 69:293-299
- Karlson P, Luscher M (1959) 'Pheromones': a New Term for a Class of Biologically Active Substances *Nature* 183:55-56 doi:10.1038/183055a0
- Keeling CI, Slessor KN, Higo HA, Winston ML (2003) New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone *Proc Natl Acad Sci U S A* 100:4486-4491 doi:10.1073/pnas.0836984100
- Keller L, Nonacs P (1993) The Role of Queen Pheromones in Social Insects - Queen Control or Queen Signal *Anim Behav* 45:787-794 doi:DOI 10.1006/anbe.1993.1092
- Kilner R, Johnstone RA (1997) Begging the question: are offspring solicitation behaviours signals of need? *Trends Ecol Evol* 12:11-15
doi:[https://doi.org/10.1016/S0169-5347\(96\)10061-6](https://doi.org/10.1016/S0169-5347(96)10061-6)

- Kocher SD, Grozinger CM (2011) Cooperation, conflict, and the evolution of queen pheromones *J Chem Ecol* 37:1263-1275 doi:10.1007/s10886-011-0036-z
- Kolliker M (2007) Benefits and costs of earwig (*Forficula auricularia*) family life *Behavioral Ecology and Sociobiology* 61:1489-1497 doi:10.1007/s00265-007-0381-7
- Kronauer DJ, Libbrecht R (2018) Back to the roots: the importance of using simple insect societies to understand the molecular basis of complex social life *Curr Opin Insect Sci* 28:33-39 doi:10.1016/j.cois.2018.03.009
- Le Conte Y, Arnold G, Trouiller J, Masson C, Chappe B (1990) Identification of a brood pheromone in honeybees *Naturwissenschaften* 77:334-336
- Le Conte Y, Mohammedi A, Robinson GE (2001) Primer effects of a brood pheromone on honeybee behavioural development *Proc Biol Sci* 268:163-168 doi:10.1098/rspb.2000.1345
- Leibig J, peeters C, Oldham NJ, Markstadter C, Holldobler B (2000) Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*? proceeding of the Nationa Acadamy of Science USA 97:4124-4131
- Leonhardt SD, Menzel F, Nehring V, Schmitt T (2016) Ecology and Evolution of Communication in Social Insects *Cell* 164:1277-1287 doi:10.1016/j.cell.2016.01.035
- Liebig J, Eliyahu D, Brent CS (2009) Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis* *J Behav Ecol Sociobiol* 63:1799-1807 doi:10.1007/s00265-009-0807-5

- Liebig J, Peeters C, Hölldobler B (1999) Worker policing limits the number of reproductives in a ponerine ant 266:1865-1870 doi:doi:10.1098/rspb.1999.0858
- Linksvayer TA, Wade MJ (2005) The evolutionary origin and elaboration of sociality in the aculeate Hymenoptera: maternal effects, sib-social effects, and heterochrony Q Rev Biol 80:317-336 doi:10.1086/432266
- Maisonnasse A, Lenoir JC, Beslay D, Crauser D, Le Conte Y (2010) E-beta-ocimene, a volatile brood pheromone involved in social regulation in the honey bee colony (*Apis mellifera*) PLoS One 5:e13531 doi:10.1371/journal.pone.0013531
- Mas F, Haynes KF, Kolliker M (2009) A chemical signal of offspring quality affects maternal care in a social insect Proc Biol Sci 276:2847-2853 doi:10.1098/rspb.2009.0498
- Matsuura K, Himuro C, Yokoi T, Yamamoto Y, Vargo EL, Keller L (2010) Identification of a pheromone regulating caste differentiation in termites PNAS 107:12963-12968 doi:10.1073/pnas.1004675107 %J Proceedings of the National Academy of Sciences
- Melgarejo V, Wilson Rankin EE, Loope KJ (2018) Do queen cuticular hydrocarbons inhibit worker reproduction in *Bombus impatiens*? Insectes Sociaux 65:601-608 doi:10.1007/s00040-018-0651-6
- Mohammedi A, Paris A, Crauser D, Le Conte Y (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera* L) Naturwissenschaften 85:455-458 doi:DOI 10.1007/s001140050531
- Monnin T, Jones G, Ratnieks FLW, Beard R (2002) Pretender punishment induced by chemical signaling in a queenless ant Nature 419:61-65

- Monnin T, Peeters C (1999) Dominance hierarchy and reproductive conflicts among subordinates in a monogynous queenless ant Behav Ecol 10:323-332 doi:DOI 10.1093/beheco/10.3.323
- Morel L, Meer RKV (1988) Do Ant Brood Pheromones Exist? Annals of the Entomological Society of America 81:705-710 doi:10.1093/aesa/81.5.705
- Noonan KC (1986) Recognition of Queen Larvae by Worker Honey-Bees (*Apis mellifera*) Ethology 73:295-306 doi:10.1111/j.1439-0310.1986.tb00811.x
- Oi CA, Millar JG, van Zweden JS, Wenseleers T (2016) Conservation of Queen Pheromones Across Two Species of Vespine Wasps J Chem Ecol 42:1175-1180 doi:10.1007/s10886-016-0777-9
- Padilla M, Amsalem E, Altman N, Hefetz A, Grozinger CM (2016) Chemical communication is not sufficient to explain reproductive inhibition in the bumblebee *Bombus impatiens* Royal Society Open Science 3:160576
- Page RE, Robinson GE, Fondrk MK (1989) Genetic specialists, kin recognition and nepotism in honey-bee colonies Nature 338:576-579 doi:10.1038/338576a0
- Pemnath SS, A; Gadagkar, R. (1996) Dominance relationship in the establishment of reproductive division of labour in a primitively eusocial wasp (*Ropalidia marginata*) Behav Ecol Sociobiol 39:125-132
- Pereboom JJM, Velthuis HHW, Duchateau MJ (2003) The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation Insect Soc 50:127-133 doi:10.1007/s00040-003-0639-7

- Pirk CW, Neumann P, Hepburn R, Moritz RF, Tautz J (2004) Egg viability and worker policing in honey bees *Proc Natl Acad Sci U S A* 101:8649-8651
doi:10.1073/pnas.0402506101
- Queller DC (1994) Extended parental care and the origin of eusociality *Proceedings of the Royal Society of London Series B: Biological Sciences* 256:105-111
doi:10.1098/rspb.1994.0056
- Ratnieks FL, Foster KR, Wenseleers T (2006) Conflict resolution in insect societies *Annu Rev Entomol* 51:581-608 doi:10.1146/annurev.ento.51.110104.151003
- Ratnieks FLW (1988) Reproductive harmony via mutual policing by workers in eusocial Hymenoptera *American Naturalist* 132:217-236 doi:Doi 10.1086/284846
- Ratnieks FLW, Visscher PK (1989) Worker Policing in the Honeybee *Nature* 342:796-797 doi:DOI 10.1038/342796a0
- Rauter CM, Moore AJ (1999) Do honest signalling models of offspring solicitation apply to insects? *Proceedings of the Royal Society of London Series B: Biological Sciences* 266:1691-1696 doi:10.1098/rspb.1999.0833
- Reeve HG, GJ. (1983) Colony activity integration in primitively eusocial wasps: the role of the queen (*Polistes fuscatus*, Hymenoptera: Vespidae) *Behav Ecol Sociobiol* 13:63-74
- Robinson GE (1992) Regulation of division of labor in insect societies *Annu Rev Entomol* 37:637-665 doi:10.1146/annurev.en.37.010192.003225
- Robinson GE, Vargo EL (1998) Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioral pacemaker *Arch Insect Biochem Physiol* 35:559-583

- Roseler PF, Roseler I, Vanhonk CGJ (1981) Evidence for Inhibition of Corpora Allata Activity in Workers of *Bombus-Terrestris* by a Pheromone from the Queens Mandibular Glands *Experientia* 37:348-351
- Rottler-Hoermann AM, Schulz S, Ayasse M (2016) Nest wax triggers worker reproduction in the bumblebee *Bombus terrestris* *R Soc Open Sci* 3:150599 doi:10.1098/rsos.150599
- Schultner E, Oettler J, Helantera H (2017) The role of brood in eusocial Hymenoptera *Q Rev Biol* 92:39-78 doi:10.1086/690840
- Seppa P, Queller DC, Strassmann JE (2002) Reproduction in foundress associations of the social wasp, *Polistes carolina*: conventions, competition, and skew *Behav Ecol* 13:531-542 doi:DOI 10.1093/beheco/13.4.531
- Sibbald ED, Plowright CMS (2013) On the relationship between aggression and reproduction in pairs of orphaned worker bumblebees (*Bombus impatiens*) *Insect Soc* 60:23-30 doi:10.1007/s00040-012-0261-7
- Sibbald ED, Plowright CMS (2014) Social interactions and their connection to aggression and ovarian development in orphaned worker bumblebees (*Bombus impatiens*) *Behav Processes* 103:150-155 doi:10.1016/j.beproc.2013.11.012
- Sledge MF, Boscaro F, Turillazzi S (2001) Cuticular hydrocarbons and reproductive status in the social wasp *Polistes dominulus* *Behavioral Ecology and Sociobiology* 49:401-409 doi:10.1007/s002650000311
- Smedal B, Brynem M, Kreibich CD, Amdam GV (2009) Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*) *The Journal of experimental biology* 212:3795-3801 doi:10.1242/jeb.035063

- Smith JM (1964) Group selection and kin selection *Nature* 201:1145
- Smith JM (1994) Must reliable signals always be costly? *Anim Behav* 47:1115-1120
- Sramkova A, Schulz C, Twele R, Francke W, Ayasse M (2008) Fertility signals in the bumblebee *Bombus terrestris* (Hymenoptera: Apidae) *Naturwissenschaften* 95:515-522 doi:10.1007/s00114-008-0353-4
- Tallamy DW (1984) Insect Parental Care *Bioscience* 34:20-24 doi:Doi 10.2307/1309421
- Toth AL et al. (2007) Wasp gene expression supports an evolutionary link between maternal behavior and eusociality *Science* 318:441-444 doi:10.1126/science.1146647
- Trivers R (1972) Parental investment and sexual selection vol 136. *Biological Laboratories, Harvard University Cambridge, MA,*
- Trivers RL, Hare H (1976) Haplodiploidy and the evolution of the social insects. *Science* 191:249-263
- Van Oystaeyen A et al. (2014) Conserved class of queen pheromones stops social insect workers from reproducing *Science* 343:287-290 doi:10.1126/science.1244899
- van Zweden JS, Heinze J, Boomsma JJ, d’Ettorre P (2009) Ant queen egg-marking signals: matching deceptive laboratory simplicity with natural complexity *PLoS One* 4 doi:<https://doi.org/10.1371/journal.pone.0004718>
- Vargo EH, CD. (1999) Multiple glandular origins of queen pheromones in the fire ant *Solenopsis invicta* *J Insect Physiol* 46:1151-1159
- Visscher PK (1996) Reproductive conflict in honey bees: A stalemate of worker egg-laying and policing *Behavioral Ecology and Sociobiology* 39:237-244 doi:DOI 10.1007/s002650050286

- Warner MR, Kovaka K, Linksvayer TA (2016) Late-instar ant worker larvae play a prominent role in colony-level caste regulation *Insect Soc* 63:575-583
- Wenseleers T, Helantera, H., Hart, A., Ratnieks, F.L.W. (2004) Worker reproduction and policing in insect societies: an ESS analysis *J Chem Ecol* 17:1035-1047
- West-Eberhard MJ (1996) Wasp societies as microcosms for the study of development and evolution *J Natural history and evolution of paper wasps* 290:317
- Wilson EO (1971) *The insect societies*. Belknap Press of Harvard University press, Cambridge, Massachusetts
- Winston ML, Slessor K, Willis L, Naumann K, Higo H, Wyborn M, Kaminski L (1989) The influence of queen mandibular pheromones on worker attraction to swarm clusters and inhibition of queen rearing in the honey bee (*Apis mellifera* L.) *Insect Soc* 36:15-27
- Zink AG (2003) Quantifying the costs and benefits of parental care in female treehoppers *Behav Ecol* 14:687-693 doi:10.1093/beheco/arg044

Chapter 2

The road to sociality: brood regulation of worker reproduction in the simple eusocial bee *Bombus impatiens*

Accepted for publication in the *Journal of Animal Behavior*, April 26, 2019

Introduction

Reproductive division of labor, in which one or few females produce offspring while the remaining females function as helpers, is a key element in social evolution (Wilson, 1971). Regulators of reproduction in social insects are likely to derive from regulators of reproduction in solitary ancestors (Leonhardt, et al., 2016; Stokl and Steiger, 2017) and thus are critical for understanding how sociality has evolved and is maintained.

Sociality has evolved multiple times during the evolution of insects (Danforth, et al., 2013) but was always rooted in a simple family structure, in which offspring receive parental care and/or remain with their mothers to provide help (Queller, 1994; Schultner, et al., 2017; Tallamy, 1984). The role of brood in regulating female reproduction is not only intuitive but is also one of the most common mechanisms regulating reproduction across insects (Schultner, et al., 2017). Young offspring may influence food allocation by begging, which often results in females caring for brood at the expense of future reproduction. Such a trade-off has been broadly shown across insects of different social organizations, including solitary (Hunt and Simmons, 2004; Kölliker, 2007; Zink, 2003), subsocial (Engel, et al., 2016; Tallamy and Denno, 1982) and advanced social

lifestyles (Bigley and Vinson, 1975; Ebie, et al., 2015; Endler, et al., 2004; Maisonnasse, et al., 2010; Villalta, et al., 2015; Woodard, et al., 2013). Nevertheless, and despite several studies demonstrating the regulatory role of brood on worker reproduction, the study of reproductive division of labor in social insects has remained focused on signals produced by adult queens and workers (Schultner, et al., 2017).

Caring for offspring may be perceived as a direct manifestation of self-reproduction; however, parent–offspring relationships are not conflict-free, since offspring are selected to demand more parental investment than parents are selected to provide. Offspring are always more related to themselves than to their sibling, whereas parents are equally related to all of their offspring, resulting in a conflict over the division of resources between offspring and their future siblings (‘interbrood conflict’) and also among members of the current brood (‘intra-brood conflict’). Offspring of holometabolous insects are often stationary and depend on progressive care, and they are therefore predicted to develop means to increase parental care. The parents, however, may want to minimize care to maximize the survival of all offspring as well as their own future reproduction (Kilner and Johnstone, 1997; Trivers, 1972).

Conflicts between offspring and parents are even stronger in social insect colonies, where the caregivers (i.e. workers) are often not the parent and have interests that may not align with those of the brood (Trivers and Hare, 1976). Workers in social hymenopteran colonies gain higher inclusive fitness from rearing their sisters than they do from rearing their own offspring, but they may not have the same preference for rearing brothers or nephews to whom they are less related compared to their own offspring (Hamilton, 1964). Thus, they are predicted to develop means to recognize kin over nonkin and females over males. Honey bee workers, for example, can discriminate between queen-laid and worker-laid eggs (Ratnieks, 2015); but see (Pirk, et al., 2004), and pupae of different ages (Free and Winder, 1983). They further show nepotism in the

rearing of queens (Page, et al., 1989) and a stronger preference for larvae of their own patriline (Noonan, 2010), demonstrating the ability to recognize and prioritize brood care according to their own preferences. A similar ability to distinguish between queen eggs and worker eggs exists in workers of *Camponotus floridanus* (Endler, et al., 2004) and *Bombus terrestris* (Zanette, et al., 2012), and early sex discrimination has been shown in several other species of ants (Helms, et al., 2000; Passera and Aron, 1996). Such conflicts between parents and offspring may be mediated using a signaling system serving both the caregiver and the brood. Indeed, offspring often communicate their presence to nearby adults using various signals conveying information about the brood identity, need and quality (Mas and Kolliker, 2008; Rauter and Moore, 1999).

Brood care behavior plays a dominant role in the social evolution of insects (Kronauer and Libbrecht, 2018). However, the role of brood has been examined in only a handful of species, mostly exhibiting advanced eusocial behavior, and most work in other social species has focused on traits exhibited by adults. Here we examine the effect of brood on worker reproduction in the simple eusocial bumble bee *B. impatiens*. Unlike species in previous studies, bumble bees form relatively small colonies in which reproduction is first monopolized by the queen but later in the season is performed by both queen and workers (Duchateau and Velthuis, 1988). Deciphering the regulators of reproduction in simple species compared with solitary and advanced eusocial species can provide valuable insights into one of the major transitions in social evolution (Amsalem, et al., 2015; Kronauer and Libbrecht, 2018). Additionally, the regulation of bumble bee reproduction has been a source of debate with conflicting results, and despite extensive research it remains unclear how reproduction is regulated in bumble bee colonies.

Previous studies in bumble bees focused mostly on queen superiority in *B. terrestris* and *B. impatiens*. Inhibition of worker reproduction was attributed to a change in queen behavior (Amsalem, et al., 2017; Padilla, et al., 2016), although this has never been examined directly.

Other studies have focused on the impacts of queen chemical secretion on workers. Several gland sources (Bloch and Hefetz, 1999; Honk van, et al., 1980) and compounds (Sramkova, et al., 2008; Van Oystaeyen, et al., 2014) have been suggested to inhibit worker reproduction (i.e. the ‘precompetition phase’, during which only the queen reproduces) but not reduce worker ovary size (Amsalem, et al., 2015; Bloch and Hefetz, 1999; Van Oystaeyen, et al., 2014). Wax from precompetition colonies reduced aggression and ovary size in *B. terrestris* workers, but only in the presence of the queen (Rottler-Hoermann, et al., 2016). Further studies suggested that workers delay egg laying until new queens are produced, in line with the competition phase proceeding in the presence of new queens (Alaux, et al., 2006) but not in the presence of males (Duchateau and Velthuis, 1988). Finally, several more studies supported the idea that the queen loses her superiority due to an increase in worker population (Amsalem and Hefetz, 2011; Bloch, 1999). Overall, despite much effort to identify the queen traits responsible for changes in worker reproduction, the mechanistic bases of reproductive inhibition remain elusive. Only the direct presence of the queen has been found to reduce ovarian activation in workers and no volatile or nonvolatile compounds were found to decrease worker ovary size (Amsalem, et al., 2017; Melgarejo, et al., 2018; Padilla, et al., 2016).

Many of the aforementioned studies included brood in their treatments. However, results provided only anecdotal observations about the role of brood in regulating worker reproduction, and sometimes conflicting results regarding its impacts on workers (Sibbald and Plowright, 2012; Sibbald and Plowright, 2014). Several studies have demonstrated a clear impact of brood on worker behavior, but they did not investigate how brood affects worker reproduction (Boer and Duchateau, 2006; Pereboom, et al., 2003). Some of these observations were inconclusive due to lack of separation between the different developmental phases of brood and the distinct effects they may have on worker reproduction.

Here we provide a first detailed examination of the effect of brood on worker reproduction in *B. impatiens* workers. First, we tested how different developmental stages of brood affect egg laying and ovary size in workers. We then examined whether the observed effects depend on previous worker experience, worker relatedness to the brood, the number of larvae or the parentage/sex of the brood.

Methods

General bumble bee rearing

Colonies of *B. impatiens* were obtained from BioBest (Canada) and were maintained in laboratory nestboxes under constant darkness, at a temperature of 28–30 °C and 60% relative humidity, and supplied ad libitum with a sugar solution and fresh pollen (Light spring bee pollen, 911Honey). These colonies were used as a source of callows (newly emerged workers <24 h), random workers of an unknown age and brood. All workers were sampled from young, precompetition colonies containing a queen. At this phase of the colony cycle, adult workers have inactive ovaries (Cnaani, et al., 2002). In all the experiments, workers were separated from their parental colonies and placed in pairs in small plastic cages (11 × 11 cm and 7 cm high; Fig. A1) for 7 days, after which they were frozen at –20 °C until further analysis. Small groups containing two to three workers are a well-established model to examine questions related to reproductive division of labor in bumble bees (Amsalem, et al., 2015). Unlike full-size colonies, small groups of workers can be controlled for multiple parameters such as age, size and parental colony, which have been shown to affect reproduction in bumblebee workers (Amsalem and Hefetz, 2010; Amsalem, et al., 2015). To account for variation in worker egg laying between colonies, we ensured that each experiment was replicated using several source colonies, equally representing

both treatment and control groups. We statistically controlled for colony effect whenever such an effect was found. For each pair, we collected the following data: parental colony, developmental stage of the brood at the onset and termination of the experiment, worker ovarian activation and the cumulative number of eggs found in the cage by the end of the experiment.

