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

Jesse Starkey, Ahja Brown, Etya Amsalem*

Department of Entomology, Center for Chemical Ecology, Center for Pollinator Research, Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA, U.S.A.

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Eusocial societies, in which egg laying is monopolized by one or a few females, have evolved multiple times during the evolution of insects but were always rooted in a simple family structure. Female reproduction in such families is often characterized by a trade-off between reproduction and brood care, yet most work on the regulation of reproduction in social insects has focused on signals and traits exhibited by adults: particularly on the behaviour and chemical signals produced by the queen and nestmate workers. Here we examined the role of brood in regulating worker reproduction in Bombus impatiens, an annual eusocial species whose reproduction is monopolized by the queen via an unknown mechanism. We found that the presence of young larvae reduced workers' egg laying, whereas the presence of pupae stimulated egg laying. The effect of young larvae was quantity dependent, with nearly complete suppression of egg laying in cages containing a pair of workers and more than 10 young larvae, and replicable regardless of worker age, relatedness to brood or brood parentage/sex. The findings that any larvae can regulate worker reproduction in this simple, yet eusocial, species highlight the role of brood in the evolution of advanced eusocial insects as a mechanism for regulating worker sterility. These findings also provide the first holistic explanation for the regulation of worker reproduction in B. impatiens, suggesting that the queen inhibits worker reproduction through her brood.

Reproductive division of labour, 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, Menzel, Nehring, & Schmitt, 2016; Stokl & 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, Cardinal, Praz, Almeida, & Michez, 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, Oettler, & Helantera, 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 & Simmons, 2004; Kolliker, 2007; Zink, 2003€), subsocial (Engel et al., 2016; Tallamy & Denno, 1982) and advanced social lifestyles (Bigley & Vinson, 1975; Ebie, Holldobler, & Liebig, 2015; Endler et al., 2004; Maisonnasse, Lenoir, Beslay, Crauser, & Le Conte, 2010; Villalta, Angulo, Devers, Cerda, & Boulay, 2015; Woodard, Bloch, Band, & Robinson, 2013). Nevertheless, and despite several studies demonstrating the regulatory role of brood on worker reproduction, the study of reproductive division of labour 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, parenteoffspring 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 ('intrabrood 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 & 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 & 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, Neumann, Hepburn, Moritz, & Tautz, 2004), and pupae of different ages (Free & Winder, 1983). They further show nepotism in the rearing of queens (Page, Robinson, & Fondrk, 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

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* Correspondence: E. Amsalem, Department of Entomology, Pennsylvania State University, University Park, PA, 16802, U.S.A.
E-mail address: eua6@psu.edu (E. Amsalem).
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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, Fewell, & Rissing, 2000; Passera & Aron, 1996). Such conflicts between parents and offspring may be mediated using a signalling 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 & Kolliker, 2008; Rauter & Mooref, 1999).

Brood care behaviour plays a dominant role in the social evolution of insects (Kronauer & Libbrecht, 2018). However, the role of brood has been examined in only a handful of species, mostly exhibiting advanced eusocial behaviour, 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 bumblebee Bombus impatiens. Unlike species in previous studies, bumblebees form relatively small colonies in which reproduction is first monopolized by the queen
(the 'precompetition phase', during which only the queen reproduces) but later in the season is performed by both queen and workers (the 'competition phase') (Duchateau & 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 & Libbrecht, 2018). Additionally, the regulation of bumblebee reproduction has been a source of debate with conflicting results, and despite extensive research it remains unclear how reproduction is regulated in bumblebee colonies.

Previous studies in bumblebees focused mostly on queen superiority in B. terrestris and B. impatiens. Inhibition of worker reproduction was attributed to a change in queen behaviour (Amsalem et al., 2017; Padilla, Amsalem, Altman, Hefetz, & Grozinger, 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 & Hefetz,1999a; van Honk, Velthuis, Roseller,€ & Malotaux, 1980) and compounds (Sramkova, Schultz, Twele, Francke, & Ayasse, 2008; Van Oystaeyen et al., 2014) have been suggested to inhibit worker reproduction but not reduce worker ovary size (Amsalem et al., 2015; Bloch & Hefetz, 1999b; 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, Schulz, & Ayasse, 2016). Further studies suggested that workers delay reproduction (the 'competition' phase) until new queens are produced, in line with the competition phase proceeding in the presence of new queens (Alaux, Jaisson, & Hefetz, 2006) but not in the presence of males (Duchateau & Velthuis, 1988). Finally, several more studies supported the idea that the queen loses her superiority due to an increase in worker population (Amsalem & 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, Wilson Rankin, & Loope, 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 & Plowright, 2012, 2014). Several studies have demonstrated a clear impact of brood on worker behaviour, but they did not investigate how brood affects worker reproduction (den Boer & Duchateau, 2006; Pereboom, Duchateau, & Velthuis, 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. 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 Bumblebee Rearing

