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# Do Bumble Bees Produce Brood Pheromones?

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## Abstract

Reproductivedivisionoflabor, a definingfeature ofsocial insects,isoften regulated bya combinationofbehavioral and chemical means. 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 sized, advanced eusocial species. Here we examined if worker reproduction in the primitively eusocial species Bombus impatiens is regulated by brood pheromones. We recently demonstrated that worker egg laying in this species is inhibited by young larvae and triggered by pupae. However, the mechanism by which the brood communicates its presence and whether brood or hunger pheromones are involved remain unknown. We found that workers were behaviorally attracted to 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. We further show that the youngest larvae are particularly vulnerable to starvation, however, despite a slight attraction and fewer eggs laid by workers in the presence of starved compared with fed larvae, these effects were insignificant. Our study demonstrates that workers can differentiate between larvae and pupae, but not between starved and fed larvae based on olfactory information. However, these signals alone do not explain the reduction in worker egg laying previously found. Bumble bee workers may use information from multiple sources 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.

Keywords Reproduction . Pheromones . Bumblebees . Brood . Eusociality

## 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 ofone orfew 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).Incontrast, advancedeusocial species are characterized by extreme reproductive skew (usually in favor of one female), extensive use of pheromones and little to no overt aggression over

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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. 2013; Monnin et al. 2002). While these interactions are more common in the absence of the queen, they may occur also in the presence of the queen. For example, dominant females of the social wasp Ropalidia marginata use aggression to force subordinates to perform maintenance or risky tasks such as foraging (Premnath et al. 1995). Aggressive interactions in more advanced eusocial insects such as the honey bee, are rare, but 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. 1999; Premnath et al. 1995; Premnath et al. 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 and behavior were found also in termites (Funaro et al. 2018; Liebig et al. 2009; Matsuura et al. 2010) 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 quantitydependent 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 pheromones remains unexplored.

Larvae of many solitary or family-structured 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 David 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 preferthe rearing ofqueens overworkers (Page et al. 1989) and prioritize care of more related brood (Noonan 2010).

Bumble bees are traditionally defined as primitively eusocial (Michener 1974) since they present characteristics of both primitively and advanced eusocial species with colonies exhibiting both harmony and competition over reproduction (Amsalem et al. 2015a). Reproduction in the first 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. 2015b; Amsalem et al. 2017; Michener 1974; Padilla et al. 2016). Previous studies on bumble bee reproduction have mostly focused on the impact that 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 activation (Amsalem et al. 2017; Melgarejo et al. 2018; Padilla et al. 2016). Recently, we have shown that worker egg-laying behavior in Bombus impatiens is reduced in the presence of young larvae in a quantity-dependent manner, while the presence of old larvae and pupae increased egglaying (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 communicatestheir presenceorneedtoworkers,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 cuticularwashes, whole-bodyextracts 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 60% 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, precompetition 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 using forceps and used only if they remained intact during collection. Egg cells are usually laid on top of pupae cells, and were removed by carefully cutting the entire egg batch from the pupae cell using dissection scissors. In order to avoid damaging the brood, we separated the batches of brood as much as possible. In experiments containing live brood, we grouped workers with about 1–2 batches of eggs, several larvae or several pupae (exact numbers are described below), all kept in their own wax cover. In experiments conducted with extracts, we separated the brood from their wax cover and immediately extracted them in a solvent. 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**

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. The first two bioassays included a 2-way choice between larvae and control, or between pupae and control. The third bioassay included a 3-way choice between larvae, pupae and control. In total we conducted 84 bioassays (n = 28/choice bioassay). Olfactometers were fashioned from petri dishes (150 × 15 mm) where equidistant holes (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 min to make a choice, after which they were considered non-responders. Bioassays were conducted under a red light and olfactometers were washed between runs. All behavioral assays were conducted under the same rearing conditions between noon to 4 PM. 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 the brood produces 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 cages (11 cm diameter × 7 cm height), after which they were frozen at −20 °C. To account for variation in worker egg laying between colonies (Amsalem et al. 2015a, b), we ensured that each experiment was replicated using workers from several colonies, equally representingboth 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 min, 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 min, during which they were frequently mixed. Ground samples were then centrifuged at 3500 g for 3 min and the supernatant was transferred into new glass vials. 1–2 ml of 1:1 of hexane and methanol solvent were 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 min after applying the extracts to allow for excess solvent to evaporate. Cuticular washes wereapplied daily in a dose of 1.4 larval equivalent 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 the 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 (Fig. S1). Two 16 oz. (500 ml) 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). The experiment was conducted in constant darkness, 60% humidity and 28– 30 °C. 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/min 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 ofB. impatienslarvae 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 leaving behind them the wax cover (PW). Newly-emerged 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 h until all larvae died.