Brood and Wax Collection

Larvae, pupae and wax were gently removed from their mother colonies. Eggs are typically laid on top of pupal cells, and they were gently separated from their host cell using dissection scissors without opening the cell. Batches of eggs, larvae or pupae were used only if they remained intact during collection. Unless noted otherwise, all the brood that was used in this study was laid by a queen in young colonies with no signs of worker reproduction. Larvae weight was used as a proxy for instar based on preliminary data we collected. Small larvae weighting below 50 mg roughly corresponded to instars 1 and 2 ('young larvae') and larvae weighting above 50 mg roughly corresponded to instars 3 and 4 ('old larvae'). Live brood developed throughout the course of the experiment (7 days), often transitioning between different developmental phases (Cnaani, et al., 2002). Brood development was monitored and the overall treatment was defined according to the initial brood stage (eggs, larvae, pupae) and terminal brood stage (larvae, pupae, 'wax'). Eggs (E) hatch within 5–6 days, so all eggs turned into larvae within 7 days (EL). The feeding period of *B. impatiens* larvae lasts 9–11 days, so the larvae remained larvae (LL) or turned into pupae (LP) during the experiment. Pupation takes 11–12 days, so pupae either remained pupae (PP) or emerged as adults and therefore were only exposed to wax (PW). Pupae that emerged into adult workers were immediately removed from the cages.

Measurement of ovarian activation

After bees were collected, each bee was placed in a separate tube and received an

individual number corresponding to their cage and treatment. Thus, dissections were performed blindly. Ovaries were dissected under a stereomicroscope and placed into drops of distilled water. The length of the terminal oocyte in the three largest ovarioles was measured with a micrometer eyepiece embedded into the lens. Workers possess four ovarioles per ovary, and at least one ovariole per ovary was measured. Mean terminal oocyte length for each bee was used as an index of ovarian activation (Amsalem, et al., 2009).

Egg laying

All cages were scanned for the presence of newly laid eggs daily. The cumulative number of eggs (or larvae, if eggs hatched) was counted on the day of collection (day 7). Although egg oophagy does exist in bumble bees, it is often performed in queen-right colonies and rarely occurs in small queenless groups (Amsalem, et al., 2015). We did not see evidence for oophagy (such as open egg cells, etc.) that could affect the results.

Experimental procedure

The effect of brood on worker reproduction was examined by grouping pairs of random-age workers ($N = 159$ cages, 6 colonies) or pairs of callow workers ($N = 61$ cages, 4 colonies) with eggs, young larvae (<50 mg), old larvae (>50 mg), pupae or wax. The sister workers in each cage were grouped with brood from their parental colonies for 7 days. Bumble bee females lay 6–15 eggs in one batch. Thus, it is impossible to know the exact number of eggs or larvae in a batch unless the larvae are already matured or pupated. If the brood is separated from its case, workers will not take care of it. To avoid damaging the brood, we separated the batches of brood as much as possible and grouped workers with one to two batches of eggs or larvae or 6–10 pupae per cage. The precise number of brood and their developmental phase were counted by the end of the experiment.

The effect of relatedness was examined by repeating the previous experiment but with pairs of random-age workers grouped with unrelated brood by swapping the workers and the brood of three colonies ($N = 93$ cages, 3 colonies).

To test the effect of parentage/sex, we compared reproduction in a pair of random-age workers that were grouped with wax (control), larvae laid by the queen (female brood) or larvae laid by workers (male brood). Female brood was taken from young colonies containing a queen. Male brood was taken from laboratory-reared queenless groups. We further examined whether worker reproduction is regulated by odors from the wax by adding a second control of a pair of workers that were grouped with wax from a foreign colony ($N = 40$ cages, 2 colonies).

To examine whether the brood effect is quantity dependent, we grouped pairs of random-age workers with varying amounts of young brood (1–34 young larvae per cage) for 7 days. These data were combined with data from the first experiment (random-age workers with related brood) and the third experiment (random-age workers with unrelated brood) for cages where larvae were present at the onset and termination of the experiment (overall: $N = 149$ cages, 13 colonies). This criterion was necessary to ensure constant larval presence throughout the experiment.

Statistics

Statistics were done using JMP Pro 14.1 (SAS Institute Inc., Cary, NC, U.S.A.). The egg-laying data did not distribute normally (goodness-of-fit test: $P < 0.001$). We therefore analyzed it using a generalized linear model (GLM) with ‘treatment’ as the fixed effect, a Poisson distribution and log as link function. Ovary size was normally distributed following log transformation (goodness-of-fit test: $P > 0.05$) and was tested using an ANOVA mixed model with ‘treatment’ as the fixed effect and ‘cage’ as a random factor. The effect of ‘parental colony’

was examined and included as a random factor if found significant. Post hoc tests were performed using contrast (following GLM) or using Tukey test (following ANOVA mixed model) between all pairs. To account for multiple testing, we used a Bonferroni correction and provide the corrected P value for each experiment.

Results

The type of brood significantly affected the number of eggs laid by workers (GLM: $\chi^2_5 = 66.7$, $P < 0.001$; Fig. 1a). Pairwise contrasts between all treatments showed that workers in the EL (eggs that developed into larvae) and LL (young larvae that remained larvae) groups laid significantly fewer eggs compared with all other treatments ($P < 0.001$). Workers in the PP (pupae that remained pupae) groups laid significantly more eggs compared with EL, LL and wax treatments ($P < 0.001$), and workers in the LP (larvae that developed into pupae) and PW (pupae that emerged during the experiment) groups did not differ from the wax group ($P = 0.15$, $P = 0.25$, respectively). All pairwise comparisons are provided in Table 1; significance was accepted at an adjusted P value of 0.003 to account for multiple testing. The parental colony did not affect the number of eggs laid by workers and was not included in the model (GLM: $\chi^2_5 = 10.7$, $P = 0.06$). Worker ovary size (Table 1) was significantly affected by parental colony (ANOVA mixed model: $F_{5,313} = 3.66$, $P = 0.003$), but not by treatment (ANOVA mixed model: $F_{5,313} = 2.16$, $P = 0.06$).

Next, we repeated the experiment using callow (newly emerged, <24 h) workers. On average, callow workers laid fewer eggs (2.2 ± 0.3 eggs, $N = 61$ cages) compared with random-age workers (11.7 ± 0.6 , $N = 158$ cages) over 7 days. Despite that, similar results were obtained with a significant effect of brood type on the cumulative number of eggs laid by workers (GLM:

$\chi^2_5 = 43.5$, $P < 0.001$; Fig. 1b, Table 4) and no significant effect by parental colony ($\chi^2_3 = 6.6$, $P = 0.08$). The number of eggs laid by workers kept with EL was significantly lower compared with all other groups ($P < 0.001$), except the wax group ($P = 0.06$), and the cumulative number of eggs laid by workers kept with PP was higher than all other groups (although not significantly higher than LL, LP and PW). The main difference compared with the first experiment was the lower number of eggs laid by workers in the wax treatment (WW versus EL: $\chi^2_1 = 3.3$, $P = 0.06$; WW versus LL: $\chi^2_1 = 5.01$, $P = 0.02$; WW versus LP: $\chi^2_1 = 10.9$, $P < 0.001$; WW versus PP: $\chi^2_1 = 18.4$, $P < 0.001$; WW versus PW: $\chi^2_1 = 5.57$, $P = 0.01$). Worker ovary size was not affected by parental colony (ANOVA mixed model: $F_{3,119} = 1.01$, $P = 0.39$) or by treatment (ANOVA mixed model: $F_{5,117} = 0.82$ $P = 0.53$; Table 1).

To examine the importance of relatedness, we compared worker reproduction in the presence of brood from a foreign colony. Here, we found similar results to those obtained with brood from the mother colony (Fig. 2a versus Fig. 1a) with a significant effect of brood type on the number of eggs laid by workers (GLM: $\chi^2_4 = 72.1$, $P < 0.001$; Table S3). Workers in the EL/LL groups (in only one cage larvae remained larvae throughout the experiment and therefore EL and LL groups were combined) laid significantly fewer eggs compared with all other groups ($P < 0.001$) while workers from the PP and PW groups laid significantly more eggs compared with the wax group ($P < 0.001$). The parental colony did not affect the number of eggs laid by workers (GLM: $\chi^2_2 = 2.28$, $P = 0.32$; Table 5). Worker ovary size was not affected by parental colony (ANOVA mixed model: $F_{2,184} = 0.7$, $P = 0.49$) and did not differ across treatment groups (ANOVA mixed model: $F_{4,182} = 0.89$ $P = 0.47$; Table 1).

To test the effect of brood parentage/sex, we compared worker reproduction in the presence of larvae laid by the queen (female), in the presence of larvae laid by workers (males) and in the presence of wax. Workers grouped with any larvae (laid by the queen or the workers)

were found to lay fewer eggs compared with workers grouped either with wax from their own colony or with wax from a foreign colony (GLM: $\chi^2_3 = 33.7$, $P < 0.001$, followed by contrasts $P < 0.001$; Fig. 2b, Table 6). No significant differences were found between worker- and queen-laid larvae ($P = 0.44$), or between the two different wax groups ($P = 0.49$). Ovary size was significantly affected by parental colony (ANOVA mixed model: $F_{1,79} = 4.22$, $P = 0.04$; Table 1) but not by treatment (ANOVA mixed model: $F_{3,77} = 2.34$, $P = 0.08$).

Finally, we examined the quantitative effect of larvae. The number of young larvae housed with workers significantly affected egg laying (GLM: $\chi^2_3 = 259.2$, $P < 0.001$; Fig. 2c, Table 7). Workers housed with wax laid significantly more eggs compared with workers housed with any number of young larvae ($P < 0.001$), and the impact of young larvae was quantity dependent, with a 39% reduction in egg laying in the presence of one to two young larvae (6.12 ± 1.6 compared with 9.91 ± 0.6 eggs on average) and nearly complete suppression of egg laying in workers housed with more than 11 young larvae (1.96 ± 0.7 eggs compared with 9.91 ± 0.6 eggs). Worker ovary size was significantly affected by parental colony (ANOVA mixed model: $F_{11,287} = 2.58$, $P = 0.003$; Table 1). However, no significant difference in ovary size was found between treatment groups (ANOVA mixed model: $F_{3,295} = 2.51$, $P = 0.059$).

Discussion

The results of the current study demonstrate that worker reproduction in *B. impatiens* is not only regulated by the queen (Alaux, et al., 2004; Amsalem, et al., 2017; Padilla, et al., 2016) and the nestmates (Bloch and Hefetz, 1999) but also by the brood, with opposing effects of young larvae and pupae on worker egg laying but not on worker ovary size. Regulation of female reproduction by offspring has been demonstrated in various insect species and typically

represents a trade-off between brood care and future reproduction (Engel, et al., 2016; Maisonnasse, et al., 2010; Schultner, et al., 2017; Tallamy and Denno, 1982; Ulrich, et al., 2016). However, in social species, the brood can also function as a social signal, which provides workers with information about the status of the queen and the health and nutritional state of the colony. Such information can assist workers with reproductive decisions in simple eusocial societies where workers retain the ability to reproduce. For example, *B. terrestris* workers eavesdrop on a queen signal by monitoring larvae development and initiate reproduction as soon as gyne larvae are produced (Alaux, et al., 2006). In *Polistes exclamans* wasps, females develop characteristics more typical of future queens in the absence of brood (Solis and Strassmann, 1990), and in several species of queenless ants, workers forgo reproduction in the presence of larvae (Heinze, et al., 1996; Ulrich, et al., 2016). In advanced eusocial species where workers have lost the ability to reproduce, such as the honey bee, signals produced by the brood may regulate collective behaviors performed by workers, such as brood care and foraging (Maisonnasse, et al., 2010), and hygienic behavior (Wagoner, et al., 2018). Similarly, in advanced eusocial ant colonies, brood has various signaling roles. In fire ants, and possibly also in army ants, brood induce tending behavior, ensuring the proper function of the colony (Bigley and Vinson, 1975; Glancey, et al., 1970). In monogynous and polydomous colonies of the ant *Novomessor cockerelli*, where a large proportion of nest workers are physically isolated from the queen for extended periods, the brood serve to signal the presence of the queen (Ebie, et al., 2015). In another ant species, *Aphaenogaster senilis*, worker reproduction is inhibited by the brood, providing the workers the opportunity to prolong the life of the colony after the queen's death (Villalta, et al., 2015).

Our results show that young larvae consistently affected egg laying, but not ovary size, of workers across all experiments. Since both callow and random-age workers responded similarly to the presence of brood (Fig. 1), it is unlikely that these responses were due to the age of the

worker. However, this may indicate that ovary size and egg laying are regulated separately. Bumble bee workers that are kept with the queen do not activate their ovaries or lay eggs either in small groups (Alaux, et al., 2006; Amsalem, et al., 2017; Padilla, et al., 2016) or in full-size colonies during the precompetition phase (Duchateau and Velthuis, 1988). However, in all these studies, the queen was always presented to workers together with her brood, making it impossible to know whether the queen directly inhibited worker reproduction using behavioral or chemical means, or indirectly through her brood. Future studies examining the effects of the queen and her brood separately can clarify whether the roles of the queens and the brood are complementary or additive. The complementary roles explanation aligns well with several previous findings in bumble bees, such as the ability of dominant, egg-laying workers (Bloch and Hefetz, 1999) or virgin, egg-laying queens (Amsalem, et al., 2017) to inhibit reproduction in workers, the ability of brood to inhibit egg laying but not ovary size (this paper), and the ability of old, egg-laying queens that are no longer capable of inhibiting worker reproduction in a full-sized colony (Cnaani, et al., 2002) to do so when grouped with few workers (Amsalem, et al., 2017). This explanation agrees with the lack of correlation between the queen switching to laying haploid male eggs and the initiation of the competition phase (Duchateau and Velthuis, 1988), since even male brood postpones worker reproduction. In fact, the combination of behavioral coercion by the queen (either combined or not combined with fertility signals) in small groups and the quantity-dependent inhibitory effect of young larvae provides the only holistic explanation for the regulation of worker sterility in bumble bee colonies thus far.

The opposing effects of larvae and pupae on worker reproduction fit a mechanism of activator-suppressor, where two conflicting signals are produced at the same time, and the behavioral output depends on the ratio between them. A similar mechanism was shown in *Monomorium* ants that mark rewarding and unrewarding trails with attractant and repellent

pheromones, respectively (Robinson, et al., 2005), in *Camponotus floridanus* ants in which queen eggs induce the opposite effects of larvae and pupae (Endler, et al., 2004), and in the honey bee where two components of the brood pheromone are produced by larvae: E- β -ocimene is produced by young brood and accelerates behavioral maturation, whereas brood ester pheromone is produced by old brood and delays behavioral maturation in workers (Maisonasse, et al., 2010). Such a mechanism maintains a balance of supply and demand and suits systems that are highly dynamic.

The workers in the present study exhibited similar reproductive responses to queen- and worker-laid brood, and to related (full sisters) and unrelated brood. This suggests that they were unable to differentiate between diploid and haploid brood or between kin and nonkin, as would be predicted by kin selection theory. Previous studies in *B. terrestris* found that workers discriminate between queen- and worker-laid eggs using signals on eggs and egg cells, and police them accordingly (Zanette, et al., 2012). However, such policing occurs soon after the eggs are laid (during the first 24 h) and rarely occurs later. The eggs and the larvae in the present study were introduced to workers several days after they were laid. The present data demonstrate that workers either lack a discriminatory ability towards brood or their responses to brood are not motivated by relatedness. Workers may still exhibit a preference towards relatives if given a choice, as shown in honey bees (Noonan, 1986; Page, et al., 1989). However, our experimental design did not include such a choice.

The reproductive responses of callow and random-age workers were similar in the presence of young larvae and pupae, but the number of eggs laid was substantially different in the presence of wax (Fig. 1a, b). Previous work in *B. terrestris* (Rottler-Hoermann, et al., 2016; Rottler, et al., 2013) has shown that wax from young precompetition colonies, but not from old, competition-phase colonies (where worker reproduction occurs) inhibits worker reproduction.

These authors suggested that queen odors are embedded in the wax and that these odors provide workers with information about the status of the colony and the best time to reproduce.

Interestingly, the workers in Rottler-Hoermann et al.'s (2016) study were sampled as callows.

Differential responses of callows and random-age workers were also found in a previous study (Amsalem, et al., 2015). Response to wax may depend on the worker's previous experience with wax odors. In the present study, random-age workers had the opportunity to learn the wax odors and did not respond to it, while callow workers were sampled prior to acquiring such knowledge. This may indicate that the response to wax is innate but can be overwritten by an extended exposure to the signal.

Finally, an intriguing question is the mechanistic basis of the inhibitory effect of young larvae on worker egg laying. While this is beyond the scope of the current study, brood pheromone emitted by eggs or larvae has been shown in the honey bee and in several ant species (Bigley and Vinson, 1975; Endler, et al., 2004; Maisonnasse, et al., 2010). However, primitively eusocial species such as bumble bees tend to rely more heavily on behavioral rather than chemical mechanisms to regulate reproductive division of labor (Kocher and Grozinger, 2011), and despite extensive research in the field, there is no evidence thus far that chemicals alone can significantly inhibit worker reproduction in bumblebees (Amsalem, et al., 2015). This, however, awaits further studies.

Conclusions

The results of this study indicate that the presence of young larvae significantly reduces worker egg laying in *B. impatiens*. This effect is replicable regardless of the workers' age, their relatedness to the brood or the parentage/sex of the brood, and it is strongly dependent on the number of larvae. These findings demonstrate that the queen inhibits worker reproduction both directly and indirectly, via her brood. These findings also highlight the role of brood in regulating

reproduction in simple eusocial species such as bumble bees. Previous studies on the role of brood focused mostly on solitary or advanced eusocial insects, demonstrating either a simple trade-off between brood care and reproduction in solitary species or the regulation of collective behaviors (such as foraging and brood care) in advanced eusocial insects. Studying the role of brood in primitively eusocial insects may fill the gap in knowledge of the process by which brood evolved to regulate social behavior. The use of simple insect societies to understand the behavioral, chemical and molecular regulation of advanced eusocial societies has been repeatedly highlighted in the past few years and has proven to be a powerful method to study the mechanistic basis of social behavior (Amsalem, et al., 2015; Jandt, et al., 2013; Kronauer and Libbrecht, 2018). Brood care is one of the trajectories by which eusociality has evolved in holometabolous insects (Kronauer and Libbrecht, 2018), and the findings that the social organization of simple eusocial insects such as bumble bees still mostly relies on brood presence is striking and opens new realms of opportunities to study the evolution of brood care.

Table and figure captions

Table 1: The average terminal oocyte size of workers and statistical analyses of the effect of brood on workers ovary size

Experiment	Treatment	Oocyte size (mm)±S.E.M	Sample size	Statistical differences	
				Colony effect	Brood type effect
1a. The effect of related brood on random-age worker ovary size	EL	1.64±0.15	38	f=3.66, p=0.003	f=2.16, p=0.06
	LL	2.08±0.15	24		
	LP	2.22±0.09	83		
	PP	1.98±0.13	59		
	PW	1.91±0.19	24		
	WW	2.01±0.09	90		
1b. The effect of related brood on 7-days old workers ovary size	EL	1.73±0.17	22	f=1.01, p=0.39	f=0.82, p=0.53
	LL	1.84±0.23	18		
	LP	1.99±0.19	22		
	PP	2.12±0.14	20		
	PW	1.84±0.19	20		
	WW	1.98±0.18	20		
2a. The effect of unrelated brood on random-age worker ovary size	EL/LL	2.14±0.14	28	f=0.7, p=0.49	f=0.89, p=0.47
	LP	2.41±0.13	40		
	PP	2.46±0.12	30		
	PW	2.45±0.1	42		
	WW	2.37±0.1	46		
2b. The effect of brood parentage/sex and wax on 7-days old worker ovary size	Queen-laid larvae	1.98±0.25	20	f=4.22, p=0.04	f=2.34, p=0.08
	Worker-laid larvae	1.99±0.19	20		
	Wax	2.6±0.19	20		
	Foreign wax	2.4±0.19	20		
2c. The quantitative effect of larvae on 7-days old worker ovary size	1-2 Larvae	2.54±0.12	34	f=2.58, p=0.003	f=2.51, p=0.059
	3-10 Larvae	1.85±0.13	34		
	More than 11 larvae	2±0.1	48		
	Wax	2.16±0.06	182		

Figure 1

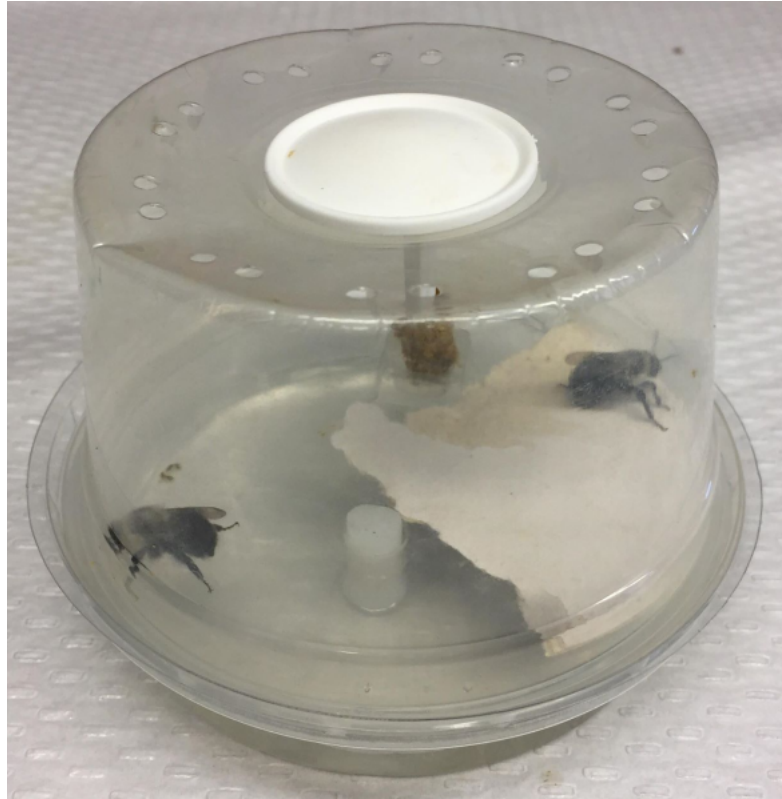


Figure 1: **An image of the custom plastic cages used in experiments.** Pairs of bees in all experiments (excluding experiment 3.3) were placed in plastic cages containing unlimited 60% sugar solution and Light Spring Harvested Bee Pollen (purchased from 911 Honey). The bees and the brood or wax were placed on top of a cardboard. Cages were kept under constant dark, 28-30° C, 60%humidity and were maintained under red light.