Colonies of B. impatiens were obtained from BioBest (Canada) and were maintained in laboratory nestboxes under constant darkness, at a temperature of 28e30 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, Schmid-Hempel, & Schmidt, 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 e20 C until further analysis. Small groups containing two to three workers are a wellestablished model to examine questions related to reproductive division of labour in bumblebees (Amsalem et al., 2015). Unlike fullsize 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 et al., 2015; Amsalem & Hefetz, 2010). 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 5e6 days, so all eggs turned into larvae within 7 days (EL). The feeding period of B. impatiens larvae lasts 9e11 days, so the larvae remained larvae (LL) or turned into pupae (LP) during the experiment. Pupation takes 11e12 days, so pupae either remained pupae (PP) or emerged as adults (PW). Pupae that emerged into adult workers were immediately removed from the cages.

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, Twele, Francke, & Hefetz, 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 bumblebees, it is often performed in queenright 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 ¼ 159 cages, 6 colonies) or pairs of callow workers (N ¼ 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. Bumblebee females lay 6e15 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 6e10 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 ¼ 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), young larvae laid by the queen (female brood) or young 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 odours from the wax by adding a second control of a pair of workers that were grouped with wax from a foreign colony (N ¼ 40 cages, 2 colonies).

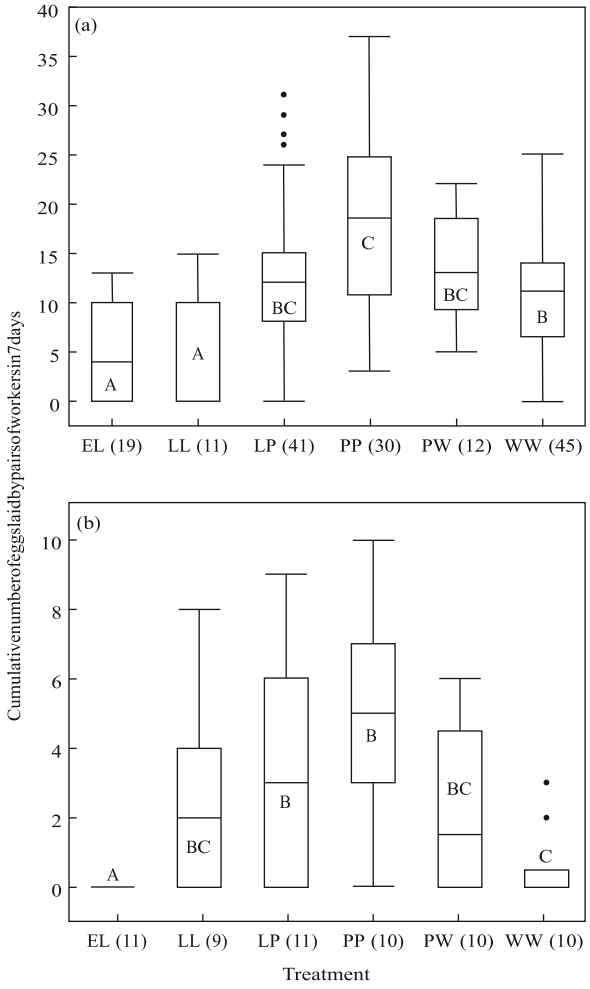


Figure 1. The effect of related brood and wax on the cumulative number of eggs laid by B. impatiens workers. Workers were randomly sampled either (a) from young queenright colonies, or (b) upon emergence and kept in pairs with brood at different developmental phases for 7 days. Treatment was defined according to the initial (eggs ¼ E; larvae ¼ L; pupae ¼ P) and terminal (larvae ¼ L; pupae ¼ P; wax ¼ W) brood stage. Numbers in parentheses represent the number of cages. All workers were kept in pairs of sisters with female brood from their mother colony. Box plots show the area between the first and third quartiles, minimum and maximum values, outliers and medians. Letters within columns denote statistical differences at a¼ 0.003 following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Tables A1 and A2.