### 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, worker responses to starved and fed, same-age, larvae were examined using a 2-way choice bioassays. We introduced workers to starved and fed larvae in olfactometer bioassay arenas as in the first experiment. Approximately 7 larvae that had just hatched (5–7 days after eggs laid; Cnaani et al. 2002) werecollected from their parental colony and placed in a small plastic cage for 16 h either with or without 10 nursing workers and unlimited food. 16 h 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 Larvae Produce a Hunger Signal Regulating Worker Reproduction

To determine if B. impatiens larvae produce 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 equivalent 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 oocyte 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 $\chi^2$ 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. Oocyte 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 percentages or as boxplots featuring the minimum and maximum values, outliers and medians.

## Results

### Examining the Attraction of Bombus impatiens Workers to Brood

This experiment demonstrated a clear preference ofworkers to pupae over larvae or controls. When workers were introduced to larvae versus controls, they preferred the larvae (67% vs 33%, n = 18, Fig. 1), However these observed values did not differ from the expected values ($\chi^2_1 = 2.03$, $p = 0.153$) 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 ($\chi^2_1 = 13.63$, $p = 0.001$), 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%) ($\chi^2_2 = 5$, $p = 0.02$). 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 s). However, these differences were not significant ($\chi^2_2 = 4.39$, $p = 0.11$).

## 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 oocyte 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 worker egg laying or oocyte size as compared to the solvent control (egg laying: GLM, $\chi^2_4 = 2.88$, $p = 0.57$, Fig. 2, Table S2; oocyte size: $f_{4,167} = 0.67$, $p = 0.61$, Table S1).

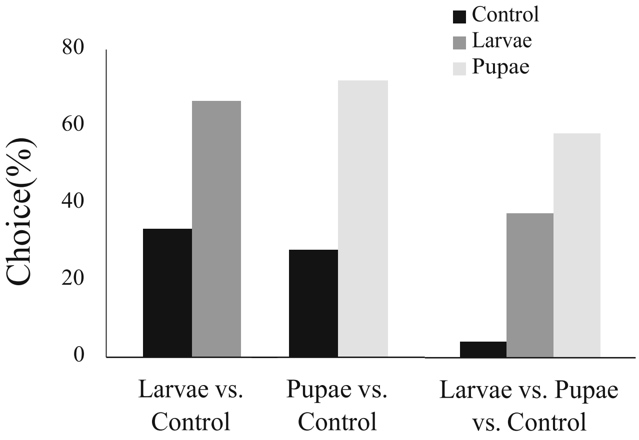


Fig. 1 The percent of choice made by B. impatiens workers in a choice bioassay between a young larvae and control, b pupae and control, and c young larvae, pupae, and control. Random-age workers were equally sampled from four young queen-right colonies, placed in an olfactometer, and given 30 min to make a choice, after which they were considered non-responders (36%,11% and 14% non-responders in a-c, respectively). Eachbioassay was repeated 28times andeachworkerwas onlyusedonce

Similarly, whole body extracts (1.4 and 7 brood-equivalent in Fig. 3a and b, respectively) did not affect either egg laying or oocyte size (1.4 brood-equivalent, egg laying: GLM, $\chi^2_4 = 8.74$, $p = 0.06$, Fig. 3a, Table S3; oocyte size: ANOVA Mixed model, $f_{4,139} = 1.07$, $p = 0.37$, Table S1; 7 brood-equivalent, egg laying: GLM, $\chi^2_4 = 5.82$, $p = 0.21$, Fig. 3b, Table S4; oocyte size: ANOVA Mixed model, $f_{4,130} = 1.01$, $p = 0.4$, Table S1).