Figure 2

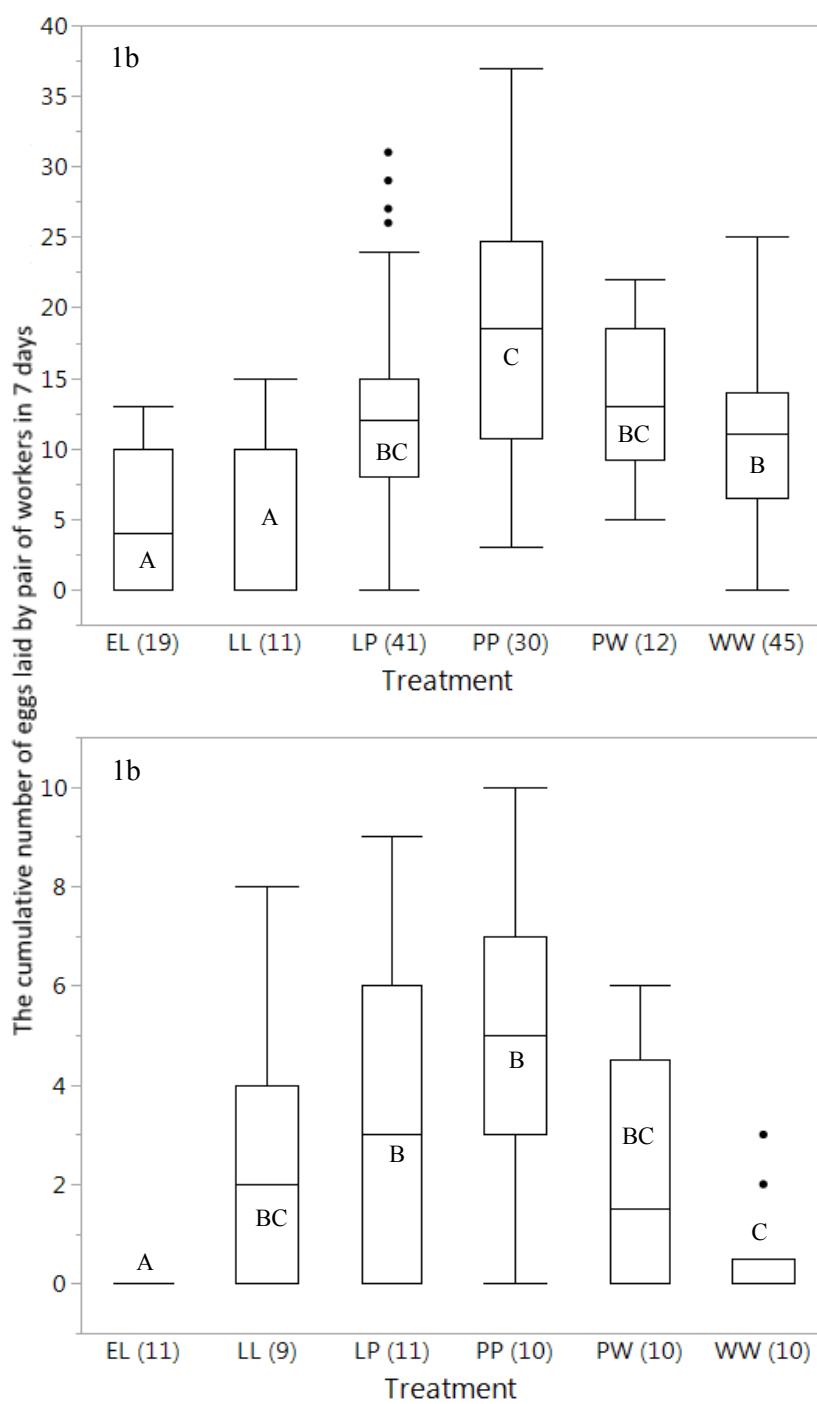


Figure 2: **The effect of related brood and wax on the cumulative number of eggs laid by *B. impatiens* workers.** Workers were either randomly sampled from young queen-right colonies (1a, or upon emergence (1b) and kept in pairs with brood at different developmental phases for seven days. The brood is expressed as the developmental stage at the onset (Eggs, Larva, Pupa, Wax; first letter) and termination (second letter) of the experiment. All workers were kept in pairs of sisters with female brood from their mother colony. Numbers in brackets represent the number of cages. Letters within columns denote statistical differences at $\alpha=0.003$ following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Tables 3 and 4.

Figure 3

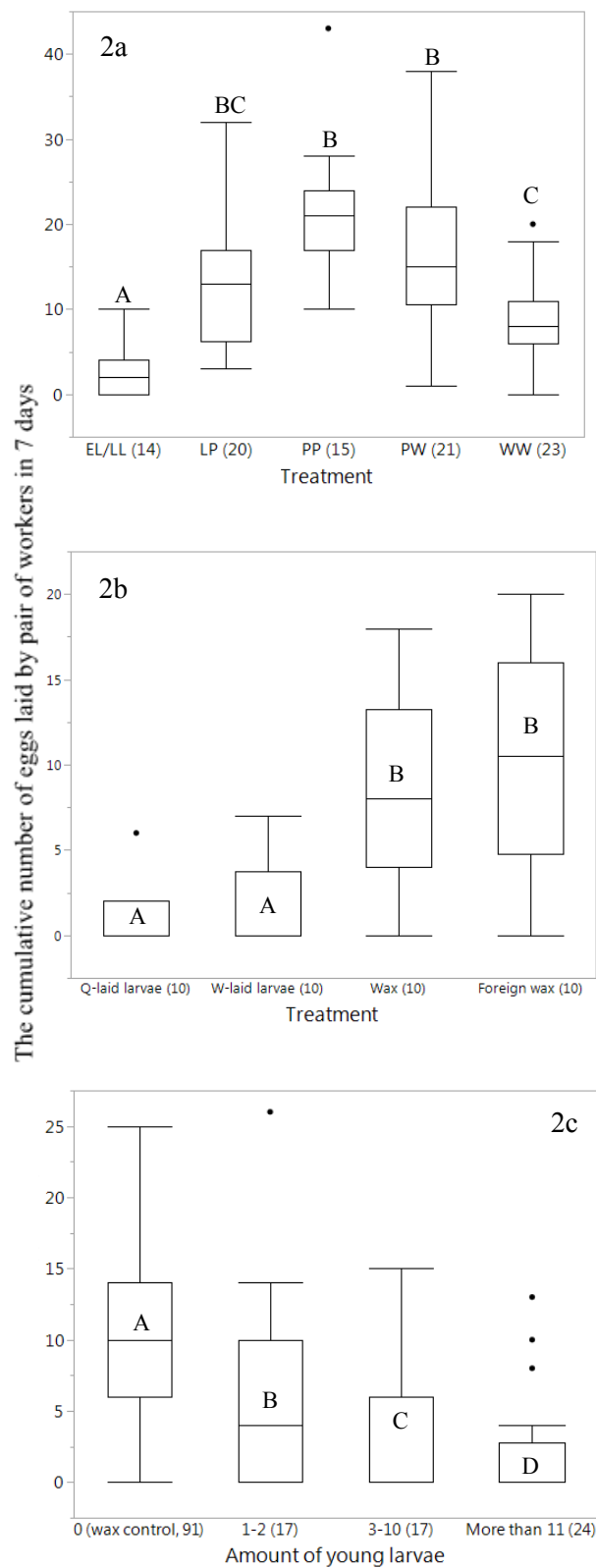


Figure 3: **The effect of relatedness, parentage/sex and amount of brood on the cumulative number of eggs laid by *B. impatiens* workers.** Random-age workers were randomly sampled from young queen-right colonies and kept in pairs with brood at different developmental phases for seven days. Workers were kept with brood from a foreign colony (2.1), with young larvae laid by the queen (females), young larvae laid by queen-less workers (males), wax from their own colony or from a foreign colony (2.2), or with varying numbers of young larvae (2.3). Numbers in brackets represent the number of cages. Letters within columns denote statistical differences at $\alpha=0.005$ (2.1) or at $\alpha=0.008$ (2.2 and 2,3) following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Tables 5-7.

References

- Alaux, C., Jaisson, P., & Hefetz, A. (2004). Queen influence on worker reproduction in bumblebees (*Bombus terrestris*) colonies. *Insectes Sociaux*, *51*(3), 287–293.
- Alaux, C., Jaisson, P., & Hefetz, A. (2006). Regulation of worker reproduction in bumblebees (*Bombus terrestris*): Workers eavesdrop on a queen signal. *Behavioral Ecology and Sociobiology*, *60*(3), 439–446.
- Amsalem, E., Grozinger, C.M., Padilla, M., & Hefetz, A. (2015). The physiological and genomic bases of bumble bee social behaviour. *Advances in Insect Physiology*, *48*, 37–93.
- Amsalem, E., & Hefetz, A. (2010). The appeasement effect of sterility signaling in dominance contests among *Bombus terrestris* workers. *Behavioral Ecology and Sociobiology*, *64*(10), 1685–1694.
- Amsalem, E., & Hefetz, A. (2011). The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris*. *PLoS One*, *6*(3), e18238.
- Amsalem, E., Orlova, M., & Grozinger, C.M. (2015). A conserved class of queen pheromones? Re-evaluating the evidence in bumblebees (*Bombus impatiens*). *Proceedings of the Royal Society B: Biological Sciences*, *282*(1817), 20151800.
- Amsalem, E., Padilla, M., Schreiber, P.M., Altman, N.S., Hefetz, A., & Grozinger, C.M. (2017). Do bumble bee, *Bombus impatiens*, queens signal their reproductive and mating status to their workers? *Journal of Chemical Ecology*, *43*(6), 563–572.
- Amsalem, E., Twele, R., Francke, W., & Hefetz, A. (2009). Reproductive competition in the bumble-bee *Bombus terrestris*: Do workers advertise sterility? *Proceedings of the Royal Society B: Biological Sciences*, *276*(1660), 1295–1304.
- Bigley, W.S., & Vinson, S.B. (1975). Characterization of a brood pheromone isolated from the

- sexual brood of the imported fire ant, *Solenopsis invicta*. *Annals of the Entomological Society of America*, 68(2), 301–304.
- Bloch, G. (1999). Regulation of queen–worker conflict in bumble-bee (*Bombus terrestris*) colonies. *Proceedings of the Royal Society B: Biological Sciences*, 266(1437), 2465–2469.
- Bloch, G., & Hefetz, A. (1999). Reevaluation of the role of mandibular glands in regulation of reproduction in bumblebee colonies. *Journal of Chemical Ecology*, 25(4), 881–896.
- Bloch, G., & Hefetz, A. (1999). Regulation of reproduction by dominant workers in bumblebee (*Bombus terrestris*) queenright colonies. *Behavioral Ecology and Sociobiology*, 45(2), 125–135.
- den Boer, S.P.A., & Duchateau, M.J.H.M. (2006). A larval hunger signal in the bumblebee *Bombus terrestris*. *Insectes Sociaux*, 53(3), 369–373.
- Cnaani, J., Schmid-Hempel, R., & Schmidt, J.O. (2002). Colony development, larval development and worker reproduction in *Bombus impatiens* Cresson. *Insectes Sociaux*, 49(2), 164–170.
- Danforth, B.N., Cardinal, S., Praz, C., Almeida, E.A., & Michez, D. (2013). The impact of molecular data on our understanding of bee phylogeny and evolution. *Annual Review of Entomology*, 58, 57–78.
- Duchateau, M.J., & Velthuis, H.H.W. (1988). Development and reproductive strategies in *Bombus terrestris* colonies. *Behaviour*, 107, 186–207.
- Ebie, J.D., Holldobler, B., & Liebig, J. (2015). Larval regulation of worker reproduction in the polydomous ant *Novomessor cockerelli*. *Naturwissenschaften*, 102(11–12), 72.
- Endler, A., Liebig, J., Schmitt, T., Parker, J.E., Jones, G.R., Schreier, P., et al. (2004). Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect. *Proceedings of the National Academy of Sciences of the United States of America*, 101(9), 2945–2950.

- Engel, K.C., Stökl, J., Schwizer, R., Vogel, H., Ayasse, M., Ruther, J., . (2016). A hormone-related female anti-aphrodisiac signals temporary infertility and causes sexual abstinence to synchronize parental care. *Nature Communications*, 7, 11035.
- Free, J.B., & Winder, M.E. (1983). Brood recognition by honeybee (*Apis mellifera*) workers. *Animal Behaviour*, 31(2), 539–545.
- Glancey, B.M., Stringer, D.E., Craig, C.H., Bishop, P.M., & Martin, B.B. (1970). Pheromone may induce brood tending in the fire ant, *Solenopsis saevissima*. *Nature*, 226(5248), 863–864.
- Hamilton, W.D. (1964). The genetical evolution of social behaviour I. *Journal of Theoretical Biology*, 7, 1–16.
- Heinze, J., Trunzer, B., Oliveira, P.S., & Hölldobler, B. (1996). Regulation of reproduction in the neotropical ponerine ant, *Pachycondyla villosa*. *Journal of Insect Behavior*, 9(3), 441–450.
- Helms, K.R., Fewell, J.H., & Rissing, S.W. (2000). Sex ratio determination by queens and workers in the ant *Pheidole desertorum*. *Animal Behaviour*, 59(3), 523–527.
- van Honk, C.G.J., Velthuis, H.H.W., Röseller, P.-F., & Malotau, M.E. (1980). The mandibular glands of *Bombus terrestris* queens as a source of queen pheromone. *Entomologia Experimentalis et Applicata*, 28, 191–198.
- Hunt, J., & Simmons, L.W. (2004). Optimal maternal investment in the dung beetle *Onthophagus taurus*? *Behavioral Ecology and Sociobiology*, 55(3), 302–312.
- Jandt, J.M., Tibbetts, E.A., & Toth, A.L. (2013). *Polistes* paper wasps: A model genus for the study of social dominance hierarchies. *Insectes Sociaux*, 61(1), 11–27.
- Kilner, R., & Johnstone, R.A. (1997). Begging the question: Are offspring solicitation behaviours signals of need? *Trends in Ecology & Evolution*, 12(1), 11–15.
- Kocher, S.D., & Grozinger, C.M. (2011). Cooperation, conflict, and the evolution of queen

- pheromones. *Journal of Chemical Ecology*, 37(11), 1263–1275.
- Kölliker, M. (2007). Benefits and costs of earwig (*Forficula auricularia*) family life. *Behavioral Ecology and Sociobiology*, 61(9), 1489–1497.
- Kronauer, D.J., & Libbrecht, R. (2018). Back to the roots: The importance of using simple insect societies to understand the molecular basis of complex social life. *Current Opinion in Insect Science*, 28, 33–39.
- Leonhardt, S.D., Menzel, F., Nehring, V. & Schmitt, T. (2016). Ecology and evolution of communication in social insects. *Cell*, 164(6), 1277–1287.
- Maisonnasse, A., Lenoir, J.C., Beslay, D., Crauser, D., & Le Conte, Y. (2010). E- β -ocimene, a volatile brood pheromone involved in social regulation in the honey bee colony (*Apis mellifera*). *PLoS One*, 5(10), e13531.
- Mas, F., & Kölliker, M. (2008). Maternal care and offspring begging in social insects: Chemical signalling, hormonal regulation and evolution. *Animal Behaviour*, 76(4), 1121–1131.
- Melgarejo, V., Wilson Rankin, E.E., & Loope, K.J. (2018). Do queen cuticular hydrocarbons inhibit worker reproduction in *Bombus impatiens*? *Insectes Sociaux*, 65(4), 601–608.
- Noonan, K.C. (1986). Recognition of queen larvae by worker honey bees (*Apis mellifera*). *Ethology*, 73(4), 295–306.
- Padilla, M., Amsalem, E., Altman, N., Hefetz, A., & Grozinger, C.M. (2016). Chemical communication is not sufficient to explain reproductive inhibition in the bumblebee *Bombus impatiens*. *Royal Society Open Science*, 3(10), 160576.
- Page, R.E., Robinson, G.E., & Fondrk, M.K. (1989). Genetic specialists, kin recognition and nepotism in honey-bee colonies. *Nature*, 338(6216), 576–579.
- Passera, L., & Aron, S. Early sex discrimination and male brood elimination by workers of the Argentine ant. (1996). *Proceedings of the Royal Society B: Biological Sciences*, 263(1373), 1041–1046.

- Pereboom, J.J.M., Duchateau, M.J., & Velthuis, H.H.W. (2003). The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation. *Insectes Sociaux*, 50(2), 127–133.
- Pirk, C.W.W., Neumann, P., Hepburn, R., Moritz, R.F.A., & Tautz, J. (2004). Egg viability and worker policing in honey bees. *Proceedings of the National Academy of Sciences of the United States of America*, 101(23), 8649–8651.
- Queller, D.C. (1994). Extended parental care and the origin of eusociality. *Proceedings of the Royal Society B: Biological Sciences*, 256(1346), 105–111.
- Ratnieks, F.L.W. (2015). Evidence for a queen-produced egg-marking pheromone and its use in worker policing in the honey bee. *Journal of Apicultural Research*, 34(1), 31–37.
- Rauter, C.M., & Moore, A.J. (1999). Do honest signalling models of offspring solicitation apply to insects? *Proceedings of the Royal Society B: Biological Sciences*, 266(1429), 1691–1696.
- Robinson, E.J.H., Jackson, D.E., Holcombe, M., & Ratnieks, F.L.W. (2005). ‘No entry’ signal in ant foraging. *Nature*, 438, 442.
- Rottler-Hoermann, A.M., Schulz, S., & Ayasse, M. (2016). Nest wax triggers worker reproduction in the bumblebee *Bombus terrestris*. *Royal Society Open Science*, 3(1), 150599.
- Rottler, A.M., Schulz, S., & Ayasse, M. (2013). Wax lipids signal nest identity in bumblebee colonies. *Journal of Chemical Ecology*, 39(1), 67–75.
- Schultner, E., Oettler, J., & Helantera, H. (2017). The role of brood in eusocial Hymenoptera. *Quarterly Review of Biology*, 92(1), 39–78.
- Sibbald, E.D., & Plowright, C.M.S. (2012). On the relationship between aggression and reproduction in pairs of orphaned worker bumblebees (*Bombus impatiens*). *Insectes Sociaux*, 60(1), 23–30.