To examine whether the brood effect is quantity dependent, we grouped pairs of random-age workers with varying amounts of young brood (1e34 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 ¼ 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 analysed 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: c^2_5 ¼ 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 ¼ 0.15, P ¼ 0.25, respectively). All pairwise comparisons are provided in the Appendix, Table A1; 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: c^2_5 ¼ 10.7, P ¼ 0.06). Worker ovary size (Table 1) was significantly affected by parental colony (ANOVA mixed model: $F_{5,313}$ ¼ 3.66, P ¼ 0.003), but not by treatment (ANOVA mixed model: $F_{5,313}$ ¼ 2.16, P ¼ 0.06).

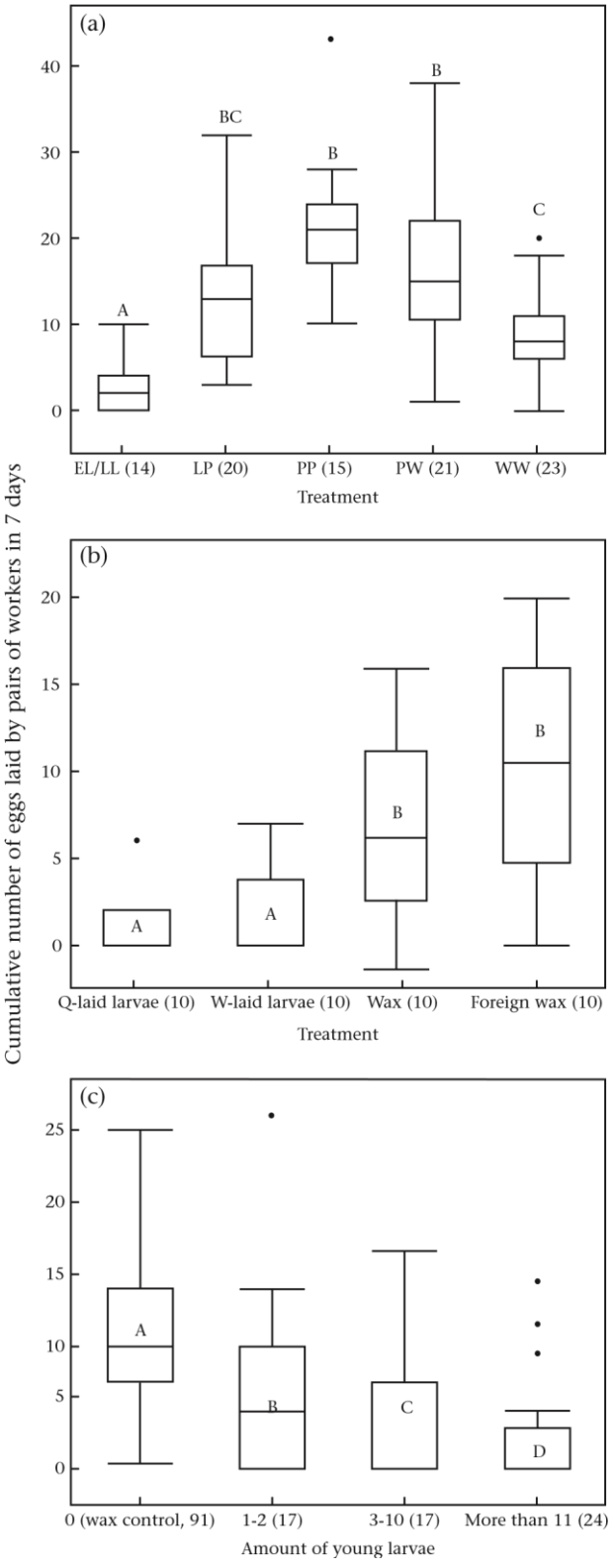
Next, we repeated the experiment using callow (newly emerged, <24 h) workers. On average, callow workers laid fewer eggs (2.2 ± 0.3 eggs, N ¼ 61 cages) compared with random-age workers (11.7 ± 0.6, N ¼ 158 cages) over 7 days. Despite that, similar results were obtained with a significant effect of brood type on the cumulative number of eggs laid by workers (GLM: c^2_5 ¼ 43.5, P < 0.001; Fig. 1b, Table A2) and no significant effect by parental colony (c^2_3 ¼ 6.6, P ¼ 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 ¼ 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