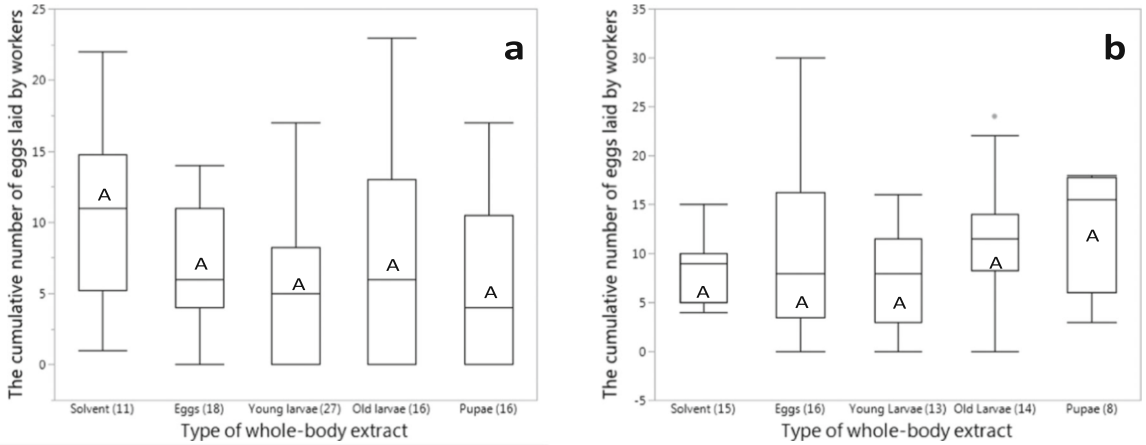
## Examining whether Bombus impatiens Brood Produces a Volatile Pheromone Regulating Worker Reproduction

To examine the effects of brood volatiles on worker reproduction (Fig. 4), 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, $t = -6.52$, $p < 0.001$), while egg laying was significantly greater in direct LP and PP groups (larvae that became pupae or pupae that remained pupae)comparedto 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 (Fig. 4, Table S5).

Worker oocyte 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 S1). In both groups however, workers had fully developed ovaries.

## Examining the Survival Rate of Starved Larvae



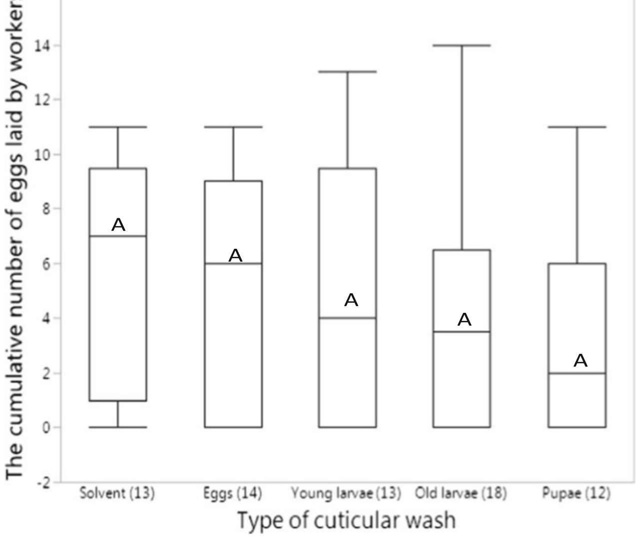


Fig. 2 The effect of brood cuticular washes on the cumulative number of eggs laid by B. impatiens workers. Random-age workers from young queen-right colonies were kept in pairs for 7 days with a filter containing cuticular washes of eggs, young larvae (<50 mg), old larvae (>50 mg) or pupae as compared to solvent control. Extracts were applied in a dose of 1.4 brood equivalent 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 Table S2

We examined the survival time (in hours) of larvae as function of their body mass (in grams) 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 ($r = 0.73$, $n = 171$, $p < 0.001$, Fig. 5), with the smallest larvae (<10 mg, $n = 41$, roughly corresponding to instar 1) surviving on average 72 ± 7 h and the largest larvae (50– 100 mg, $n = 26$, roughly corresponding to instar 3) surviving on average 252 ± 13 h without food or brood care. The average survival time of all the larvae we considered as young larvae in our study (<50 mg) was 158 ± 6 h, meaning that, on average, all larvae capable of reducing egg laying in workers (Starkey et al. 2019) will die within 6.5 days without brood care or under nutritional stress.

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

In this experiment workers were introduced to either fed or starved young larvae of approximately the same age in a 2way choice bioassay. Workers were slightly more attracted to starved compared to fed brood (Fig. 6), however this preference was insignificant compared to the expected results ($\chi^2_1 = 0.89$, $p = 0.34$).