- Sibbald, E.D., & Plowright, C.M.S. (2014). Social interactions and their connection to aggression and ovarian development in orphaned worker bumblebees (*Bombus impatiens*). *Behaviour Processes*, *103*, 150–155.
- Solis, C.R., & Strassmann, J.E. (1990). Presence of brood affects caste differentiation in the social wasp, *Polistes exclamans viereck* (Hymenoptera: Vespidae). *Functional Ecology*, *4*(4), 531–541.
- Sramkova, A., Schultz, C., Twele, R., Francke, W., & Ayasse, M. (2008). Fertility signals in the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). *Naturwissenschaften*, *95*(6), 515–522.
- Stokl, J., & Steiger, S. (2017). Evolutionary origin of insect pheromones. *Current Opinion in Insect Science*, *24*, 36–42.
- Tallamy, D.W. (1984). Insect parental care. *BioScience*, *34*(1), 20–24.
- Tallamy, D.W., & Denno, R.F. (1982). Life history trade-offs in *Gargaphia solani* (Hemiptera: Tingidae): The cost of reproduction. *Ecology*, *63*(3), 616–620.
- Trivers, R.L. (1972). *Parental investment and sexual selection*. Cambridge, MA: Harvard University Press.
- Trivers, R.L., & Hare, H. (1976). Haplodiploidy and the evolution of the social insects. *Science*, *191*, 249–263.
- Ulrich, Y., Burns, D., Libbrecht, R., & Kronauer, D.J. (2016). Ant larvae regulate worker foraging behavior and ovarian activity in a dose-dependent manner. *Behavioral Ecology and Sociobiology*, *70*(7), 1011–1018.
- Van Oystaeyen, A., Oliveira, R.C., Homan, L., van Zweden, J.S., Romero, C., Oi, C.A., et al. (2014). Conserved class of queen pheromones stops social insect workers from reproducing. *Science*, *343*(6168), 287–290.
- Villalta, I., Angulo, E., Devers, S., Cerdá, X. & Boulay, R. (2015). Regulation of worker egg

- laying by larvae in a fission-performing ant. *Animal Behaviour*, *106*, 149–156.
- Wagoner, K.M., Spivak, M., & Rueppell, O. (2018). Brood affects hygienic behavior in the honey bee (Hymenoptera: Apidae). *Journal of Economic Entomology*, *111*(6), 2520–2530.
- Wilson, E.O. (1971). *The insect societies*. Cambridge, Massachusetts: Belknap Press of Harvard University Press.
- Woodard, S.H., Bloch, G., Band, M.R., & Robinson, G.E. (2013). Social regulation of maternal traits in nest-founding bumble bee (*Bombus terrestris*) queens. *Journal of Experimental Biology*, *216*(Pt 18), 3474–3482.
- Zanette, L.R., Miller, S.D., Faria, C.M., Almond, E.J., Huggins, T.J., Jordan, W.C., et al. (2012). Reproductive conflict in bumblebees and the evolution of worker policing. *Evolution*, *66*(12), 3765–3777.
- Zink, A.G. (2003). Quantifying the costs and benefits of parental care in female treehoppers. *Behavioral Ecology*, *14*(5), 687–693.

Chapter 3

Do Bumblebees produce brood pheromones?

Introduction

Reproductive division of labor is a hallmark of social insect societies, but the mechanisms that regulate it are complex and not fully understood (Fletcher and Ross 1985; Kocher and Grozinger 2011). The end result of a reproductive skew in favor of one or few dominant females can be achieved through either behavioral enforcement or behavior-inducing pheromones produced by the queen, workers, or the brood (Fletcher and Ross 1985; Le Conte and Hefetz 2008; Peeters and Liebig 2009). The extent to which these mechanisms are employed can vary depending on the social structure of the species and their colony size. Social insects exhibit remarkable diversity of social organizations from subsocial, family-structured species to insects exhibiting primitive and advanced eusociality. Primitive eusocial insects are typically characterized by varying levels of reproductive skew with workers retaining their ability to reproduce, limited use of pheromones and overt conflicts over reproduction (Michener 1974). In contrast, advanced eusocial species are characterized by extreme reproductive skew (usually in favor of one female), extensive use of pheromones and little to no overt aggression over reproduction. These fundamentally different social organizations often vary in group size from dozens (primitive) to up to hundreds of thousands (advanced) of individuals. Differences in colony size may directly influence the effectiveness of behavioral or pheromonal means in regulating female reproduction (Beshers and Fewell 2001; Naug 2009).

Behavioral means, usually in the form of aggressive behavior, are hypothesized to play a more important role in regulating reproduction in smaller-sized, primitive compared to advanced eusocial insect species (Kocher and Grozinger 2011). Primitively eusocial females competing for reproductive dominance often engage in non-lethal bouts of “mauling”, biting, or pouncing to establish reproductive hierarchies (Amsalem and Hefetz 2010; Jandt et al. 2014; Monnin et al. 2002). Dominant females of the social wasp *Ropalidia marginata* use aggression to force subordinates to perform maintenance or risky tasks such as foraging (Premnath 1996), and in more advanced eusocial insects such as the honey bee, aggression, although rare, may be exhibited by sterile individuals towards egg laying females (policing behavior), enforcing the monopolization of the queen (Ratnieks and Visscher 1989). These behavioral interactions have physiological consequences, inhibiting ovarian activation in the loser or leading to sterility due to a tradeoff between maintenance tasks (e.g., foraging) and reproduction (Liebig et al. 1999a; Premnath et al. 1995; Premnath 1996).

In contrast, pheromones regulating reproduction are typical of large, advanced eusocial insect colonies where the dominant female is unable to physically interact with all the individuals in the colony. For example, *Apis mellifera* queens produce queen mandibular pheromones that induce immediate (releaser) retinue behavior, in addition to long-term (primer) physiological effects inhibiting ovarian activation in workers (Winston et al. 1989). Queen-produced signals regulating worker reproduction were found also in termites (Funaro et al. 2018; Liebig et al. 2009; Matsuura et al. 2010b) and in several ant and wasp species (Bhadra et al. 2010; Holldobler and Wilson 1983; Oi et al. 2015). These signals have similar physiological consequences to aggression, leading to worker sterility by providing information about the queen’s presence or an honest signal of her fecundity (Keller and Nonacs 1993).

Studies examining the interplay between behavioral and chemical means in regulating reproductive division of labor have traditionally focused on adult traits, mainly behavioral means and pheromones exhibited by the queen or nestmates (Fletcher and Ross 1985). However, most social insect species are rooted in a simple family structure showing a tradeoff between brood care and reproduction (reviewed in [Schultner et al. 2017](#)), highlighting the role of young in shaping the social organization. Indeed, recent studies have shown brood presence to inhibit worker reproduction in a quantity-dependent manner in several primitively eusocial species (Starkey et al. 2019; Ulrich et al. 2016). These findings support previous research in advanced eusocial species, showing both releaser and primer effects mediated by pheromones produced by the brood. For example, brood of *Apis mellifera* produce *E-β-Ocimene* and a blend of esters, which act as both primer and releaser pheromones that alter worker behavior and reduce ovarian activation (Le Conte et al. 2001; Maisonnasse et al. 2010), and brood of the fire ant *Solenopsis invicta* produce triolein, a brood-tending pheromone (Bigley and Vinson 1975). However, the extent to which the effect of the brood in primitively eusocial species is also mediated by a pheromone remains unexplored.

Larvae of many solitary or family-structure species use simple behavioral means to communicate with adults. For example, larvae of the burying beetle *Nicrophorus vespilloides* convey their hunger by rearing up and waving their legs (Engel et al. 2016). However, unlike solitary species that tend a limited number of offspring, social insects may have to tend large number of larvae at the same time. These larvae may differ in their quality or identity (i.e., they may be more or less related to the caregiver, of different age or health condition) (Haig 1990; Trivers and Hare 1976), and may also differ in their need (i.e., some may be hungrier than others as function of the last time they were fed or as function of their developmental phase) (Godfray 1991; Kilner and Johnstone 1997). In these societies, brood signals may be especially useful since the caregiver is

often not the parent and could prioritize care or produce their own offspring based on relatedness (Queller 1994). For example, larvae of *Apis mellifera* produce larger amounts of *E-β-Ocimene* when they are starved (He et al. 2016), and as they age (Maisonnasse et al. 2010). Workers prefer the rearing of queens over workers (Page et al. 1989) and prioritize care for more related brood (Noonan 1986).

Bumble bees are traditionally defined as primitively eusocial (Michener 1974), however they in fact present an interesting intermediate phase during the evolution of eusociality with colonies exhibiting both harmony and competition over reproduction (Amsalem et al. 2015a).

Reproduction in the first harmonious half of the annual life cycle is solely controlled by a single queen and is followed by a competition phase over reproduction between the queen and the workers (Duchateau and Velthuis 1988). In accordance with their social organization, it was suggested that bumble bee worker reproduction is regulated by a mix of behavioral and chemical signals (Amsalem et al. 2015c; Amsalem et al. 2017; Michener 1974; Padilla et al. 2016).

Previous studies on bumble bee reproduction have mostly focused on the impact queen-produced pheromones have on worker reproduction. However, overall, only the direct presence of the queen has been found to reduce ovarian activation in workers and no volatile or non-volatile compounds produced by the queen were found to decrease worker ovary size (Amsalem et al. 2017; Melgarejo et al. 2018; Padilla et al. 2016). Recently, we have shown that worker egg-laying behavior is reduced in the presence of young larvae in a quantity-dependent manner, while the presence of old larvae and pupae increases egg-laying (Starkey et al. 2019). However, the mechanism underlying brood regulation of worker reproduction and whether pheromones mediate these effects remain unknown.

Here we investigated the short- and long-term effects of brood odors on *Bombus impatiens* workers. We hypothesize that 1) young larvae produce a chemical signal that communicates their

presence or need to workers, and 2) young larvae produce a chemical signal that has a primer effect on worker reproduction. We first examined the releaser effects of odors produced by live brood on worker attraction. We then examined the primer effects of these odors by testing if cuticular washes, whole-body extracts and volatiles drawn from the brood affect worker reproduction. Next, we examined the survival of larvae as function of their body mass, hypothesizing that young larvae are more vulnerable to starvation and are thus more dependent on worker care. We then examined the releaser effects of starved and fed larvae on worker attraction and whether odors extracted from starved and fed larvae have a primer effect on worker reproduction.

Methods

Insects

Colonies of *B. impatiens* were obtained from Koppert Biological Systems (Howell Michigan, USA) and maintained in nest-boxes under constant darkness, a temperature of 28–30°C, 60% relative humidity, and supplied *ad libitum* with a sugar solution and fresh pollen (Light spring bee pollen, 911Honey, West Grove, PA). These colonies were used as a source of workers and brood in all experiments. Workers, brood of different developmental phases (eggs, larvae, pupae), and wax were sampled from young, queenright, pre-competition colonies, a phase of the colony cycle during which adult workers have inactive ovaries (Cnaani et al. 2002). Brood cells of eggs, larvae, or pupae were carefully removed from the colony and used only if they remained intact during collection. Larval weight was used as a proxy for instar (Starkey et al. 2019). Larvae weighing less than 50 mg roughly correspond to 1st and 2nd instars and were

classified as ‘young larvae’, while larvae weighing greater than 50 mg roughly correspond to 3rd and 4th instars and were classified as ‘old larvae’.

Examining the Attraction of *Bombus impatiens* Workers to Brood.

Worker responses to brood were examined using *choice bioassays*. We created still-air olfactometer bioassay arenas where bees were presented with either 2 or 3 choices (control versus larvae; control versus pupae; and control versus larvae versus pupae, n=28/choice bioassay, total of 84 bioassays). Olfactometers were fashioned from petri dishes (150 x 15 mm) where equidistant holes (either 2 or 3, 2 cm diameter) led to small plastic cups that held treatment or control stimuli. A 3 cm section of plastic straw glued to the hole created a chute that prevented the responding bee from returning to the main arena after a choice was made. Pollen was provided in all choices to control for worker hunger. All larval treatments contained approximately 10 young larvae (<50 mg). Pupae treatments contained 4-6 pupae and control contained no brood. Brood in all bioassays was unrelated to workers and its location in the arena was randomized between runs. Workers were placed in the center of the olfactometer and were given 30 minutes to make a choice, after which they were considered non-responders. Bioassays were conducted under red light and olfactometers were washed between runs. Both choice and response time were measured. Each bioassay was conducted using a single bee that was sampled from her parental colony prior to the test. Workers were used only once. We controlled for colony effect by repeating each bioassay with workers from four different colonies.

Examining whether *Bombus impatiens* Brood Produces a Contact Pheromone

Regulating Worker Reproduction.

To determine if brood produce a contact pheromone that reduces worker reproduction, pairs of workers were exposed to either whole-body extracts or cuticular washes of brood at

different developmental stages. Workers were kept for 7 days in small plastic micro-colonies (11 cm diameter x 7 cm height), after which they were frozen at -20° C. To account for variation in worker egg laying between colonies (Amsalem et al. 2015; Amsalem et al. 2009), we ensured that each experiment was replicated using workers from several colonies, equally representing both treatment and control groups. We statistically controlled for a colony effect whenever such effect was found.

Preparation of Cuticular Washes

Brood of different developmental stages (eggs, young larvae (<50 mg), old larvae (>50 mg), pupae) were collected from their parental colony and placed in glass vials containing 1-2 ml of a 1:1 mixture of hexane and methanol. We used a mix of polar and non-polar solvents in order to optimize the extraction process. Cuticular compounds were extracted from brood at room temperature for 10 minutes, after which the solvent was transferred to a clean vial and its volume adjusted to 1.4 ml.

Preparation of Whole-body Extracts

Brood of different developmental stages were placed in glass vials containing 1-2 ml of a 1:1 mixture of hexane and methanol. The samples were ground using a glass pipette and left in room temperature for 30 minutes, during which they were frequently mixed. Ground samples were then centrifuged at 3500g for 3 minutes and the supernatant was transferred into new glass vials. 1-2 ml of 1:1 of hexane and methanol solvent was then added to the precipitate and the extraction process was repeated 2 more times.

Washes or extracts, as well as solvent controls, were applied daily in a volume of 200 µl per cage for 7 days into a small filter (Zen Regular Cigarette Filters). Filters were kept outside the cage for 10 minutes after applying the extracts to allow for excess solvent to evaporate. Cuticular

washes were applied daily in a dose of 1.4 larval equivalents per cage (88 cages, 3 colonies). Whole-body extracts were applied daily in a dose of 1.4 and 7 larval equivalents per cage in two consecutive experiments (a total of 136 cages taken from 6 colonies). Egg laying by workers was monitored daily and workers were frozen by the end of the 7th day. The cumulative number of eggs and average terminal oocyte size of workers were compared between workers exposed to extracts of brood at different developmental stage versus solvent control.

Examining whether *Bombus impatiens* Brood Produces a Volatile Pheromone

Regulating Worker Reproduction

To determine if brood produces a volatile pheromone that reduces worker reproduction, we exposed workers to brood volatiles using a previously described vacuum air-flow system (Padilla et al. 2016) with slight modifications (Figure 1). Two 16 oz. glass mason jars were connected in a row to a vacuum pump, allowing air to be drawn from jars containing workers and brood to jars containing only workers. Each pair of jars were assigned to a specific treatment (eggs, larvae <50 mg, larvae >50 mg, pupae, or wax). This system enabled us to test 10 pairs of cages at once (2/treatment). In total, we tested 80 cages in four consecutive runs; each contains workers from a single colony. Air entering the volatile system was filtered through activated-carbon and the air flow held constant at 800 ccm/s for each pair of cages. The cumulative number of eggs and average terminal oocyte size were compared between workers that were exposed directly to brood or indirectly to brood volatiles within the same treatment. The brood in this experiment was alive and thus developed throughout the course of the experiment (7 days), often transitioning between different developmental phases (Cnaani et al. 2002). Brood development was monitored as in (Starkey et al. 2019), and the overall treatment was defined according to the initial (eggs, larvae, pupae) and terminal brood stage (larvae, pupae, “wax”). Eggs (E) hatch within 5-6 days, thus all eggs turned into larvae within 7 days (EL). The feeding period of *B.*

impatiens larvae lasts 9-11 days, thus larvae remained larvae (LL) or turned into pupae (LP) during the experiment. Pupation takes 11-12 days, thus, pupae either remained pupae (PP) or emerged as adults and therefore were only exposed to wax (PW). Pupae that emerged into adult workers were immediately removed from the cages.

Examining the Survival Rate of Starved Larvae

To determine the larval survival rate under starvation, larvae were collected from their parental colonies, separated from their cells, individually weighed and placed in 48 well plate (n=171). The plates were placed on top of a water bath within the rearing chamber (28-30° C and 60% humidity) to maintain humidity close to 100% around the developing larvae. This was critical in order to compensate for the incubating behavior typically performed by workers and that is necessary for larvae survival. Larvae that were held under similar conditions with unlimited food were developed normally. Starved larvae were inspected for signs of life, movements, or change in color under a stereomicroscope every 12 hours until all larvae died.

Examining the Attraction of *Bombus impatiens* Workers to Starved Larvae

Worker responses to starved and fed brood were examined using choice bioassays. We introduced workers to starved and fed larvae in olfactometer bioassay arenas as in experiment 1. Approximately 7 larvae that had just hatched (5-7 days after eggs laid; (Cnaani et al. 2002) were collected from their parental colony and placed in a small plastic cage for 16 hours either with or without 10 nursing workers and unlimited food. 16 hours later, larvae were checked for signs of life and were used only if they were still alive. Queen-right workers in 20 separated choice bioassays were given 30 min to choose between starved and fed larvae, after which they were considered non-responders. We controlled for colony effect by repeating the bioassay with workers from four different colonies.

Examining whether *Bombus impatiens* Brood Produces a Hunger Signal Regulating Worker Reproduction

To determine if *B. impatiens* brood produces a hunger pheromone regulating worker reproduction, starved and fed larvae were reared as in the previous experiment and whole-body extracts of fed and starved larvae were prepared as described above. Whole-body extracts, as well as solvent controls, were applied daily in a dose of 7 larval equivalents per cage for 7 days (a total of 30 cages taken from 3 colonies). Workers were frozen by the end of the 7th day. The cumulative number of eggs and average terminal oocyte size of workers were compared between workers exposed to extracts of starved and fed larvae versus solvent control.

Assessment of Worker Reproduction

Ovarian activation. To perform blind measurements, each bee was placed in a separate tube and received an individual number corresponding with their cage and treatment prior to dissections. Ovaries were dissected under a stereomicroscope and placed into drops of distilled water. The length of the terminal oocyte in the three largest ovarioles was measured with a micrometer eyepiece embedded into the lens. Workers possess four ovarioles per ovary and at least one ovariole per ovary was measured. Mean terminal oocyte length for each bee was used as an index of ovarian activation (Amsalem et al. 2009).

Egg laying. The cumulative number of eggs (or larvae, if eggs hatched) was counted on the day of collection (day 7). While egg oophagy does occur in bumble bees, it is often performed in queen-right colonies and rarely occurs in small queen-less groups (Amsalem et al. 2015a). We did not see evidence of oophagy (such as open egg cells, etc.) that could affect the results.

Statistics

Statistics were performed using JMP Pro 14.1. Choice bioassays were analyzed using a χ^2 test. The egg laying data were not normally distributed (Goodness of fit test $p < 0.001$) and were therefore analyzed using a Generalized Linear Model (GLM) with the ‘treatment’ as the fixed effect, a Poisson distribution, and log as link function. Ovary size data were normally distributed following a log-transformation (Goodness of fit test $p > 0.05$) and were analyzed using an ANOVA Mixed Model with ‘treatment’ as the fixed effect and ‘cage’ as a random factor. The effect of the ‘parental colony’ was examined and included as a random factor if found significant. Post-hoc tests were performed using contrast (following GLM) or using Tukey’s test (following ANOVA Mixed model) between all pairs. To account for multiple testing, we used a Bonferroni correction and provide the corrected p-value for each experiment. To analyze the survival of starved brood over time, a Pearson correlation was performed. To compare the effect of brood volatiles on workers egg laying we used a Matched Pairs test to compare differences within treatments. Data are presented as means \pm S.E.M or as boxplots featuring the minimum and maximum values, outliers and medians. Boxplot columns represent the area between the first and third quartiles.

Results

*Examining the Attraction of *Bombus impatiens* Workers to Brood.*

In order to examine the short-term behavioral responses of workers to brood, we introduced workers to young larvae and pupae in three consecutive choice bioassays: larvae versus control, pupae versus control, or all three options at the same time. This experiment demonstrated a clear preference of workers for pupae over larvae or controls. When workers were

introduced to larvae versus controls, they preferred the larvae (67% vs 33%, n=18, Figure 5), However these observed values did not differ from the expected values ($\chi^2=2.03$, $df=1$, $p=0.153$) and the non-responders' rate was relatively high (36%, 10 out of 28 assays). When workers were introduced to pupae versus controls, their preference for pupae (72% vs 28%, n=24) was significantly higher compared to the expected results ($\chi^2=13.63$, $df=1$, $p=0.001$), and the rate of non-responders was much lower (11%). When workers were introduced to all options (n=25), they significantly preferred the pupae (58%) over larvae (38%) or control (4%), with a relatively low rate of non-responders (14%) ($\chi^2=5$, $df=2$, $p=0.02$). The time to respond in the bioassay that included larvae versus control, was as twice as high compared to the two other choice experiments (730 ± 96 vs 464 ± 95 and 453 ± 51 seconds). However, these differences were not significant ($\chi^2=4.39$, $df=2$, $p=0.11$).

Examining whether *Bombus impatiens* Brood Produces a Contact Pheromone

Regulating Worker Reproduction.

To test whether the short-term behavioral responses to brood translate into long term physiological effects on worker reproduction, we extracted low-volatility compounds from brood and tested their effect on egg laying and ovary size in 7 days long trails. Cuticular washes of brood (1.4 brood-equivalent per day) at different developmental phases (eggs, young larvae, old larvae, pupae) did not affect either workers egg laying or ovary size as compared to the solvent control (egg laying: GLM, $\chi^2=2.88$, $df=4$, $p=0.57$, Figure 6, Table 8; ovary size: $f=0.67$, $df=4$, $p=0.61$, Table 1).