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

Experiment	Treatment	Oocyte size± SEM (mm)	Sample size (workers)	Statistical differences	
				Colony effect	Brood type effect
The effect of related brood on random-age worker ovary size (N ¼ 159 cages, 318 EL workers, 6 colonies; Fig. 1a)		1.64 ± 0.15 2.08 ± 0.15	38 24	$F_{5,313}$ ¼ 3.66, P ¼ 0.003	$F_{5,313}$ ¼ 2.16, P ¼ 0.06
	LL	2.22 ± 0.09	83		
	LP				
	PP	1.98 ± 0.13	59		
	PW	1.91 ± 0.19	24		
	WW	2.01 ± 0.09	90		
The effect of related brood on 7-day-old worker ovary size (N ¼ 61 cages, 122 workers, EL 4 colonies; Fig. 1b)		1.73 ± 0.17 1.84 ± 0.23	22 18	$F_{3,119}$ ¼ 1.01, P ¼ 0.39	$F_{5,117}$ ¼ 0.82, P ¼ 0.53
	LL	1.99 ± 0.19	22		
	LP				
	PP	2.12 ± 0.14	20		
	PW	1.84 ± 0.19	20		
	WW	1.98 ± 0.18	20		
The effect of unrelated brood on random-age worker ovary size (N ¼ 93 cages, 186 colonies; Fig. 2a)	EL/LL workers, 3	2.14 ± 0.14	28	$F_{2,184}$ ¼ 0.7, P ¼ 0.49	$F_{4,182}$ ¼ 0.89, P ¼ 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		
The effect of brood parentage/sex and wax on random-age worker ovary size (N ¼ 40 Queen-laid cages, 80 workers, 2 colonies; Fig. 2b)	larvae	1.98 ± 0.25	20	$F_{1,79}$ ¼ 4.22, P ¼ 0.04	$F_{3,77}$ ¼ 2.34, P ¼ 0.08
		1.99 ± 0.19	20		
	Worker-laid larvae				
	Wax	2.6 ± 0.19	20		
	Foreign wax	2.4 ± 0.19	20		
The quantitative effect of larvae on random-age worker ovary size (N ¼ 149 cages, 298 1e2 larvae workers, 13 colonies; Fig. 2c)		2.54 ± 0.12 1.85 ± 0.13	34 34 48	$F_{11,287}$ ¼ 2.58, P ¼ 0.003	$F_{3,295}$ ¼ 2.51, P ¼ 0.059
	3e10 larvae				
	More than 11 larvae	2 ± 0.1			
	Wax	2.16 ± 0.06	182		

E ¼ eggs; L ¼ larvae; P ¼ pupae; W ¼ wax. In the first three experiments, treatment was defined according to the initial (eggs, larvae, pupae) and terminal (larvae, pupae, wax brood stage.

was the lower number of eggs laid by workers in the wax treatment (WW versus EL: c^2_1 ¼ 3.3, ^{P} ¼ 0.06; WW versus LL: c^2_1 ¼ 5.01, P ¼ 0.02; WW versus LP: c^2_1 ¼10.9, P < 0.001; WW versus PP: c^2_1 ¼18.4, P < 0.001; WW versus PW: c^2_1 ¼ 5.57, P ¼ 0.01). Worker



(Q-laid female larvae), young larvae laid by queenless workers (W-laid male larvae),