Fig. 3 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 7 days with a filter containing whole-body extracts of eggs, young larvae (<50 mg), old larvae (>50 mg), pupae or solvent control. Extracts were applied in a dose

## 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: $\chi^2_2 = 6.82$, $p = 0.033$). A post-hoc test showed significant differences between the number of eggs laid by workers after exposure to fed and starved larvae ($\chi^2_2 = 6.625$, $p = 0.01$) but not between the treatments and the solvent control (fed versus solvent: $\chi^2_2 = 2.64$, $p = 0.104$; starved versus solvent: $\chi^2_2 =$

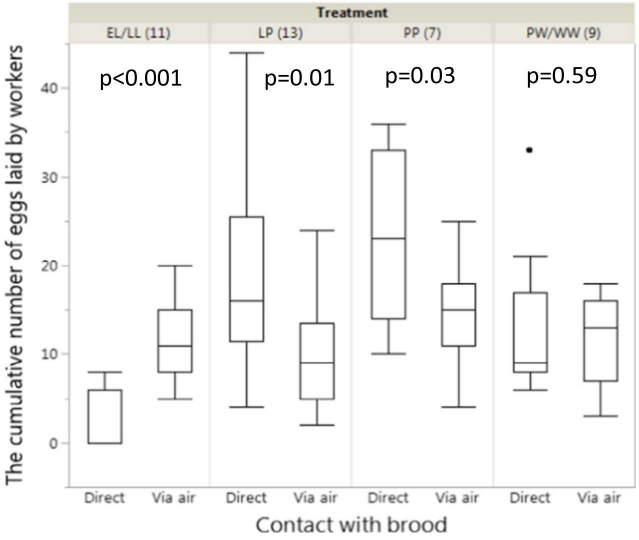
of 1.4 (a) or 7 (b) brood equivalent per day per cage. Numbers in brackets represent the number of cages. Letters within columns denote statistical differences at α = 0.005 following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Tables S3–4

0.9, p = 0.342, Fig. 7, Table S6). The treatment did not affect the oocyte size of workers ($f_{2,58}$ = 0.19, p = 0.82, Table S1).

## Discussion

The results of this study demonstrate that while B. impatiens workers are attracted to olfactory 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 support previous studies

Fig. 4



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 and Fig. S1) for 7 days. Numbers in brackets represent the number of cages. Detailed statistics for all comparisons are provided in Tables S5

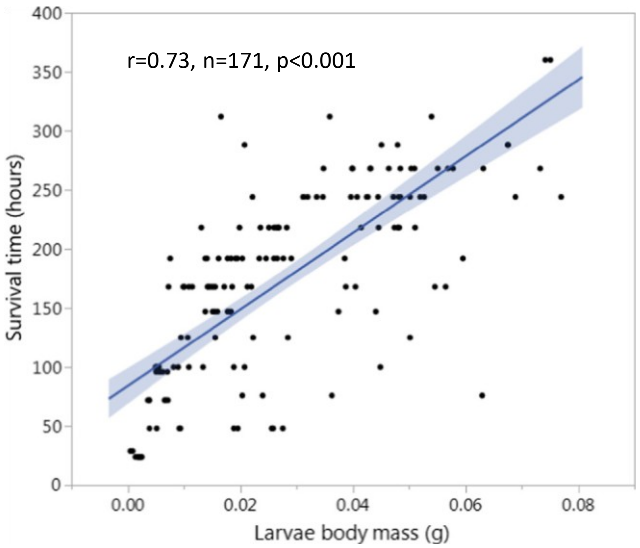


Fig. 5 Survival of B. impatiens larvae as a function of their body mass in the absence of food. Larvae were separated from their cases, individually weighed and placed in 48-well plates in a climate-controlled room (28 °C, 60% humidity and constant darkness) on top of a water bath, creating a microclimate with nearly 100% humidity. Survival was examined under a microscope (see methods) every 12 h until all larvae died

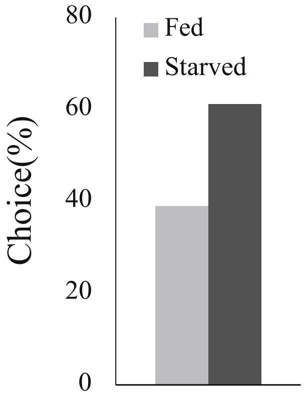


Fig. 6 The effect of larval starvation on worker attraction. The percent of choice made by B. impatiens workers in a choice bioassay between starved and fed young larvae. Random-age workerswereequallysampled from 3 young queen-right colonies, placed in an olfactometer and were given 30 min to decide, after which they were considered non-responders (90% responserate). Thebioassay wasrepeated20timesand each worker was only used once

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 the simple social organization of bumble bees.