Similarly, whole body extracts (1.4 and 7 brood-equivalent in Figures 5A and 5B, respectively) did not affect either egg laying or ovary size (1.4 brood-equivalent, egg laying: GLM, $\chi^2=8.74$, $df=4$, $p=0.06$, Figure 7A, Table 9; ovary size: ANOVA Mixed model, $f=1.07$,

$df=4$, $p=0.37$, Table 2; 7 brood-equivalent, egg laying: GLM, $\chi^2=5.82$, $df=4$, $p=0.21$, Figure 7B, Table 10; ovary size: ANOVA Mixed model, $f=1.01$, $p=0.4$, Table 2).

Examining whether *Bombus impatiens* Brood Produces a Volatile Pheromone

Regulating Worker Reproduction.

To examine the effects of brood volatiles on worker reproduction (Figure 8), worker egg laying was compared between paired cages containing workers housed with live brood ('direct') or workers housed with no brood who received brood volatiles via air system ('air'). This experiment was repeated 4 times with 4 different colonies, however, no parental effect on egg laying was found ($p>0.11$ for each of the colonies included in this experiment). Comparison of 'air' versus 'direct' within the PW/WW group (pupae that emerged during the duration of the experiment turning into a control wax, and wax groups) showed no significant differences (Matched Pairs test, $t=0.55$, $p=0.59$). However, in all other groups we found significant differences between 'air' versus 'direct'. Egg laying was significantly lower in direct EL/LL groups (eggs that developed to larvae or larvae that remained larvae during the 7 days of the experiment) compared to their air counterparts (Matched Pairs test, $t=-6.52$, $p<0.0001$), while egg laying was significantly greater in direct LP and PP groups (larvae that became pupae or pupae that remained pupae) compared to their air counterparts (Matched Pairs test, LP: $t=2.93$, $p=0.01$, PP: $t=2.8$, $p=0.03$). Overall, these results replicated the findings in our previous study (Starkey et al. 2019) where contact with brood was direct and resulted in suppression of egg laying in the presence of young larvae, but show no differences between treatments when the contact with brood was via air (Figure 8, Table 11).

Worker ovary size also did not differ between 'direct' and 'air' in the groups EL/LL ($t= -0.04$, $p=0.96$), PP ($t= -0.83$, $p=0.43$) and WW ($t= 0.69$, $p=0.5$), but was significantly higher in 'air' versus 'direct' in the LP treatment ($t= -2.84$, $p=0.01$) with exposure to air resulting in

slightly more developed ovaries compared to direct contact (Table 2). In both groups however, workers had fully developed ovaries.

Examining the Survival Rate of Starved Larvae.

We examined the survival time of larvae as function of their body mass and found a positive correlation between larvae mass and duration of survival in hours. Young larvae were more vulnerable to starvation compared to larger larvae ($r=0.73$, $n=171$, $p<0.001$, Figure 9), with the smallest larvae (<10 mg, $n=41$) surviving on average 72 ± 7 hours and the largest larvae (>50 mg, $n=26$) surviving on average 252 ± 13 hours without food or brood care.

Examining the Attraction of *Bombus impatiens* Workers to Starved Larvae.

To examine whether the larval effect on worker reproduction is due to their need (hunger) rather than to their presence, we conducted a choice experiment where workers were introduced to either fed or starved young larvae of approximately the same age. Workers were slightly more attracted to starved compared to fed brood (Figure 10), however this preference was insignificant compared to the expected results ($\chi^2=0.89$, $df=1$, $p=0.34$).

Examining whether *Bombus impatiens* Brood Produces a Hunger Signal Regulating Worker Reproduction.

Daily exposure of workers to whole body extracts of fed larvae, starved larvae, or solvent control resulted in overall significant differences between all three groups (GLM: $\chi^2=6.82$, $p=0.033$). A post-hoc test showed significant differences between the number of eggs laid by workers after exposure to fed and starved larvae ($\chi^2=6.625$, $df=2$, $p=0.01$) but not between any of the treatments and the solvent control (fed versus solvent: $\chi^2=2.64$, $df=2$, $p=0.104$; starved versus

solvent: $\chi^2=0.9$, $df=x$, $p=0.342$, Figure 11, Table 12). The treatment did not affect the ovary size of workers ($f=0.19$, $df=2$, $p=0.82$) (Table 1).

Discussion

The results of this study demonstrate that while *B. impatiens* workers are attracted to olfactory cues or signals from the brood, chemical signals (contact or volatile) alone that are associated with either the brood presence or its hunger, do not explain the reduction in worker egg laying previously observed (Starkey et al. 2019). These findings join previous studies demonstrating that bumble bee worker reproduction is not regulated solely by chemical signals produced by the queen (Amsalem et al. 2014; Amsalem et al. 2017; Bloch and Hefetz 1999; Melgarejo et al. 2018; Padilla et al. 2016), and in line with the small colony size and simple social organization of bumble bees.

While olfactory cues/signals from the brood were not sufficient to regulate worker reproduction, they do seem to be recognized and induce short term behavioral attraction in workers. Our previous study (Starkey et al. 2019) showed that worker egg laying is reduced in the presence of young larvae but is increased in the presence of pupae. Thus, in the absence of brood (likely female larvae produced by the queen, to whom workers are more closely related (Hamilton 1964a)), workers attempt to increase their fitness by producing their own offspring (males). These preferences were also reflected in the choice made by workers: they were more attracted to larvae (insignificantly), when introduced with larvae or control, but were more significantly attracted to pupae when introduced with all three options. These responses suggest that workers may prefer the social option over the non-social alternative (ie, lack of brood), but prefer self-reproduction over brood care when given the choice. It may also suggest that the

presence of pupae is necessary for worker reproduction to occur. Indeed, our studies show that while both workers and queens are able to lay eggs when provided only pollen, they do it much faster when fresh pupae are provided, and sometimes do not reproduce at all if pupae are not provided.

In the olfactometers used in this study, workers did not have clear sight of the brood, indicating that the observed attraction is based on olfactory information. The identity of the compounds inducing this effect is unknown, though a previous study in *Bombus terrestris* has suggested that workers are attracted to brood sprayed with cuticular hydrocarbons of starved brood (Boer and Duchateau 2006). Hydrocarbon composition is likely to change with the brood age and developmental phase as was shown in many insect species (Blomquist and Bagnères 2010) and provide workers with information that is critical for making reproductive decisions. For example, *B. terrestris* workers were shown to eavesdrop on a queen signal by differentiating between queen and worker brood and begin reproducing when queen larvae are produced (Alaux et al. 2005). Hydrocarbons were shown to have releaser effects in several social species, for example heneicosane was shown to induce shaking and antennation behavior associated with queen presence in termites (Funaro et al. 2018). Our study did not identify a primer effect induced by brood signals. However, we cannot preclude the possibility that the brood signals may induce a primer effect when provided in a relevant social context or in conjunction with other visual or behavioral signals. Thus far, the only species where brood has been shown to have both releaser and primer effects is the honeybee, an advanced eusocial species with colonies containing thousands of individuals. There, brood pheromone affects both worker foraging behavior and also reduces worker ovarian activation (Maisonnasse et al. 2010).

Our results show that while workers were slightly more attracted to starved over fed larvae, these preferences were not significantly different. Given that younger larvae are more

vulnerable to starvation than old larvae (which often simply pupate in the absence of food or brood care), we expected a greater attraction to starved larvae. In a previous study with *B. terrestris* (Boer and Duchateau 2006; Pereboom et al. 2003), starved larvae received more feedings by adults and larvae that were sprayed with cuticular washes of starved larvae increased the visitation rate of workers. However, in both cases the bioassay was performed with live larvae and allowed physical contact between the nursing bees and the brood. Overall, previous and current studies suggest that while workers can differentiate between larvae and pupae, the actual act of feeding and differentiating between larvae in need versus fed larvae requires physical contact with the brood.

There are several aspects to consider before dismissing the idea of a brood-produced pheromones regulating worker reproduction in bumble bees. A chemical signal may still exist as maybe evidenced by the nearly insignificant effect of whole-body extracts of young larvae (Figure 7, Table S9). However, even if such signal exists, its effect on egg laying is likely to be context-dependent and may only operate together with additional cues. The role of context in pheromone regulation was highlighted in several recent studies (Amsalem et al. 2015c; Amsalem et al. 2017; Melgarejo et al. 2018; Smith and Liebig 2017), including in bumble bees. Indeed, it makes little sense to respond to a costly signal (inhibiting reproduction) without additional information from the environment.

Additionally, although highly unlikely, the extracts we used may have not captured the active compounds of this putative signal. In this study, we used a mix of hexane and methanol solvents to extract compounds with a wide range of polarity. Various solvents were used in previous studies examining queen signals, and in several cases, were able to show an effect on worker reproduction regardless of the solvent used (Hoover et al. 2003; Lofgren et al. 1983; Nunes et al. 2014). This, however, depends on the identity of the active compounds and whether

they were masked by more abundant, noisy compounds. The dose used in this study could be another issue to consider, although we ensured that the brood-equivalent dose was biologically relevant. Both 1.4 and 7 brood-equivalents were shown to significantly reduce egg laying in workers as live brood, with 30% reduction in egg laying at the presence of 1-2 larvae grouped with 2 workers (similar to the lower dose in this study) and nearly complete suppress of egg laying when 2 workers were grouped with 10 young larvae (close to the higher dose in this study) (Starkey et al. 2019). Thus, hypothetically, if the larvae communicate their presence via a low-volatility pheromone, these doses should be sufficient to replicate the effect. On the same note, while the role of a low-volatility signal is questionable, volatile compounds produced in bumblebee colonies can be excluded since their effect was examined in a paired study where the physical interaction with live brood was sufficient to reduce egg laying while the volatiles from the same larvae were insufficient to replicate the same effect. These findings are in line with previous study showing that neither volatiles from the queen nor volatiles from the entire colonies (containing both queen and brood at all developmental stages) were able to inhibit worker reproduction (Padilla et al. 2016).

Overall, our study shows a releaser effect of olfactory cues or signals by larvae and pupae in *B. impatiens* on worker attraction, but not on worker reproduction. Workers seem unable to differentiate between starved and fed brood based on olfactory cues. While it is unlikely that bumble bee brood produce pheromones that regulate worker reproduction, the physical interaction with young larvae reduced egg laying by workers, likely via a behavioral mechanism, supporting predictions based on the level of social organization and colony size of bumble bee colonies.

Table and figure captions

Table 2: The average terminal oocyte size of workers and statistical analyses of the effect of brood on workers ovary size

Experiment	Treatment	Oocyte size (mm)±S.E.M	Sample size	Statistical differences	
				Colony effect	Brood type effect
The effect of brood cuticular washes (1.4/day) on random-age worker ovary size	Eggs	2.78±0.11	22	f=0.77, p=0.46	f=0.67, p=0.61
	Young larvae	2.51±0.14	36		
	Old larvae	2.34±0.1	54		
	Pupae	2.57±0.12	32		
	Solvent	2.51±0.12	25		
The effect of brood whole-body extracts (1.4/day) on random-age worker ovary size	Eggs	2.37±0.12	29	f=0.5, p=0.6	f=1.07, p=0.37
	Young larvae	2.29±0.14	26		
	Old larvae	2.2±0.12	36		
	Pupae	2.42±0.16	24		
	Solvent	2.31±0.14	26		
The effect of brood whole-body extracts (7/day) on random-age worker ovary size	Eggs	2.89±0.09	32	f=2.77, p=0.06	f=1.01, p=0.4
	Young larvae	3.00±0.09	26		
	Old larvae	2.73±0.1	28		
	Pupae	2.95±0.15	16		
	Solvent	2.99±0.06	30		
The effect of brood volatiles on worker ovary size	EL/LL (direct contact)	2.61±0.19	22	Within pairs: f=0.94, p=0.42 Among pairs: f=3.43, p=0.02	t= -0.04, p=0.96
	EL/LL (via air)	2.51±0.14	22		t= -2.84, p=0.01
	LP (direct contact)	2.51±0.11	26		
	LP (via air)	2.96±0.06	26		t= -0.83, p=0.43
	PP (direct contact)	2.6±0.13	14		
	PP (via air)	2.8±0.15	14		t= 0.69, p=0.5
	PW/WW (direct contact)	2.66±0.11	18		
	PW/WW (via air)	2.56±0.13	18		
The effect of brood whole body extracts of fed/starved larvae on workers ovary size	Solvent	2.56±0.17	20	f=0.77, p=0.46	f=0.19, p=0.82
	Young larvae fed	2.69±0.13	20		
	Young larvae starved	2.51±0.21	20		

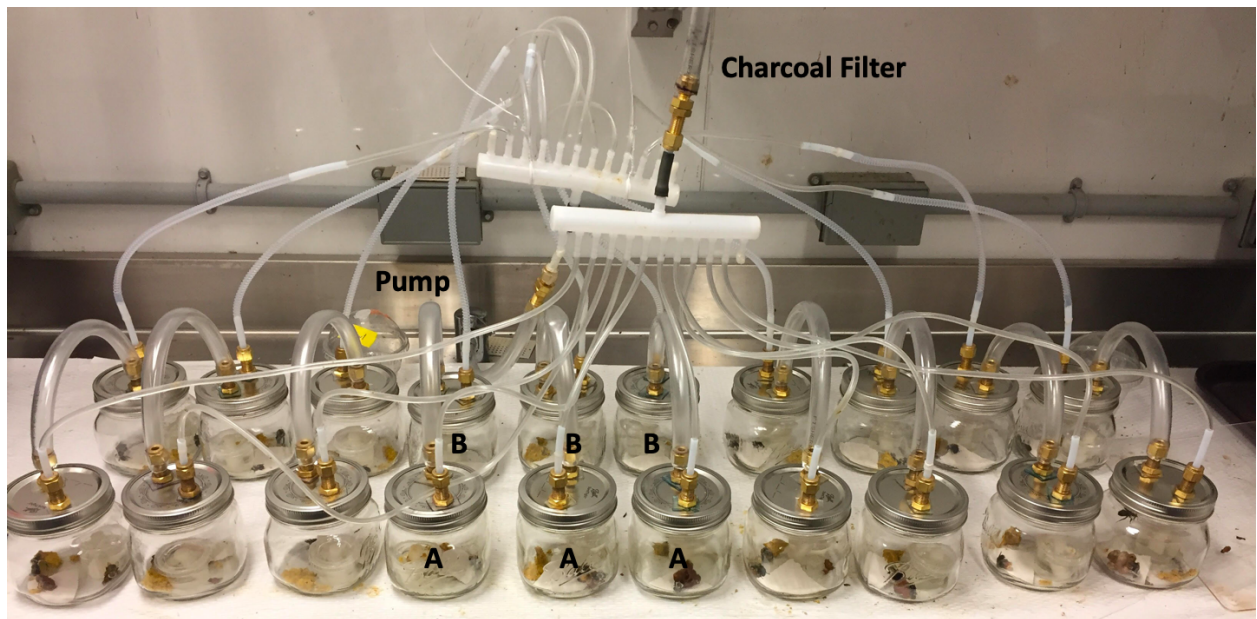
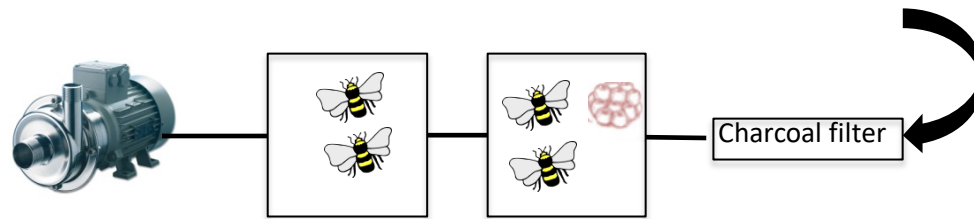


Figure 4: **An image of the vacuum air-flow system that was used for the volatile experiment.** Air was drawn from the room using an air pump through a charcoal filter into a glass jar containing brood or wax and nursing workers (marked as 'A'). This jar was connected via tygon tubing into a second glass jar containing a pair of workers with no brood (marked as 'B'). The air-flow system was fixed at 800 ccm/s for each pair of cages to allow for constant air flow throughout the experiment (7 days).

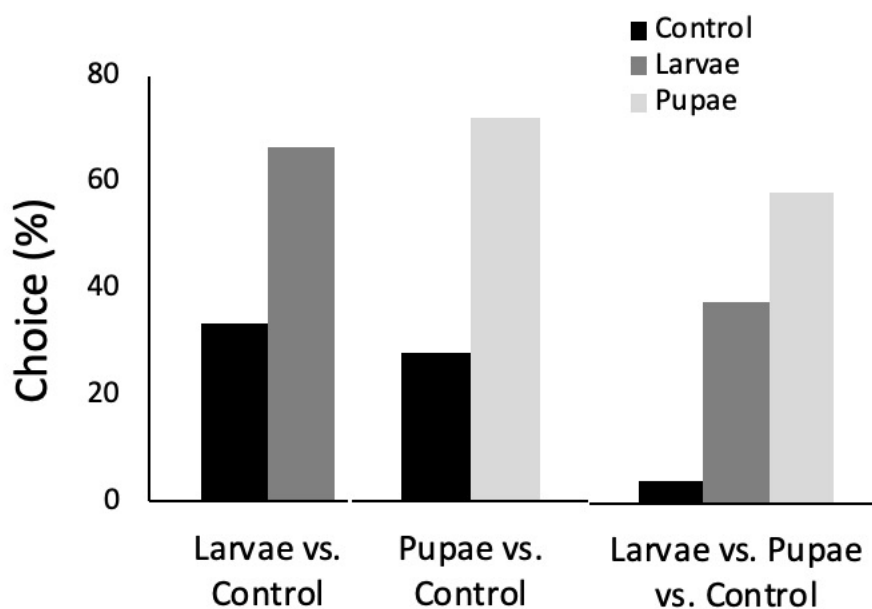


Figure 5: The percent of choice made by *Bombus impatiens* workers in choice bioassays between (A) young larvae and control, (B) pupae and control, and (C) young larvae, pupae and control. Random-age workers were equally sampled from four young queen-right colonies, placed in an olfactometer and were given 30 minutes to decide, afterward they were considered as non-responders (36%, 11% and 14% non-responders in A-C, respectively). Each bioassay was repeated 28 times. Each worker was only used once.