Figure 2. 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 sampled from young queenright colonies and kept in pairs with brood at different developmental phases for 7 days. Workers were kept (a) with brood from a foreign colony (brood stage abbreviations as in Fig. 1), (b) with young larvae laid by the queen

ovary size was not affected by parental colony (ANOVA mixed model: $F_{3,119}$ ¼ 1.01, P ¼ 0.39) or by treatment (ANOVA mixed model: $F_{5,117}$ ¼ 0.82 P ¼ 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: c^2_4 ¼ 72.1, P < 0.001; Table A3). 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: c^2_2 ¼ 2.28, P ¼ 0.32; Table A3). Worker ovary size was not affected by parental colony (ANOVA mixed model: $F_{2,184}$ ¼ 0.7, P ¼ 0.49) and did not differ across treatment groups (ANOVA mixed model: $F_{4,182}$ ¼ 0.89 P ¼ 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: c^2_3 ¼ 33.7, P < 0.001, followed by contrasts P < 0.001; Fig. 2b, Table A4). No significant differences were found between worker- and queen-laid larvae (P ¼ 0.44), or between the two different wax groups (P ¼ 0.49). Ovary size was significantly affected by parental colony (ANOVA mixed model: $F_{1,79}$ ¼ 4.22, P ¼ 0.04; Table 1) but not by treatment (ANOVA mixed model: $F_{3,77}$ ¼ 2.34, P ¼ 0.08).

Finally, we examined the quantitative effect of larvae on worker reproduction. The number of young larvae housed with workers significantly affected egg laying (GLM: c^2_3 ¼ 259.2, P < 0.001; Fig. 2c, Table A5). 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: $F_{11,287}$ ¼ 2.58, P ¼ 0.003; Table 1). However, no significant difference in ovary size was found between treatment groups (ANOVA mixed model: $F_{3,295}$ ¼ 2.51, P ¼ 0.059).

Discussion

The results of the current study demonstrate that worker reproduction in B. impatiens is not only regulated by the queen (Alaux, Jaisson, & Hefetz, 2004; Amsalem et al., 2017; Padilla et al., 2016) and the nestmates (Bloch & Hefetz, 1999a) 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 & Denno, 1982; Ulrich, Burns,

wax from their own colony or from a foreign colony, or (c) with varying numbers of young larvae. Box plots show the area between the first and third quartiles, minimum and maximum values, outliers and medians. Numbers in parentheses represent the number of cages. Letters within columns denote statistical differences at a¼ 0.005 (a) or at a¼ 0.008 (b, c) following Bonferroni correction for multiple testing. Detailed statistics for all comparisons are provided in Tables A3eA5.

Libbrecht, & Kronauer, 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 & Strassmann, 1990), and in several species of queenless ants, workers forgo reproduction in the presence of larvae (Heinze, Trunzer, Oliveira, & Holldobler, 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 behaviours performed by workers, such as brood care and foraging (Maisonnasse et al., 2010), and hygienic behaviour (Wagoner, Spivak, & Rueppell, 2018). Similarly, in advanced eusocial ant colonies, brood has various signalling roles. In fire ants, and possibly also in army ants, brood induce tending behaviour, ensuring the proper function of the colony (Bigley & Vinson, 1975; Glancey, Stringer, Craig, Bishop, & Martin, 1970). In 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. Bumblebee 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 & 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 behavioural and 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 bumblebees, such as the ability of dominant, egg-laying workers (Bloch & Hefetz,1999b) 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 & Velthuis, 1988), since even male brood postpones worker reproduction. In fact, the combination of behavioural 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 bumblebee colonies thus far.

The opposing effects of larvae and pupae on worker reproduction fit a mechanism of activatoresuppressor, where two conflicting signals are produced at the same time, and the behavioural 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, Jackson, Holcombe, & Ratnieks, 2005), in C. 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-b-ocimene is produced by young brood and accelerates behavioural maturation, whereas brood ester pheromone is produced by old brood and delays behavioural maturation in workers (Maisonnasse 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, 2010; 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 and b). Previous work in B. terrestris (Rottler, Schulz, & Ayasse, 2013; Rottler-Hoermann et al., 2016) 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 odours are embedded in the wax and that these odours provide workers with information about the status of the colony and the best time to reproduce. Interestingly, the workers in RottlerHoermann 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 odours. In the present study, random-age workers had the opportunity to learn the wax odours 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 & Vinson, 1975; Endler et al., 2004; Maisonnasse et al., 2010). However, primitively eusocial species such as bumblebees tend to rely more heavily on behavioural rather than chemical mechanisms to regulate reproductive division of labour (Kocher & 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 bumblebees. 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 behaviours (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 behaviour. The use of simple insect societies to understand the behavioural, 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 behaviour (Amsalem et al., 2015; Jandt, Tibbetts, & Toth, 2013; Kronauer & Libbrecht, 2018). Brood care is one of the trajectories by which eusociality has evolved in holometabolous insects (Kronauer & Libbrecht, 2018), and the findings that the social organization of simple eusocial insects such as bumblebees still mostly relies on brood presence is striking and opens new realms of opportunities to study the evolution of brood care.