While olfactory 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.

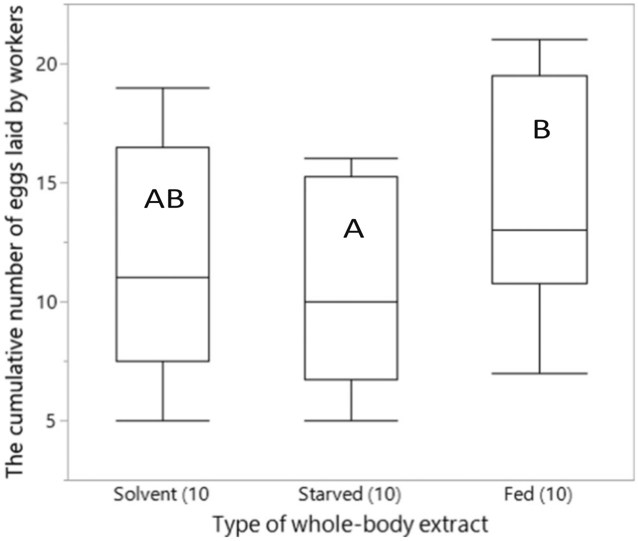


Fig. 7 The effect of starved and fed larvae whole-body extracts on the cumulative number of eggs laid by B. impatiens workers. Random-age workers were sampled from young queen-right colonies and kept in pairs for 7 days with a filter containing whole-body extracts of either 16 h starved or fed young larvae (<50 mg) compared to solvent control. Extracts were applied in a dose of 7 brood equivalent per day per cage. Letters within columns denote statistical differences at α = 0.017 following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Tables S6

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, 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 larvae, pupae and control. These responses suggest that workers may prefer the social option over the non-social alternative (ie, lack of brood), but prefer selfreproduction over brood care when are 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 (personal observation, EA).

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 larvae sprayed with cuticular hydrocarbons of starved larvae (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 (Orlova and Amsalem 2019). Thus far, the only species where brood has been shown to have both releaser and primer effects is the honey bee, an advance 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 workers were no more attracted to starved over fed larvae. 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 bioassays were 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. The importance of physical contact between workers and larvae is further emphasized by the survival rate of starved larvae showing that starvation is more detrimental for small larvae. Tiny larvae were 3.5 times faster to die compared to old larvae under starvation (Fig. 5). These extreme differences may reflect not only on the means used by tiny larvae to ensure their survival, but also on the overall resilient of the colony under conditions of nutritional stress. In conditions that do not allow foraging and in the absence of food storage, all tiny larvae will die within 3 days and all larvae capable to reduce worker egg laying will die within 6.5 days. This may affect life history traits such as colony size, transition to reproduction in workers, sex ratio and division of labor and deserves great attention when studying bumble bee sociobiology.

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 wholebody extracts of young larvae (Fig. 3, Table S3). However, even if such a signal exists, its effect on egg laying is likely to be context-dependentandmayonlyoperatetogetherwithadditional cues. The role of context in pheromone regulation was highlighted in several recent studies (Amsalem et al. 2015b; Amsalem et al. 2017; Melgarejo et al. 2018; Orlova and Amsalem 2019; Smith and Liebig 2017), including in bumble bees. Indeed, it makes little sense to respond to a costly signal (inhibiting reproduction) without collecting additional information about the social 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,dependsontheidentityoftheactivecompoundsandwhether 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-equivalent doses 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 suppression 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,thesedosesshouldbesufficienttoreplicatetheeffect.On the same note, while the role of a low-volatility signal is questionable, volatile compounds produced in bumble bee colonies can be excluded since their effectwas examined in a paired study where the physical interaction with live brood was sufficient to reduce egg laying in workers while the volatiles from the same larvae were insufficient to replicate the same effect. These findings are in line with a 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 odors produced by pupae on B. impatiens worker attraction, but not on worker reproduction. Workers seem unable to differentiate between starved and fed brood based on olfactory information. 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.

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