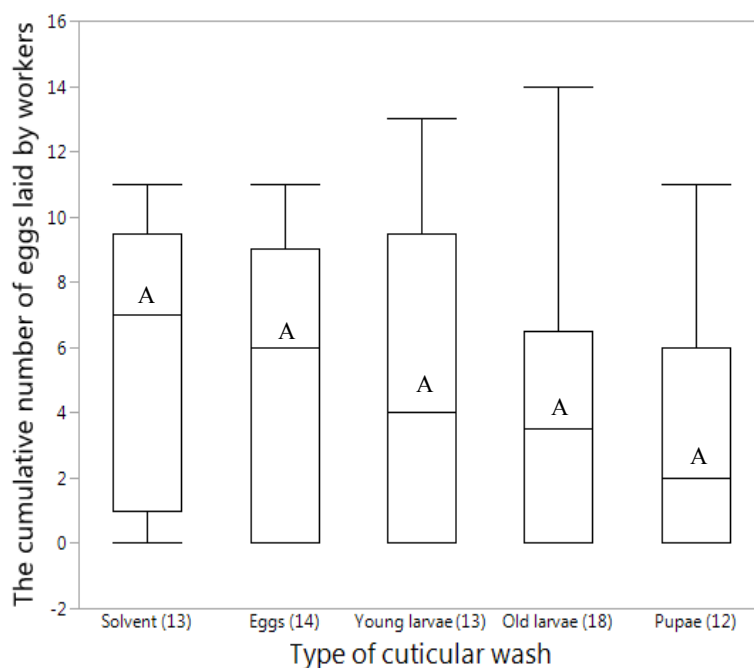


Figure 6: **The effect of brood cuticular washes on the cumulative number of eggs laid by *B. impatiens* workers.** Random-age workers from young queen-right colonies were kept in pairs for seven days with a filter containing cuticular washes of eggs, young larvae (<50 mg), old larvae (>50 mg) or pupae as compared to solvent control. Extracts were applied in a dose of 1.4 brood equivalents per day per cage. Numbers in brackets represent the number of cages. Letters within columns denote statistical differences at $\alpha=0.005$ following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Tables 12

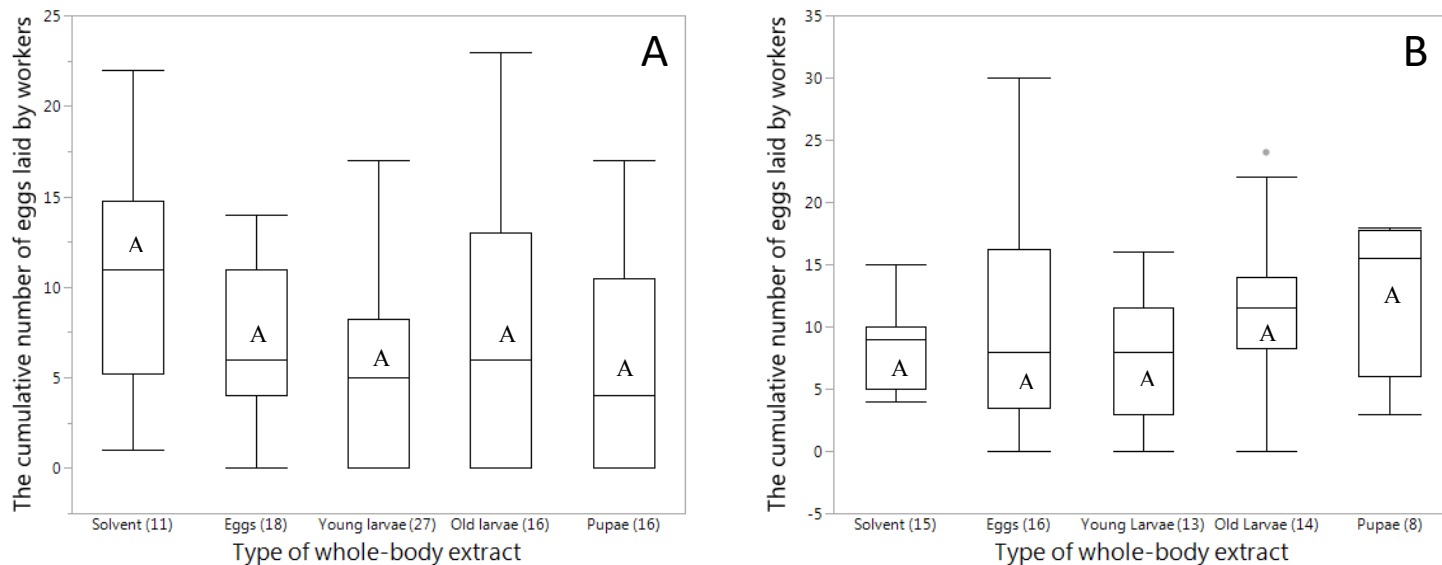


Figure 7: **The effect of brood whole-body extracts on the cumulative number of eggs laid by *B. impatiens* workers.** Random-age workers from young queen-right colonies were kept in pairs for seven days with a filter containing whole-body extracts of eggs, young larvae (<50 mg), old larvae (>50 mg) or pupae as compared to solvent control. Extracts were applied in a dose of 1.4 (a) or 7 (b) brood equivalents per day per cage. Numbers in brackets represent the number of cages. Letters within columns denote statistical differences at $\alpha=0.005$ following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Tables 13-14

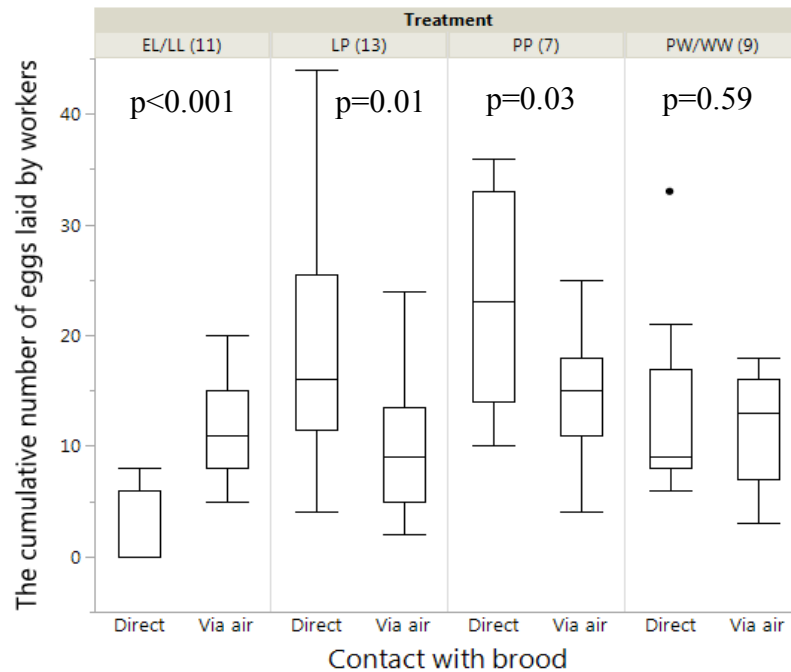


Figure 8: **The effect of brood volatiles on the cumulative number of eggs laid by *B. impatiens* workers.** Random-age workers were collected from young queen-right colonies, were matched-paired and placed with either brood or air drawn from that brood in a vacuum air-flow system (see methods Figure 1) for 7 days. Numbers in brackets represent the number of cages. Letters within columns denote statistical differences at $\alpha=0.05$. Detailed statistics for all comparisons are provided in Table 15.

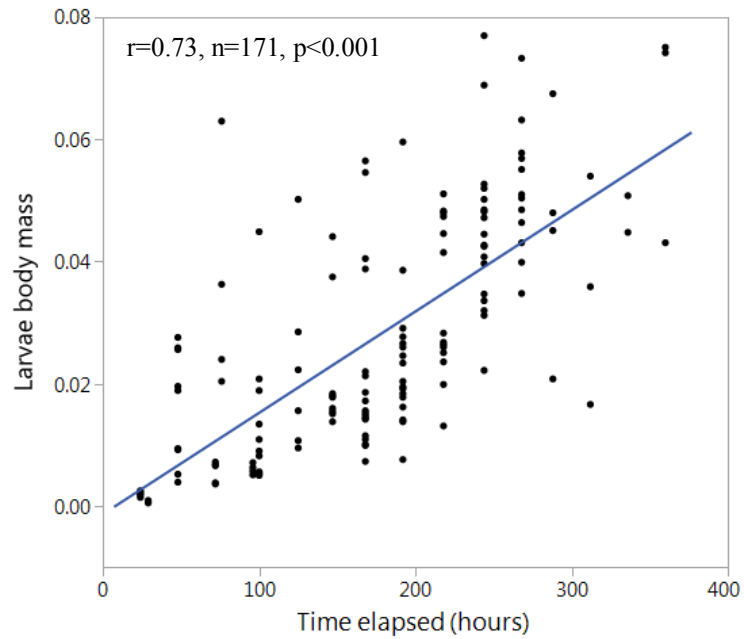


Figure 9: **Survival of *B. impatiens* larvae as function of body mass in the absence of food.**

Larvae were separated from their cases, individually weighted and placed in 48-well plates in a climate control room (28°C, 60% humidity and constant darkness) on top of a water bath, creating a micro climate with nearly 100% humidity. Survival was examined under a binocular (see methods) every 12 hours until all larvae died.

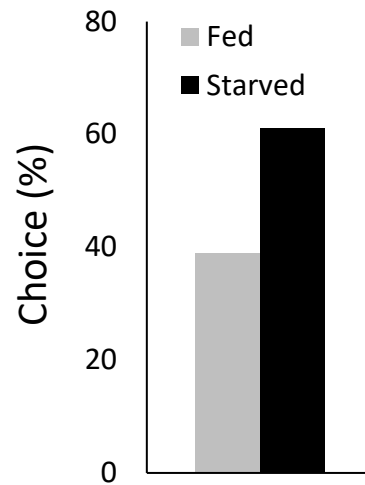


Figure 10: **The percent of choices made by *B. impatiens* workers in a choice bioassay between fed and starved larvae.** The percent of choice made by *B. impatiens* workers in choice bioassay between starved or fed young larvae. Random-age workers were equally sampled from 3 young queen-right colonies, placed in an olfactometer and were given 30 minutes to decide, afterward they were considered as non-responders (90% response rate). The bioassay was repeated 20 times. Each worker was only used once.

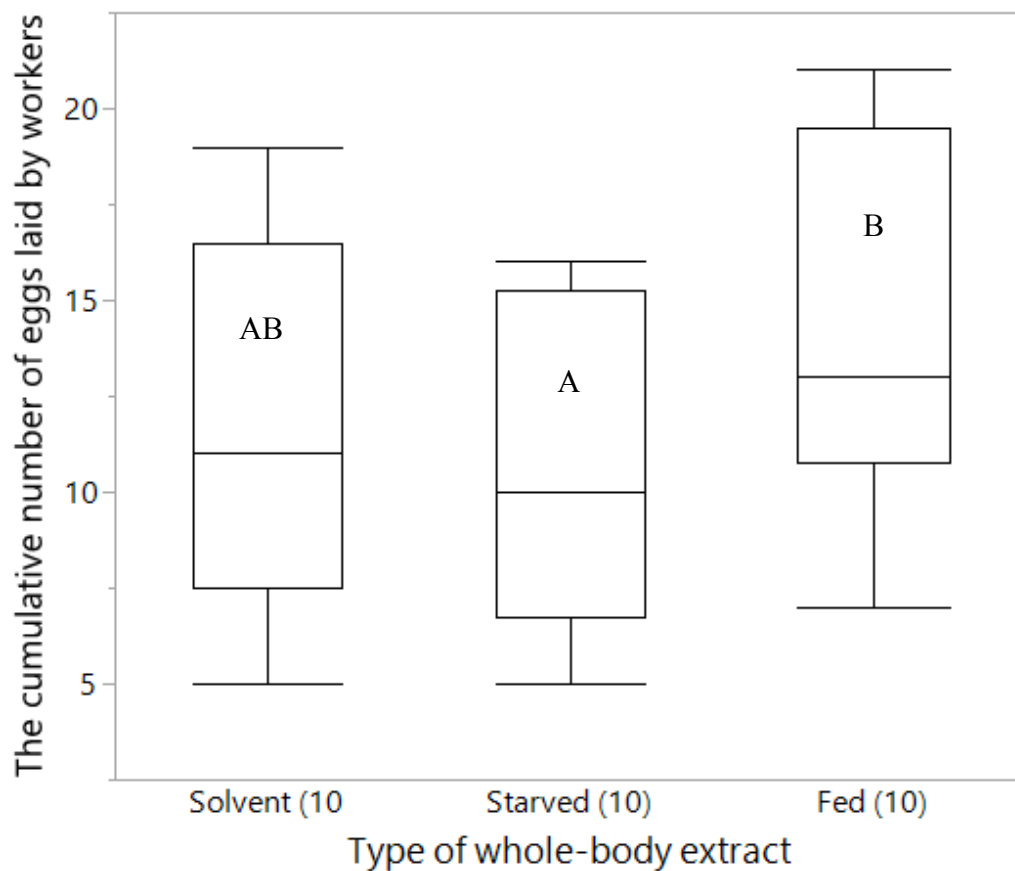


Figure 11: **The effect of starved and fed larvae whole-body extracts on the cumulative number of eggs laid by *B. impatiens* workers.** Random-age workers were sampled from young queen-right colonies and kept in pairs for seven days with a filter containing whole-body extracts of either 16 hours starved or fed young larvae (<50 mg) compared to solvent control. Extracts were applied in a dose 7 brood equivalents per day per cage. Letters within columns denote statistical differences at $\alpha=0.017$ following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Table 16.

References

- Alaux C, Jaisson P, Hefetz A (2005) Reproductive decision-making in semelparous colonies of the bumblebee *Bombus terrestris* Behavioral Ecology and Sociobiology 59:270-277 doi:10.1007/s00265-005-0035-6
- Amsalem E, Grozinger CM, Padilla M, Hefetz A (2015a) The physiological and genomic bases of bumble bee social behaviour. In: Amro Z, Clement FK (eds) Genomics, Physiology and Behaviour of Social Insects, vol 48. Academic Press, Adv In Insect Phys, pp 37-93
- Amsalem E, Hefetz A (2010) The appeasement effect of sterility signaling in dominance contests among *Bombus terrestris* workers Behavioral Ecology and Sociobiology 64:1685-1694 doi:10.1007/s00265-010-0982-4
- Amsalem E, Kiefer J, Schulz S, Hefetz A (2014) The effect of caste and reproductive state on the chemistry of the cephalic labial glands secretion of *Bombus terrestris* J Chem Ecol 40:900-912 doi:10.1007/s10886-014-0484-3
- Amsalem E, Orlova M, Grozinger CM (2015b) A conserved class of queen pheromones? Re-evaluating the evidence in bumblebees (*Bombus impatiens*) Proc Biol Sci 282:20151800 doi:10.1098/rspb.2015.1800
- Amsalem E, Padilla M, Schreiber PM, Altman NS, Hefetz A, Grozinger CM (2017) Do bumble bee, *Bombus impatiens*, queens signal their reproductive and mating status to their workers? J Chem Ecol 43:563-572 doi:10.1007/s10886-017-0858-4

- Beshers SN, Fewell JH (2001) Models of division of labor in social insects *Annu Rev Entomol* 46:413-440 doi:10.1146/annurev.ento.46.1.413
- Bhadra A, Mitra A, Deshpande SA, Chandrasekhar K, Naik DG, Hefetz A, Gadagkar R (2010) Regulation of reproduction in the primitively eusocial wasp *Ropalidia marginata*: on the trail of the queen pheromone *J Chem Ecol* 36:424-431 doi:10.1007/s10886-010-9770-x
- Bigley WS, Vinson SB (1975) Characterization of a brood pheromone isolated from the sexual brood of the imported fire ant, *Solenopsis invicta* *Ann Entomol Soc Am* 68:301-304 doi:10.1093/aesa/68.2.301
- Bloch G, Hefetz A (1999) Reevaluation of the role of mandibular glands in regulation of reproduction in bumblebee colonies *Journal of Chemical Ecology* 25:881-896 doi:10.1023/a:1020805103379
- Blomquist GJ, Bagnères A-G (2010) *Insect hydrocarbons: biology, biochemistry, and chemical ecology*. Cambridge University Press,
- Boer SPAd, Duchateau MJHM (2006) A larval hunger signal in the bumblebee *Bombus terrestris* *Insectes Sociaux* 53:369-373 doi:10.1007/s00040-006-0883-8
- Cnaani J, Schmid-Hempel R, Schmidt JO (2002) Colony development, larval development and worker reproduction in *Bombus impatiens* Cresson *Insectes Sociaux* 49:164-170 doi:10.1007/s00040-002-8297-8
- Duchateau MJ, Velthuis HHW (1988) Development and reproductive strategies in *Bombus terrestris* colonies *Behavior* 107:186-207
- Engel KC, Stokl J, Schweizer R, Vogel H, Ayasse M, Ruther J, Steiger S (2016) A hormone-related female anti-aphrodisiac signals temporary infertility and causes

sexual abstinence to synchronize parental care Nat Commun 7:11035

doi:10.1038/ncomms11035

Fletcher DJC, Ross KG (1985) Regulation of reproduction in eusocial Hymenoptera

Annu Rev Entomol 30:319-343 doi:10.1146/annurev.en.30.010185.001535

Funaro CF, Boroczky K, Vargo EL, Schal C (2018) Identification of a queen and king

recognition pheromone in the subterranean termite *Reticulitermes flavipes* Proc

Natl Acad Sci U S A 115:3888-3893 doi:10.1073/pnas.1721419115

Godfray HCJ (1991) Signalling of need by offspring to their parents Nature 352:328-330

Haig D (1990) Brood reduction and optimal parental investment when offspring differ in

quality The American Naturalist 136:550-556

Hamilton WD (1964) The genetical evolution of social behaviour I J Theor Biol 7

He XJ, Zhang XC, Jiang WJ, Barron AB, Zhang JH, Zeng ZJ (2016) Starving honey bee

(*Apis mellifera*) larvae signal pheromonally to worker bees Sci Rep 6:22359

doi:10.1038/srep22359

Holldobler B, Wilson EO (1983) Queen control in colonies of weaver ants

(Hymenoptera: Formicidae) Ann Entomol Soc Am 76:235-238

Hoover SE, Keeling CI, Winston ML, Slessor KN (2003) The effect of queen

pheromones on worker honey bee ovary development Naturwissenschaften

90:477-480 doi:10.1007/s00114-003-0462-z

Jandt JM, Tibbetts EA, Toth AL (2013) *Polistes* paper wasps: a model genus for the

study of social dominance hierarchies Insectes Sociaux 61:11-27

doi:10.1007/s00040-013-0328-0

- Keller L, Nonacs P (1993) The role of queen pheromones in social insects: queen control or queen signal? *Animal Behaviour* 45:787-794 doi:10.1006/anbe.1993.1092
- Kilner R, Johnstone RA (1997) Begging the question: are offspring solicitation behaviours signals of need? *Trends Ecol Evol* 12:11-15
doi:[https://doi.org/10.1016/S0169-5347\(96\)10061-6](https://doi.org/10.1016/S0169-5347(96)10061-6)
- Kocher SD, Grozinger CM (2011) Cooperation, conflict, and the evolution of queen pheromones *J Chem Ecol* 37:1263-1275 doi:10.1007/s10886-011-0036-z
- Le Conte Y, Hefetz A (2008) Primer pheromones in social hymenoptera *Annu Rev Entomol* 53:523-542 doi:10.1146/annurev.ento.52.110405.091434
- Le Conte Y, Mohammedi A, Robinson GE (2001) Primer effects of a brood pheromone on honeybee behavioural development *Proc Biol Sci* 268:163-168
doi:10.1098/rspb.2000.1345
- Liebig J, Eliyahu D, Brent CS (2009) Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis* *Behavioral Ecology and Sociobiology* 63:1799-1807 doi:10.1007/s00265-009-0807-5
- Liebig J, Peeters C, Holldobler B (1999) Worker policing limits the number of reproductives in a ponerine ant *Proc R Soc Lond B* 266:1865-1870
- Lofgren CS, Glancey BM, Glover A, Rocca J, Tumlinson J (1983) Behavior of Workers of *Solenopsis invicta* (Hymenoptera: Formicidae) to the Queen Recognition Pheromone: Laboratory Studies with an Olfactometer and Surrogate Queens1 *Annals of the Entomological Society of America* 76:44-50
doi:10.1093/aesa/76.1.44

- Maisonnasse A, Lenoir JC, Beslay D, Crauser D, Le Conte Y (2010) E-beta-ocimene, a volatile brood pheromone involved in social regulation in the honey bee colony (*Apis mellifera*) PLoS One 5:e13531 doi:10.1371/journal.pone.0013531
- Matsuura K, Himuro C, Yokoi T, Yamamoto Y, Vargo EL, Keller L (2010) Identification of a pheromone regulating caste differentiation in termites Proc Natl Acad Sci U S A 107:12963-12968 doi:10.1073/pnas.1004675107
- Melgarejo V, Wilson Rankin EE, Loope KJ (2018) Do queen cuticular hydrocarbons inhibit worker reproduction in *Bombus impatiens*? Insectes Sociaux 65:601-608 doi:10.1007/s00040-018-0651-6
- Michener CD (1974) The social behavior of the bees. Harvard University Press, Cambridge, Massachusetts
- Monnin T, Jones G, Ratnieks FLW, Beard R (2002) Pretender punishment induced by chemical signaling in a queenless ant Nature 419:61-65
- Naug D (2009) Structure and resilience of the social network in an insect colony as a function of colony size Behavioral Ecology and Sociobiology 63:1023-1028 doi:10.1007/s00265-009-0721-x
- Noonan KC (2010) Recognition of queen larvae by worker honey bees (*Apis mellifera*) Ethology 73:295-306 doi:10.1111/j.1439-0310.1986.tb00811.x
- Nunes TM et al. (2014) Queen signals in a stingless bee: suppression of worker ovary activation and spatial distribution of active compounds Sci Rep 4:7449 doi:10.1038/srep07449
- Oi CA, van Zweden JS, Oliveira RC, Van Oystaeyen A, Nascimento FS, Wenseleers T (2015) The origin and evolution of social insect queen pheromones: Novel

hypotheses and outstanding problems *Bioessays* 37:808-821

doi:10.1002/bies.201400180

- Padilla M, Amsalem E, Altman N, Hefetz A, Grozinger CM (2016) Chemical communication is not sufficient to explain reproductive inhibition in the bumblebee *Bombus impatiens* *Royal Society Open Science* 3:160576
- Page RE, Robinson GE, Fondrk MK (1989) Genetic specialists, kin recognition and nepotism in honey-bee colonies *Nature* 338:576-579 doi:10.1038/338576a0
- Peeters C, Liebig J (2009) Fertility signaling as a general mechanism of regulating reproductive division of labor in ants. In: Gadau J, Fewell J (eds) *In Organization of Insect Societies: From Genome to Sociocomplexity* Harvard University Press,
- Pereboom JJM, Duchateau MJ, Velthuis HHW (2003) The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation *Insectes Sociaux* 50:127-133 doi:10.1007/s00040-003-0639-7
- Premnath S, Sinha A, Gadagkar R (1995) Regulation of worker activity in a primitively eusocial wasp, *Ropalidia marginata* *Behavioral Ecology* 6:117-123
- Premnath SS, A; Gadagkar, R. (1996) Dominance relationship in the establishment of reproductive division of labour in a primitively eusocial wasp (*Ropalidia marginata*) *Behav Ecol Sociobiol* 39:125-132
- Queller David C (1994) Extended parental care and the origin of eusociality *Proceedings of the Royal Society of London Series B: Biological Sciences* 256:105-111 doi:10.1098/rspb.1994.0056
- Ratnieks FLW, Visscher PK (1989) Worker policing in the honeybee *Nature* 342:796-797 doi:10.1038/342796a0

- Schultner E, Oettler J, Helanterä H (2017) The role of brood in eusocial Hymenoptera *Q Rev Biol* 92:39-78 doi:10.1086/690840
- Smith AA, Liebig J (2017) The evolution of cuticular fertility signals in eusocial insects *Curr Opin Insect Sci* 22:79-84 doi:10.1016/j.cois.2017.05.017
- Starkey J, Brown A, Amsalem E (2019) The road to sociality: Brood regulation of worker reproduction in the simple eusocial bee *Bombus impatiens* *Anim Behav*. Accepted
- Trivers RL, Hare H (1976) Haplodiploidy and the evolution of the social insects. *Science* 191:249-263
- Ulrich Y, Burns D, Libbrecht R, Kronauer DJ (2016) Ant larvae regulate worker foraging behavior and ovarian activity in a dose-dependent manner *Behav Ecol Sociobiol* 70:1011-1018 doi:10.1007/s00265-015-2046-2
- Winston ML, Slessor KN, Willis LG, Naumann K, Higo HA, Wyborn MH, Kaminski LA (1989) The influence of queen mandibular pheromones on worker attraction to swarm clusters and inhibition of queen rearing in the honey bee (*Apis mellifera* L.). *Insectes Sociaux* 36:15-27

Chapter 4

Conclusion and Future Directions

The regulation of reproductive division of labor in social insects is one of the main questions in the study of the evolution of social behavior. Sociality has evolved multiple times in insects and most of the work examining how social behavior is regulated have focused on signals exhibited by adults. Social species are rooted in simple family structure that originated from solitary ancestors. Therefore, focusing on how adults regulate reproduction and social behavior leaves out an important aspect of this family structure; the brood, and looking into both the roles of adults and offspring in regulating reproduction is critical to fully understand how social systems regulated and evolved (Queller 1994; Tallamy 1984). The role of brood in regulating worker reproduction has been studied in several social insect species in the past few years (mostly in ants and honey bees), but remain relatively unexplored.