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Supplementary Material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anbehav.2019.06.004.

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Appendix

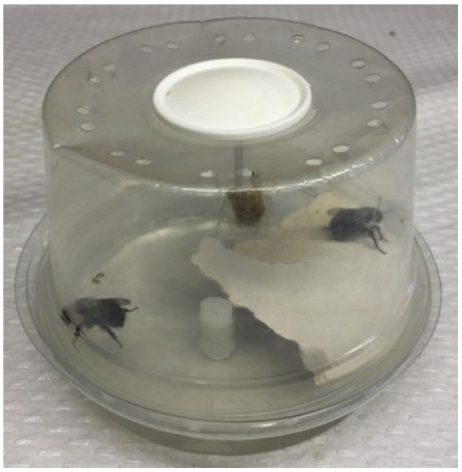

Figure A1. An image of the custom plastic cages used in this study. Pairs of bees in all experiments were placed in plastic cages containing unlimited 60% sugar solution and Light Spring Harvested Bee Pollen (purchased from 911 Honey). The bees and the brood or the wax were placed on top of a cardboard. Cages were kept in constant darkness, at 28e30 C and 60% humidity and were maintained under red light.

Table A1

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

	EL	LL	LP	PP	PW	WW
EL	e	c^2_5 ¼ 0.12, P ¼ 0.7 e	c^2_5 ¼ 17.6, P < 0.001	c^2_5 ¼ 33.5, P < 0.001	c^2_5 ¼ 15.04, P < 0.001 c^2_5 ¼	c^2_5 ¼ 13.7, P < 0.001
LL			^{2} ¼ < 0.001 c 5 17.05, P e	^{2} ¼ < 0.001 c 5 33.6, P ^{2} ¼ 8.63, P ¼ 0.003 c 5 e	13.3, P < 0.001 ^{2} ¼ 0.04, P ¼ 0.84 c 5 ^{2} c 5 ¼ 3.41, P ¼ 0.06 e	^{2} ¼ < 0.001 c 5 11.19, P ^{2} ¼ 1.97, P ¼ 0.15 c 5 c^2_5 ¼ 18.5, P < 0.001 ^{2} c 5 ¼ 1.31, P ¼ 0.25 e
LP						
PP						
PW						
WW						

E ¼ eggs; L ¼ larvae; P ¼ pupae; W ¼ wax. Treatment was defined according to the initial (eggs, larvae, pupae) and terminal brood stage (larvae, pupae, 'wax'). The effect of colony and brood type on eggs laid by workers was examined using a generalized linear model with Poisson/log link. There was no significant effect of parental colony (c^2_5 ¼ 10.7,P ¼ 0.06), but there was a significant effect of brood type (c^2_5 ¼ 66.7, P < 0.001), followed by contrast comparisons shown above. Corrected P value following Bonferroni correction was 0.003 (15 tests).P ¼ 0.06), but there was a significant effect of brood type (c^2_5 ¼ 66.7, P < 0.001), followed by contrast comparisons shown above. Corrected P value following Bonferroni correction was 0.003 (15 tests).

Table A2

The effect of related brood and wax on the cumulative number of eggs laid by 7-day-old B. impatiens workers

	EL	LL	LP	PP	PW	WW
EL	e	c^2_5 ¼ 14.1, P < 0.001 e	c^2_5 ¼ 22.8, P < 0.001	c^2_5 ¼ 32.3, P < 0.001	c^2_5 ¼ 15.1, P < 0.001 c^2_5 ¼	c^2_5 ¼ 3.29, P ¼ 0.06
LL			^{2} c 5 ¼ 1.02, P ¼ 0.3 e	^{2} c 5 ¼ 4.3, P ¼ 0.03 ^{2} 1.34, P ¼ 0.24 c 5 ¼ e	0.005, P ¼ 0.94 ^{2} 0.93, P ¼ 0.33 c 5 ¼ ^{2} c 5 ¼ 4.26, P ¼ 0.03 e	^{2} c 5 ¼ 5.01, P ¼ 0.02 ^{2} 10.9, P < 0.001 c_{25} ¼ < 0.001 c_{25} ¼ 18.4, P c 5 ¼ 5.57, P ¼ 0.01 e
LP						
PP						
PW						
WW						