Bumble bees are primitively eusocial species exhibiting characteristics of both advanced and simple eusocial insects. They present both cooperation and competition over reproduction during different phases of their life cycle (Duchateau and Velthuis 1988) and their reproductive division of labor is assumed to be regulated by a combination of behavioral and chemical means. The interplay between the behavioral and chemical means, as well as the use of a primitive eusocial species like bumble bees as a

model system to study the mechanistic basis of worker sterility may provide insight into major evolutionary transitions in the evolution of eusociality (Amsalem et al. 2015b; Jandt et al. 2014; Kronauer and Libbrecht 2018).

In chapter 2, I found that grouping workers with eggs that transitioned into larvae, or with young larvae that remained larvae during the 7-day observation period resulted in a reduction in worker egg-laying behavior. This was consistent with results from chapter 3, where young larvae were shown to be more prone to die from starvation if not fed by adults. These observations make sense, as the younger brood would have a larger incentive to communicate their need to adults in order to ensure their survival. Despite this, the presence of starved larvae did not (significantly) increase worker attraction and there was no effect of whole-body extracts of starved compared to fed larvae on worker reproduction. While I examined the effect of odors produced by starved larvae on worker attraction and reproduction, I did not examine the physical presence of starved brood on worker behavior. Designing an experiment to test this proved difficult, as I could not design an experimental setup where larvae could produce a consistent hunger signal in the presence of workers and 1) not have the workers immediately feed them to remove the signal, or 2) not have the larvae die from starvation. Designing an experiment which can capture if and how starved larvae regulate worker behavior and reproduction as compared to normally-fed brood would be the next step and truly show if there is a hunger signal that affects worker behavior and reproduction.

In chapter 3, I found that chemical signals alone from the brood did not affect worker reproduction. I predict that since chemical cues/signals from the brood

attract workers but do not reduce egg-laying or ovary size, brood utilize other signals (i.e, tactile or visual) to induce brood care, which in turn reduces worker egg laying. It has been shown in burying beetles that the larvae touch their mother's face in order to induce feeding (Engel et al. 2016). Bumble bee workers have no way of seeing the brood while they are confined within their cells. Thus, they must rely on olfactory cues/signals [or other less common auditory signals produced in *Vespula* wasps, (Ishay and Landau 1972)]. to receive the information that larvae need to be fed before they open the brood cells. This would explain why workers are still able to differentiate between larvae and pupae.

In an additional set of experiments not included in this thesis I compared the effect of both queen and young larvae on worker egg laying and ovary size. I found that queens inhibit egg laying in workers regardless of the presence of the brood, but that queen with no brood was less efficient in reducing worker ovary size compared to queen with brood. These results provide an interesting insight: in contrast to the queen that inhibits both egg laying and reduce ovary size, the presence of brood reduced egg-laying behavior in workers but not ovary size. This may indicate that the queen and brood have additive inhibitory effects on worker reproduction, and the brood act as a 'last ditch effort' in controlling worker egg-laying when the queen cannot directly control the workers by either behavioral or a combination of behavioral and chemical means. Teasing apart the differences between how brood and the queen regulate worker reproduction would be the next step in understanding the regulation of reproductive division of labor in bumble bee colonies. I had examined how the queen and her brood affect brain gene expression associated with reproduction, providing insights into the

genetic mechanisms regulated by both queen and brood and separately by the brood or the queen. Understanding how brood and the queen affect worker gene expression would inform us if the regulatory pathways in which worker reproduction is regulated are additive or complimentary, possibly suggesting that the queen inhibits worker reproduction indirectly via her brood. These data are still in progress and their completion will provide a holistic explanation of how bumblebee worker sterility is regulated.

The experiments conducted in this thesis consisted of a reduced system of two workers. Small groups of workers were shown to be an excellent model system to test questions related to workers reproduction in bumble bees (Alaux et al. 2006; Amsalem and Hefetz 2011; Bloch 1999; Padilla et al. 2016; Sibbald and Plowright 2014; Sibbald and Plowright 2013) and allowed us to control for many confounding factors such as the colony of origin and worker size. Padilla et al. 2016 used groups of three workers in order to examine the effects of colony or queen volatiles on worker reproduction. Sibbald and Plowright 2013 and 2014 both used pairs of orphaned workers to study the relationship between aggression and ovarian activation. However, in order to fully understand how worker sterility is regulated, the next step would be to test the effect of brood on a colony-wide scale. Bloch 1999 found increased worker reproduction after manipulating the ratio of workers to brood and the sex of the brood, but did not control for brood developmental stage. Alaux 2006 found that the onset of worker-queen reproductive competition occurred sooner in the presence of gyne larvae but not by any volatiles alone drawn from them. However, this study, too, used small groups of workers (around 14 workers) and did not control for brood life stages, different castes, or sexes. The effect of brood on worker reproduction on a colony-wide scale can be examined by altering the

ratios of young larvae to pupae, the two brood-types shown to have the most prominent and opposing effects on worker reproduction, and to observe if the manipulation influences the onset of workers reproduction.

Bumble bees are also important pollinators for natural and agriculture/greenhouse crops such as tomatoes (Van Ravestijn and Van Der Sande 1990). Commercial bumble bees are typically reared in small cardboard and plastic boxes which are then moved to field or greenhouse where their pollination services are needed. One of the challenges the commercial rearing of bumble bees face is the need to extend the period at which a single colony effectively pollinate in the field (around 8-12 weeks (Velthuis and van Doorn 2006). This period is limited by the onset of the reproductive competition which typically follows with chaos and production of sexuals. The effect of brood on worker reproduction can be utilized for commercial rearing and management of bumble bee colonies. Since the presence of young larvae reduces worker egg laying, the ratios of young brood within a colony can be manipulated in order to reduce worker production in colonies of *B. impatiens* workers (and possibly also of *B. terrestris*). Colonies with high ratio of young larvae to pupae will last longer in the field since the queen will be able to maintain reproductive superiority for a longer period and, as a result, produce more workers, thus extending the active life-span of commercial bumble bee colonies. By manipulating the onset of worker reproduction, commercial producers can develop protocols to increase the effectiveness of their colony production both for their own use as well as their customers to have their colonies last longer without the need for replacement.

The regulation of worker reproduction by brood is logically sound when thinking about life-history trade-offs between parental care and future reproduction. By studying reproductive division of labor in bumble bees, we found that the regulation of worker reproduction still relies on the direct presence of offspring. Additionally, since brood produced chemicals alone were insufficient to reduce worker egg laying, we provide further evidence for the theory that brood pheromones are characteristics of highly eusocial insects (Leonhardt et al. 2016). Overall, results from my thesis show previously unknown form of reproductive regulation for workers of the bumble bee *Bombus impatiens*.

References

- Abril S, Diaz M, Lenoir A, Ivon Paris C, Boulay R, Gomez C (2018) Cuticular hydrocarbons correlate with queen reproductive status in native and invasive Argentine ants (*Linepithema humile*, Mayr) PLoS One 13:e0193115
doi:10.1371/journal.pone.0193115
- Alaux C, Jaisson P, Hefetz A (2004) Queen influence on worker reproduction in bumblebees (*Bombus terrestris*) colonies Insectes Soc 51:287-293
doi:10.1007/s00040-004-0741-5
- Alaux C, Jaisson P, Hefetz A (2005) Reproductive decision-making in semelparous colonies of the bumblebee *bombus terrestris* Behav Ecol Sociobiol 59:270-277
doi:10.1007/s00265-005-0035-6
- Alaux C, Jaisson P, Hefetz A (2006) Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal Behav Ecol Sociobiol 60:439-446 doi:10.1007/s00265-006-0184-2
- Amsalem E, Grozinger CM, Padilla M, Hefetz A (2015a) The physiological and genomic bases of bumble bee social behaviour. In: Amro Z, Clement FK (eds) Genomics, Physiology and Behaviour of Social Insects, vol 48. Academic Press, Adv In Insect Phys, pp 37-93
- Amsalem E, Grozinger CM, Padilla M, Hefetz A (2015b) The physiological and genomic bases of bumble bee social behaviour. In: Amro Z, Clement FK (eds) Advances in Insect Physiology, vol 48. Academic Press, pp 37-93

- Amsalem E, Hefetz A (2010) The appeasement effect of sterility signaling in dominance contests among *Bombus terrestris* workers Behav Ecol Sociobiol 64:1685-1694 doi:10.1007/s00265-010-0982-4
- Amsalem E, Hefetz A (2011) The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris* PLoS One 6:e18238 doi:10.1371/journal.pone.0018238
- Amsalem E, Kiefer J, Schulz S, Hefetz A (2014) The effect of caste and reproductive state on the chemistry of the cephalic labial glands secretion of *Bombus terrestris* J Chem Ecol 40:900-912 doi:10.1007/s10886-014-0484-3
- Amsalem E, Orlova M, Grozinger CM (2015c) A conserved class of queen pheromones? Re-evaluating the evidence in bumblebees (*Bombus impatiens*) Proc Biol Sci 282:20151800 doi:10.1098/rspb.2015.1800
- Amsalem E, Padilla M, Schreiber PM, Altman NS, Hefetz A, Grozinger CM (2017) Do bumble bee, *Bombus impatiens*, queens signal their reproductive and mating status to their workers? J Chem Ecol 43:563-572 doi:10.1007/s10886-017-0858-4
- Amsalem E, Twele R, Francke W, Hefetz A (2009) Reproductive competition in the bumble-bee *Bombus terrestris*: do workers advertise sterility? Proc Biol Sci 276:1295-1304 doi:10.1098/rspb.2008.1688
- Arnold G, Le Conte, Y., Trouiller, J., Hervet, H., Chappe, B., Masson, C. (1994) Inhibition of worker honeybee ovaries development by a mixture of fatty acid esters from larvae Acad Sci
- Beshers SN, Fewell JH (2001) Models of division of labor in social insects Annu Rev Entomol 46:413-440 doi:10.1146/annurev.ento.46.1.413

- Bhadra A, Mitra A, Deshpande SA, Chandrasekhar K, Naik DG, Hefetz A, Gadagkar R (2010) Regulation of reproduction in the primitively eusocial wasp *Ropalidia marginata*: on the trail of the queen pheromone J Chem Ecol 36:424-431 doi:10.1007/s10886-010-9770-x
- Bigley WS, Vinson SB (1975) Characterization of a brood pheromone isolated from the sexual brood of the imported fire ant, *Solenopsis invicta*1,2 Ann Entomol Soc Am 68:301-304 doi:10.1093/aesa/68.2.301
- Bloch G (1999) Regulation of queen-worker conflict in bumble-bee (*Bombus terrestris*) colonies Proc Biol Sci 266:2465-2469 doi:10.1098/rspb.1999.0947
- Bloch G, Hefetz A (1999) Reevaluation of the role of mandibular glands in regulation of reproduction in bumblebee colonies J Chem Ecol 25:881-896 doi:10.1023/A:1020805103379
- Blomquist GJ, Bagnères A-G (2010) Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press,
- Boer SPAd, Duchateau MJHM (2006) A larval hunger signal in the bumblebee *Bombus terrestris* Insectes Soc 53:369-373 doi:10.1007/s00040-006-0883-8
- Bonabeau A, Theraulaz G, Deneubourg JL (1996) Mathematical model of self-organizing hierarchies in animal societies Bull Math Biol 58:661-717
- Boomsma JJ (2009) Lifetime monogamy and the evolution of eusociality Philos Trans Royal Soc B 364:3191-3207 doi:10.1098/rstb.2009.0101
- Bourke AF (2014) Hamilton's rule and the causes of social evolution Philos T R Soc B 369:20130362 doi:10.1098/rstb.2013.0362

- Cnaani J, Schmid-Hempel R, Schmidt JO (2002) Colony development, larval development and worker reproduction in *Bombus impatiens* Cresson Insectes Soc 49:164-170 doi:DOI 10.1007/s00040-002-8297-8
- Cuvillier-Hot V, Lenoir A, Crewe R, Malosse C, Peeters C (2004) Fertility signalling and reproductive skew in queenless ants Anim Behav 68:1209-1219 doi:10.1016/j.anbehav.2003.11.026
- Darwin C (1859) The origin of species.
- Duchateau MJ, Velthuis HHW (1988) Development and reproductive strategies in *Bombus terrestris* colonies Behavior 107:186-207
- Duchateau MJ, Velthuis HHW (1989) Ovarian development and egg-laying in workers of *Bombus terrestris* Entomol Exp Appl 51:199-213 doi:DOI 10.1111/j.1570-7458.1989.tb01231.x
- Endler A, Liebig J, Schmitt T, Parker JE, Jones GR, Schreier P, Holldobler B (2004) Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect PNAS 101:2945-2950 doi:10.1073/pnas.0308447101
- Engel KC, Stokl J, Schweizer R, Vogel H, Ayasse M, Ruther J, Steiger S (2016) A hormone-related female anti-aphrodisiac signals temporary infertility and causes sexual abstinence to synchronize parental care Nat Commun 7:11035 doi:10.1038/ncomms11035
- Fletcher DJC, Ross KG (1985) Regulation of reproduction in eusocial Hymenoptera Annu Rev Entomol 30:319-343 doi:10.1146/annurev.en.30.010185.001535
- Free JB, Winder ME (1983) Brood recognition by honeybee (*Apis mellifera*) workers Anim Behav 31:539-545 doi:Doi 10.1016/S0003-3472(83)80077-3

- Funaro CF, Boroczky K, Vargo EL, Schal C (2018) Identification of a queen and king recognition pheromone in the subterranean termite *Reticulitermes flavipes* PNAS 115:3888-3893 doi:10.1073/pnas.1721419115
- Gilbert JD, Manica A (2010) Parental care trade-offs and life-history relationships in insects Am Nat 176:212-226 doi:10.1086/653661
- Godfray HCJ (1991) Signalling of need by offspring to their parents Nature 352:328-330
- Grozinger CM, Fan Y, Hoover SE, Winston ML (2007) Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*) Mol Ecol 16:4837-4848 doi:10.1111/j.1365-294X.2007.03545.x
- Haig D (1990) Brood reduction and optimal parental investment when offspring differ in quality Am Nat 136:550-556
- Hamilton WD (1964a) The genetical evolution of social behaviour I J Theor Biol 7
- Hamilton WD (1964b) The genetical evolution of social behaviour. II J Theor Biol 7:17-52
- He XJ, Zhang XC, Jiang WJ, Barron AB, Zhang JH, Zeng ZJ (2016) Starving honey bee (*Apis mellifera*) larvae signal pheromonally to worker bees Sci Rep 6:22359 doi:10.1038/srep22359
- Holldobler B, Wilson EO (1983) Queen control in colonies of weaver ants (Hymenoptera: Formicidae) Ann Entomol Soc Am 76:235-238
- Honk van CGJ, Velthuis HHW, Roseler PF, Malotau ME (1980) The mandibular glands of *Bombus terrestris* queens as a source of queen pheromone. Entomol Exp Appl 28:191-198

- Hoover SE, Keeling CI, Winston ML, Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development *Naturwissenschaften* 90:477-480 doi:10.1007/s00114-003-0462-z
- Hunt J, Simmons LW (2004) Optimal maternal investment in the dung beetle *Onthophagus taurus*? *Behav Ecol Sociobiol* 55:302-312
- Ishay J, Landau EM (1972) *Vespa* larvae send out rhythmic hunger signals *Nature* 237:286-287 doi:10.1038/237286a0
- Jandt JM, Tibbetts EA, Toth AL (2014) *Polistes* paper wasps: a model genus for the study of social dominance hierarchies *Insectes Soc* 61:11-27 doi:10.1007/s00040-013-0328-0
- Kaptein N, Billen J, Gobin B (2005) Larval begging for food enhances reproductive options in the ponerine ant *Gnamptogenys striatula* *Anim Behav* 69:293-299
- Karlson P, Luscher M (1959) 'Pheromones': a new term for a class of biologically active substances *Nature* 183:55-56 doi:10.1038/183055a0
- Keeling CI, Slessor KN, Higo HA, Winston ML (2003) New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone *PNAS* 100:4486-4491 doi:10.1073/pnas.0836984100
- Keller L, Nonacs P (1993) The role of queen pheromones in social insects - queen control or queen signal *Anim Behav* 45:787-794 doi:DOI 10.1006/anbe.1993.1092
- Kilner R, Johnstone RA (1997) Begging the question: are offspring solicitation behaviours signals of need? *Trends Ecol Evol* 12:11-15
doi:[https://doi.org/10.1016/S0169-5347\(96\)10061-6](https://doi.org/10.1016/S0169-5347(96)10061-6)

- Kocher SD, Grozinger CM (2011) Cooperation, conflict, and the evolution of queen pheromones *J Chem Ecol* 37:1263-1275 doi:10.1007/s10886-011-0036-z
- Kolliker M (2007) Benefits and costs of earwig (*Forficula auricularia*) family life *Behav Ecol Sociobiol* 61:1489-1497 doi:10.1007/s00265-007-0381-7
- Kronauer DJ, Libbrecht R (2018) Back to the roots: the importance of using simple insect societies to understand the molecular basis of complex social life *Curr Opin Insect Sci* 28:33-39 doi:10.1016/j.cois.2018.03.009
- Le Conte Y, Arnold G, Trouiller J, Masson C, Chappe B (1990) Identification of a brood pheromone in honeybees *Naturwissenschaften* 77:334-336
- Le Conte Y, Hefetz A (2008) Primer pheromones in social hymenoptera *Annu Rev Entomol* 53:523-542 doi:10.1146/annurev.ento.52.110405.091434
- Le Conte Y, Mohammedi A, Robinson GE (2001) Primer effects of a brood pheromone on honeybee behavioural development *Proc Biol Sci* 268:163-168 doi:10.1098/rspb.2000.1345
- Leibig J, Peeters C, Oldham NJ, Markstadter C, Holldobler B (2000) Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*? *PNAS* 97:4124-4131
- Leonhardt SD, Menzel F, Nehring V, Schmitt T (2016) Ecology and evolution of communication in social insects *Cell* 164:1277-1287 doi:10.1016/j.cell.2016.01.035
- Liebig J, Eliyahu D, Brent CS (2009) Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis* *Behav Ecol Sociobiol* 63:1799-1807 doi:10.1007/s00265-009-0807-5