Abbreviations as in Table A1. The effect of colony and brood type on eggs laid by workers was examined using a generalized linear model with Poisson/log link. There was no significant effect of parental colony (c^2_3 ¼ 6.68, P ¼ 0.08), but there was a significant effect of brood type (c^2_5 ¼ 43.5, P < 0.001), followed by contrast comparisons shown above. Corrected P value following Bonferroni correction is 0.003 (15 tests).

Table A3

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

	EL/LL	LP	PP	PW	WW

EL/LL	e	c^2_4 ¼ 32.7, P < 0.001 e	c^2_4 ¼ 57.03, P < 0.001	c^2_4 ¼ 46.6, P < 0.001	c^2_4 ¼ 14.7, P < 0.001 c^2_4
LP			c^2_4 ¼ 6.12, P ¼ 0.01 e	c^2_4 ¼ 1.7, P ¼ 0.19	¼ 5.85, P ¼ 0.01
PP				c^2_4 ¼ 1.65, P ¼ 0.19 e	c^2_4 ¼ < 0.001 c^2_4 23.07, P
PW					c^2_4 ¼ < 0.001 c^2_4 14.4, P e
WW					

Abbreviations as in Table A1. The effect of colony and brood type on eggs laid by workers was examined using a generalized linear model with Poisson/log link. There was no significant effect of parental colony (c^2_2 ¼ 2.28, P ¼ 0.3), but there was a significant effect of brood type (c^2_4 ¼ 72.1, P < 0.001), followed by contrast comparisons shown above. Corrected P value following Bonferroni correction is 0.005 (10 tests).

Table A4
The effect of queen- and worker-laid larvae and wax on the cumulative number of eggs laid by random-age B. impatiens workers

	Queen larvae	Worker larvae	Wax	Foreign wax
Queen larvae	e	c^2_3 ¼ 0.57, P ¼ 0.44 e	c^2_3 ¼ 16.5, P < 0.001	c^2_3 ¼ 22.1, P < 0.001
Worker larvae			c^2_3 ¼ < 0.001 c^2_3 11.5, P e	c^2_3 ¼ < 0.001 c^2_3 16.3, P
Wax				c^2_3 ¼ 0.46, P ¼ 0.49 e
Foreign wax				

The effect of colony and brood type on eggs laid by workers was examined using a generalized linear model with Poisson/log link. There was no significant effect of parental colony (c^2_1 ¼ 0.47, P ¼ 0.49), but there was a significant effect of brood type (c^2_3 ¼ 33.7, P < 0.001), followed by contrast comparisons shown above. Corrected P value following Bonferroni correction is 0.008 (6 tests).

Table A5
The quantitative effect of larvae on the cumulative number of eggs laid by random-age B. impatiens workers

	Wax	1e2 larvae	3e10 larvae	More than 10 larvae
Wax	e	c^2_3 ¼ 24.6, P < 0.001 e	c^2_3 ¼ 89.3, P < 0.001	c^2_3 ¼ 195.4, P < 0.001
1e2 larvae			c^2_3 ¼ < 0.001 c^2_3 14.6, P e	c^2_3 ¼ < 0.001 c^2_3 46.1, P
3e10 larvae				c^2_3 ¼ 6.93, P ¼ 0.008 e
More than 10 larvae				

To examine the effect of brood amount on workers' egg laying, we combined data for random-age workers from the first experiment (6 colonies, Fig. 1a), the third experiment (3 colonies, Fig. 2a) and an additional replicate (4 colonies). Since we could only include cages with live larvae by the end of the experiment (N ¼ 149), treatment 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 showed similar trends and statistical differences between the treatment groups; we therefore combined all data. The effect of colony and brood type on eggs laid by workers was examined using a generalized linear model with Poisson/log link. The effect of brood type was significant (c^2_3 ¼ 259.2, P < 0.001), followed by contrast comparisons shown above. Corrected P value following Bonferroni correction is 0.008 (6 tests).