- Liebig J, Peeters C, Holldobler B (1999a) Worker policing limits the number of reproductives in a ponerine ant Proc R Soc Lond B 266:1865-1870
- Liebig J, Peeters C, Hölldobler B (1999b) Worker policing limits the number of reproductives in a ponerine ant 266:1865-1870 doi:doi:10.1098/rspb.1999.0858
- Linksvayer TA, Wade MJ (2005) The evolutionary origin and elaboration of sociality in the aculeate Hymenoptera: maternal effects, sib-social effects, and heterochrony Q Rev Biol 80:317-336 doi:10.1086/432266
- Lofgren CS, Glancey BM, Glover A, Rocca J, Tumlinson J (1983) Behavior of workers of *Solenopsis invicta* (Hymenoptera: Formicidae) to the queen recognition pheromone: laboratory studies with an olfactometer and surrogate queens1 Ann Entomol Soc Am 76:44-50 doi:10.1093/aesa/76.1.44
- Maisonnasse A, Lenoir JC, Beslay D, Crauser D, Le Conte Y (2010) E-beta-ocimene, a volatile brood pheromone involved in social regulation in the honey bee colony (*Apis mellifera*) PLoS One 5:e13531 doi:10.1371/journal.pone.0013531
- Mas F, Haynes KF, Kolliker M (2009) A chemical signal of offspring quality affects maternal care in a social insect Proc Biol Sci 276:2847-2853 doi:10.1098/rspb.2009.0498
- Matsuura K, Himuro C, Yokoi T, Yamamoto Y, Vargo EL, Keller L (2010a) Identification of a pheromone regulating caste differentiation in termites PNAS 107:12963-12968 doi:10.1073/pnas.1004675107 %J Proceedings of the National Academy of Sciences

- Matsuura K, Himuro C, Yokoi T, Yamamoto Y, Vargo EL, Keller L (2010b)
Identification of a pheromone regulating caste differentiation in termites Proc Natl
Acad Sci U S A 107:12963-12968 doi:10.1073/pnas.1004675107
- Melgarejo V, Wilson Rankin EE, Loope KJ (2018) Do queen cuticular hydrocarbons
inhibit worker reproduction in *Bombus impatiens*? Insectes Soc 65:601-608
doi:10.1007/s00040-018-0651-6
- Michener CD (1974) The social behavior of the bees. Harvard University Press,
Cambridge, Massachusetts
- Mohammedi A, Paris A, Crauser D, Le Conte Y (1998) Effect of aliphatic esters on ovary
development of queenless bees (*Apis mellifera* L) Naturwissenschaften 85:455-
458 doi:DOI 10.1007/s001140050531
- Monnin T, Jones G, Ratnieks FLW, Beard R (2002) Pretender punishment induced by
chemical signaling in a queenless ant Nature 419:61-65
- Monnin T, Peeters C (1999) Dominance hierarchy and reproductive conflicts among
subordinates in a monogynous queenless ant Behav Ecol 10:323-332 doi:DOI
10.1093/beheco/10.3.323
- Morel L, Meer RKV (1988) Do ant brood pheromones exist? Ann Entomol Soc Am
81:705-710 doi:10.1093/aesa/81.5.705
- Naug D (2009) Structure and resilience of the social network in an insect colony as a
function of colony size Behavioral Ecology and Sociobiology 63:1023-1028
doi:10.1007/s00265-009-0721-x
- Noonan KC (1986) Recognition of queen larvae by worker honey-bees (*Apis mellifera*)
Ethology 73:295-306 doi:10.1111/j.1439-0310.1986.tb00811.x

- Nunes TM et al. (2014) Queen signals in a stingless bee: suppression of worker ovary activation and spatial distribution of active compounds *Sci Rep* 4:7449
doi:10.1038/srep07449
- Oi CA, Millar JG, van Zweden JS, Wenseleers T (2016) Conservation of queen pheromones across two species of Vespine wasps *J Chem Ecol* 42:1175-1180
doi:10.1007/s10886-016-0777-9
- Oi CA, van Zweden JS, Oliveira RC, Van Oystaeyen A, Nascimento FS, Wenseleers T (2015) The origin and evolution of social insect queen pheromones: Novel hypotheses and outstanding problems *Bioessays* 37:808-821
doi:10.1002/bies.201400180
- Padilla M, Amsalem E, Altman N, Hefetz A, Grozinger CM (2016) Chemical communication is not sufficient to explain reproductive inhibition in the bumblebee *Bombus impatiens* *Royal Soc Open Sci* 3:160576
- Page RE, Robinson GE, Fondrk MK (1989) Genetic specialists, kin recognition and nepotism in honey-bee colonies *Nature* 338:576-579 doi:10.1038/338576a0
- Peeters C, Liebig J (2009) Fertility signaling as a general mechanism of regulating reproductive division of labor in ants. In: Gadau J, Fewell J (eds) *Organization of Insect Societies: From Genome to Sociocomplexity* Harvard University Press,
- Pereboom JJM, Velthuis HHW, Duchateau MJ (2003) The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation *Insectes Soc* 50:127-133 doi:10.1007/s00040-003-0639-7
- Pirk CW, Neumann P, Hepburn R, Moritz RF, Tautz J (2004) Egg viability and worker policing in honey bees *PNAS* 101:8649-8651 doi:10.1073/pnas.0402506101

- Premnath S, Sinha A, Gadagkar R (1995) Regulation of worker activity in a primitively eusocial wasp, *Ropalidia marginata* Behav Ecol 6:117-123
- Premnath SS, A; Gadagkar, R. (1996) Dominance relationship in the establishment of reproductive division of labour in a primitively eusocial wasp (*Ropalidia marginata*) Behav Ecol Sociobiol 39:125-132
- Queller DC (1994) Extended parental care and the origin of eusociality Proc R Soc Lond B Biol Sci 256:105-111 doi:10.1098/rspb.1994.0056
- Ratnieks FL, Foster KR, Wenseleers T (2006) Conflict resolution in insect societies Annu Rev Entomol 51:581-608 doi:10.1146/annurev.ento.51.110104.151003
- Ratnieks FLW (1988) Reproductive harmony via mutual policing by workers in eusocial Hymenoptera Am Nat 132:217-236 doi:Doi 10.1086/284846
- Ratnieks FLW, Visscher PK (1989) Worker policing in the honeybee Nature 342:796-797 doi:DOI 10.1038/342796a0
- Rauter CM, Mooref AJ (1999) Do honest signalling models of offspring solicitation apply to insects? Proc R Soc Lond B Biol Sci 266:1691-1696 doi:10.1098/rspb.1999.0833
- Reeve HG, GJ. (1983) Colony activity integration in primitively eusocial wasps: the role of the queen (*Polistes fuscatus*, Hymenoptera: Vespidae) Behav Ecol Sociobiol 13:63-74
- Robinson GE (1992) Regulation of division of labor in insect societies Annu Rev Entomol 37:637-665 doi:10.1146/annurev.en.37.010192.003225
- Robinson GE, Vargo EL (1998) Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioral pacemaker Arch Insect Biochem Physiol 35:559-583

- Roseler PF, Roseler I, Vanhonk CGJ (1981) Evidence for inhibition of corpora allata activity in workers of *Bombus terrestris* by a pheromone from the queens mandibular glands *Experientia* 37:348-351
- Rottler-Hoermann AM, Schulz S, Ayasse M (2016) Nest wax triggers worker reproduction in the bumblebee *Bombus terrestris* *R Soc Open Sci* 3:150599 doi:10.1098/rsos.150599
- Schultner E, Oettler J, Helantera H (2017) The role of brood in eusocial Hymenoptera *Q Rev Biol* 92:39-78 doi:10.1086/690840
- Seppa P, Queller DC, Strassmann JE (2002) Reproduction in foundress associations of the social wasp, *Polistes carolina*: conventions, competition, and skew *Behav Ecol* 13:531-542 doi:DOI 10.1093/beheco/13.4.531
- Sibbald ED, Plowright CM (2014) Social interactions and their connection to aggression and ovarian development in orphaned worker bumblebees (*Bombus impatiens*) *Behav Processes* 103:150-155 doi:10.1016/j.beproc.2013.11.012
- Sibbald ED, Plowright CMS (2013) On the relationship between aggression and reproduction in pairs of orphaned worker bumblebees (*Bombus impatiens*) *Insectes Soc* 60:23-30 doi:10.1007/s00040-012-0261-7
- Sledge MF, Boscaro F, Turillazzi S (2001) Cuticular hydrocarbons and reproductive status in the social wasp *Polistes dominulus* *Behav Ecol Sociobiol* 49:401-409 doi:10.1007/s002650000311
- Smedal B, Brynem M, Kreibich CD, Amdam GV (2009) Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*) *The Journal of experimental biology* 212:3795-3801 doi:10.1242/jeb.035063

- Smith AA, Liebig J (2017) The evolution of cuticular fertility signals in eusocial insects
Curr Opin Insect Sci 22:79-84 doi:10.1016/j.cois.2017.05.017
- Smith JM (1964) Group selection and kin selection Nature 201:1145
- Smith JM (1994) Must reliable signals always be costly? Anim Behav 47:1115-1120
- Sramkova A, Schulz C, Twele R, Francke W, Ayasse M (2008) Fertility signals in the
bumblebee *Bombus terrestris* (Hymenoptera: Apidae) Naturwissenschaften
95:515-522 doi:10.1007/s00114-008-0353-4
- Starkey J, Brown A, Amsalem E (2019) The road to sociality: Brood regulation of worker
reproduction in the simple eusocial bee *Bombus impatiens* Anim Behav. Accepted
- Tallamy DW (1984) Insect Parental Care Bioscience 34:20-24 doi:Doi 10.2307/1309421
- Toth AL et al. (2007) Wasp gene expression supports an evolutionary link between
maternal behavior and eusociality Science 318:441-444
doi:10.1126/science.1146647
- Trivers R (1972) Parental investment and sexual selection vol 136. Biological
Laboratories, Harvard University Cambridge, MA,
- Trivers RL, Hare H (1976) Haplodiploidy and the evolution of the social insects. Science
191:249-263
- Ulrich Y, Burns D, Libbrecht R, Kronauer DJ (2016) Ant larvae regulate worker foraging
behavior and ovarian activity in a dose-dependent manner Behav Ecol Sociobiol
70:1011-1018 doi:10.1007/s00265-015-2046-2
- Van Oystaeyen A et al. (2014) Conserved class of queen pheromones stops social insect
workers from reproducing Science 343:287-290 doi:10.1126/science.1244899

- Van Ravestijn W, Van Der Sande J Use of bumblebees for the pollination of glasshouse tomatoes. In: VI International Symposium on Pollination 288, 1990. pp 204-212
- van Zweden JS, Heinze J, Boomsma JJ, d'Ettorre P (2009) Ant queen egg-marking signals: matching deceptive laboratory simplicity with natural complexity PLoS One 4 doi:<https://doi.org/10.1371/journal.pone.0004718>
- Vargo EH, CD. (1999) Multiple glandular origins of queen pheromones in the fire ant *Solenopsis invicta* J Insect Physiol 46:1151-1159
- Velthuis HHW, van Doorn A (2006) A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination Apidologie 37:421-451 doi:10.1051/apido:2006019
- Visscher PK (1996) Reproductive conflict in honey bees: A stalemate of worker egg-laying and policing Behav Ecol Sociobiol 39:237-244 doi:DOI 10.1007/s002650050286
- Warner MR, Kovaka K, Linksvayer TA (2016) Late-instar ant worker larvae play a prominent role in colony-level caste regulation Insectes Soc 63:575-583
- Wenseleers T, Helantera, H., Hart, A., Ratnieks, F.L.W. (2004) Worker reproduction and policing in insect societies: an ESS analysis J Chem Ecol 17:1035-1047
- West-Eberhard MJ (1996) Wasp societies as microcosms for the study of development and evolution vol 290. Natural history and evolution of paper wasps. Oxford University Press,
- Wilson EO (1971) The insect societies. Belknap Press of Harvard University press, Cambridge, Massachusetts

Winston ML, Slessor K, Willis L, Naumann K, Higo H, Wyborn M, Kaminski L (1989)

The influence of queen mandibular pheromones on worker attraction to swarm clusters and inhibition of queen rearing in the honey bee (*Apis mellifera* L.)

Insectes Soc 36:15-27

Zink AG (2003) Quantifying the costs and benefits of parental care in female treehoppers

Behav Ecol 14:687-693 doi:10.1093/beheco/arg044

Appendix

Statistics used in experiments

Table 3: The effect of related brood and wax on the cumulative number of eggs laid by random-age *B. impatiens* workers.

The effect of colony and brood type on eggs laid by workers was examined using Generalized Linear Model with poisson/log link. The effect of colony was insignificant ($\chi^2=10.7$ $p=0.06$). The effect of brood type was significant ($\chi^2=66.7$, $p<0.001$) and followed by contrast comparisons (below). Corrected p-value following Bonferroni correction is 0.003 (15 tests)

	EL	LL	LP	PP	PW	WW
EL	n/a	0.12, $p=0.7$	17.6, $p<0.001$	33.5, $p<0.001$	15.04, $p<0.001$	13.7, $p<0.001$
LL		n/a	17.05, $p<0.001$	33.6, $p<0.001$	13.3, $p<0.001$	11.19, $p<0.001$
LP			n/a	8.63, $p=0.003$	0.04, $p=0.84$	1.97, $p=0.15$
PP				n/a	3.41, $p=0.06$	18.5, $p<0.001$
PW					n/a	1.31, $p=0.25$
WW						n/a

Table 4: **The effect of related brood and wax on the cumulative number of eggs laid by callow *B. impatiens* workers.**

The effect of colony and brood type on eggs laid by workers was examined using Generalized Linear Model with poisson/log link. The effect of colony was insignificant ($\chi^2=6.68$ $p=0.08$). The effect of brood type was significant ($\chi^2=43.5$, $p<0.001$) and followed by contrast comparisons (below). Corrected p-value following Bonferroni correction is 0.003 (15 tests)

	EL	LL	LP	PP	PW	WW
EL	n/a	14.1, $p<0.001$	22.8, $p<0.001$	32.3, $p<0.001$	15.1, $p<0.001$	3.29, $p=0.06$
LL		n/a	1.02, $p=0.3$	4.3, $p=0.03$	0.005, $p=0.94$	5.01, $p=0.02$
LP			n/a	1.34, $p=0.24$	0.93, $p=0.33$	10.9, $p<0.001$
PP				n/a	4.26, $p=0.03$	18.4, $p<0.001$
PW					n/a	5.57, $p=0.01$
WW						n/a

Table 5: **The effect of unrelated brood and wax on the cumulative number of eggs laid by random-age *B. impatiens* workers.**

The effect of colony and brood type on eggs laid by workers was examined using Generalized Linear Model with poisson/log link. The effect of colony was insignificant ($\chi^2=2.28$ $p=0.3$). The effect of brood type was significant ($\chi^2=72.1$, $p<0.001$) and followed by contrast comparisons (below). Corrected p-value following Bonferroni correction is 0.005 (10 tests)

	EL/LL	LP	PP	PW	WW
EL/LL	n/a	32.7, $p<0.001$	57.03, $p<0.001$	46.6, $p<0.001$	14.7, $p<0.001$
LP		n/a	6.12, $p=0.01$	1.7, $p=0.19$	5.85, $p=0.01$
PP			n/a	1.65, $p=0.19$	23.07, $p<0.001$
PW				n/a	14.4, $p<0.001$
WW					n/a

Table 6: The effect of queen- and worker-laid larvae and wax on the cumulative number of eggs laid by random-age *B. impatiens* workers.

The effect of colony and brood type on eggs laid by workers was examined using Generalized Linear Model with poisson/log link. The effect of colony was insignificant ($\chi^2=0.47$ $p=0.49$). The effect of brood type was significant ($\chi^2=33.7$, $p<0.001$) and followed by contrast comparisons (below). Corrected p-value following Bonferroni correction is 0.008 (6 tests)

	Queen larvae	Worker larvae	Wax	Foreign-wax
Queen-laid larvae	n/a	0.57, $p=0.44$	16.5, $p<0.001$	22.1, $p<0.001$
Worker-laid larvae		n/a	11.5, $p<0.001$	16.3, $p<0.001$
Wax			n/a	0.46, $p=0.49$
Foreign wax				n/a

Table 7: The quantitative effect of larvae on the cumulative number of eggs laid by random-age *B. impatiens* workers.

To examine the effect of brood amount on workers egg laying I combined data for random-age workers from 1.1 (6 colonies), 2.1 (3 colonies) and an additional replicate (4 colonies). Since I could only include cages with live larvae by the end of the experiment (n=149), treatments groups were not equally represented between colonies and both ‘colony’ and ‘experiment’ had a significant effect on the eggs laid by workers.

Examination of each experiment separately shows similar trend and similar statistical differences between the treatment groups, I therefore combined all data below.

The effect of colony and brood type on eggs laid by workers was examined using Generalized Linear Model with poisson/log link. The effect of brood type was significant ($\chi^2=259.2$, $p<0.001$) and followed by contrast comparisons (below). Corrected p-value following Bonferroni correction is 0.008 (6 tests)

	Wax	1-2 larvae	3-10 larvae	More than 10 larvae
Wax	n/a	24.6, $p<0.001$	89.3, $p<0.001$	195.4, $p<0.001$
1-2 larvae		n/a	14.6, $p<0.001$	46.1, $p<0.001$
3-10 larvae			n/a	6.93, $p=0.008$
More than 10 larvae				n/a

Table 8: The effect of brood cuticular washes (1.4 brood equivalent/day) on the cumulative number of eggs laid by random-age *B. impatiens* workers.

The effect of colony and brood type on eggs laid by workers was examined using Generalized Linear Model with poisson/log link. The effect of colony was significant ($\chi^2=29.3$, $p<0.001$). The effect of brood type was significant ($\chi^2=2.88$, $p=0.57$) and followed by contrast comparisons (below). Corrected p-value following Bonferroni correction is 0.005 (10 tests)

	Solvent	Eggs	Young Larvae	Old Larvae	Pupae
Solvent	n/a	0.12, p=0.72	0.25, p=0.61	1.3, p=0.25	2.19, p=0.13
Eggs		n/a	0.02, p=0.86	0.62, p=0.43	1.37, p=0.24
Young Larvae			n/a	0.34, p=0.55	0.97, p=0.32
Old Larvae				n/a	0.23, p=0.62
Pupae					n/a

Table 9: The effect of brood whole-body extracts (1.4 brood equivalent/day) on the cumulative number of eggs laid by random-age *B. impatiens* workers.

The effect of colony and brood type on eggs laid by workers was examined using Generalized Linear Model with poisson/log link. The effect of colony was insignificant ($\chi^2=29.3$, $p=0.23$). The effect of brood type was nearly insignificant ($\chi^2=8.74$, $p=0.06$) and followed by contrast comparisons (below). Corrected p-value following Bonferroni correction is 0.005 (10 tests)

	Solvent	Eggs	Young Larvae	Old Larvae	Pupae
Solvent	n/a	2.19, p=0.13	6.75, p=0.009	1.87, p=0.17	5.44, p=0.01
Eggs		n/a	0.65, p=0.41	0.18, p=0.66	0.38, p=0.53
Young Larvae			n/a	2.3, p=0.12	0.03, p=0.84
Old Larvae				n/a	1.57, p=0.2
Pupae					n/a

Table 10: The effect of brood whole-body extracts (7 brood equivalent/day) on the cumulative number of eggs laid by random-age *B. impatiens* workers.

The effect of colony and brood type on eggs laid by workers was examined using Generalized Linear Model with poisson/log link. The effect of colony was insignificant ($\chi^2=1.67$, $p=0.43$). The effect of brood type was insignificant ($\chi^2=5.82$, $p=.21$) and followed by contrast comparisons (below). Corrected p-value following Bonferroni correction is 0.005 (10 tests)

	Solvent	Eggs	Young Larvae	Old Larvae	Pupae
Solvent	n/a	1.19, $p=0.27$	0.06, $p=0.8$	2.18, $p=0.13$	2.82, $p=0.09$
Eggs		n/a	1.7, $p=0.19$	0.18, $p=0.67$	0.6, $p=0.43$
Young Larvae			n/a	2.78, $p=0.09$	3.41, $p=0.06$
Old Larvae				n/a	0.16, $p=0.68$
Pupae					n/a

Table 11: The effect of brood volatiles on the cumulative number of eggs laid by random-age *B. impatiens* workers.

The effects of treatment (brood type) and contact with the brood (direct vs. air) on the cumulative number of eggs laid by workers were examined using matched-pairs test.

	Direct vs. air (matched paired test)		n
	t ratio	probability of t (two tailed)	
EL/LL	-6.52	$p<0.0001$	11
LP	2.93	$p=0.01$	13
PP	2.8	$p=0.03$	7
PW/WW	0.55	$p=0.59$	9

Table 12: The effect of fed and starved larvae whole-body extracts (7 equivalent/day) on the cumulative number of eggs laid by random-age *B. impatiens* workers.

The effect of treatment (extract of starved versus fed young larvae) on cumulative number of eggs laid by workers was examined by a Generalized Linear Model with poisson/log link. The effect of colony was significant ($\chi^2=7.53$, $p=0.023$). The effect of brood type was significant ($\chi^2=6.82$, $p=0.033$) and followed by contrast comparisons demonstrating significant differences between starved and fed larvae, but not between the treatments and the solvent control. Corrected p-value following Bonferroni correction is 0.016 (3 tests)

	Solvent	Fed larvae	Starved larvae
Solvent	n/a	2.64, $p=0.104$	0.9, $p=0.342$
Fed larvae	2.64, $p=0.104$	n/a	6.625, $p=0.01$
Starved larvae	0.9, $p=0.342$	6.625, $p=0.01$	n/a