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RESEARCH ARTICLE

Do queen cuticular hydrocarbons inhibit worker reproduction in *Bombus impatiens?*

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Abstract
Social insect colonies are organized by a reproductive division of labor, in which non-reproductive workers cooperate to rear the offspring of the queen. Queen pheromones, chemical compounds produced by queens that regulate worker fertility, have been identified in a handful of bees, ants, wasps, and termites. However, recent studies on bumblebee (*Bombus* spp.) queen signals have yielded conflicting findings. Here we provide an independent investigation of experiments to test the hypothesis that queen-produced non-volatile cuticular compounds influence worker's reproductive behavior. We exposed small groups of *Bombus impatiens* workers to extracted cuticular compounds from queens collected from either mid-season (pre-reproductive) or late-season (reproductive) colonies and tracked worker reproduction and ovary development. We observed no difference in worker's ovarian development or egg production when comparing among the mid-season queen extract, late-season queen extract and the solvent control treatments. Our data replicate the finding that body size positively correlates with ovarian development in workers. These results are consistent with recent studies showing that queen cuticular compounds do not inhibit worker reproduction in *B. impatiens*.

Keywords Queen pheromone · Worker reproduction · Cuticular hydrocarbons

Introduction

A reproductive division of labor between workers and queens is a defining feature of social insect colonies (Wilson 1971). In efforts to understand the evolution of altruistic sterility, there has been substantial interest in discovering the mechanisms by which workers remain non-reproductive in the presence of the queen. Queen pheromones, chemical signals produced by queens that induce sterility in workers, are thought to be widely important in maintaining this reproductive division of labor (recently reviewed in Oi et al. 2015; Smith and Liebig 2017). Such pheromones could evolve as an honest indicator of the queen's presence and fertility, with worker's and queen's interests aligned (Keller and Nonacs 1993). Alternatively, such pheromones could be produced by queens to selfishly manipulate worker's reproductive behavior at the expense of the worker fitness (Keller and Nonacs 1993). Several queen pheromones have now been identified: most are non-volatile, cuticular hydrocarbons (CHCs) in a variety of ants (Smith et al. 2009; Holman et al. 2010, 2016; Van Oystaeyen et al. 2014) and wasps (Van Oystaeyen et al. 2014; Oi et al. 2016), though there are at least two examples of non-CHC queen pheromones, in the European Honeybee *Apis mellifera* (Hoover et al. 2003) and a termite (Matsuura et al. 2010). However, despite much study, the existence of a cuticular queen pheromone in bumblebees (genus *Bombus*) remains controversial.

Bumblebees are model organisms for studying social evolution. The chemical ecology of worker–queen interactions and worker reproduction has been extensively studied in the European species, *Bombus terrestris* (Röseler et al. 1981; Bloch and Hefetz 1999; Amsalem et al. 2009, 2014; Van Oystaeyen et al. 2014; Holman 2014; Rottler-Hoermann et al. 2016; reviewed in Amsalem et al. 2015a). Although not as well-studied, the common North-American species, *Bombus impatiens*, has similar morphological differences between queens and workers, and a typical annual colony cycle. Colonies are founded by solitary queens in the spring, producing a first generation of workers who take over non-reproductive tasks. Colonies grow in size and eventually begin to produce new queens and males before colonies die

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in the autumn. Worker reproduction is common in queenright *B. terrestris* colonies once gyne-destined brood have been produced (Duchateau and Velthuis 1988; Bourke and Ratnieks 2001), and antagonistic behavior between workers and the queen over who produces males is common, sometimes resulting in the queen's death (reviewed in Bourke 1994). In contrast, worker reproduction is rare in queenright colonies of *B. impatiens* (Cnaani et al. 2002), though workers readily reproduce following the queen's death (Cnaani et al. 2002; Jandt and Dornhaus 2011; Amsalem et al. 2015b).

Early evidence of a cuticular queen pheromone in bumblebees showed that queen cuticular extracts reduced juvenile hormone production in *B. terrestris* workers, though they did not directly measure ovary development (Röseler et al. 1981; Bloch and Hefetz 1999). More recently, it has been proposed that linear hydrocarbons on the queen cuticle prevent workers from reproducing (Van Oystaeyen et al. 2014; Holman 2014). This is accomplished by inhibiting the development of worker's ovaries, as well as by inducing secondary oocyte resorption. In particular, treatment with pentacosane (*n*-C25), which is more abundant in queens than workers, caused significant resorption of worker oocytes (but no reduction in ovary development) in one experiment (Van Oystaeyen et al. 2014), and a reduction in worker oocyte number (but no oocyte resorption) in another (Holman 2014).

Nevertheless, other studies have questioned the existence of a queen pheromone in *B. impatiens* (Amsalem et al. 2015b; Padilla et al. 2016). Pentacosane, the putative queen pheromone compound of *B. terrestris*, had no impact on worker's oocyte size, latency to lay eggs and the cumulative number of eggs produced in groups of either callow or adult *B. impatiens* workers (Amsalem et al. 2015b). Treatment with pentacosane, however, did induce oocyte regression (Amsalem et al. 2015b), as was found in *B. terrestris* (Van Oystaeyen et al. 2014). However, a reanalysis of the data suggested that pentacosane may diminish worker reproduction in *B. impatiens* (Holman et al. 2017). Recently, a series of elegant experiments also showed that neither volatile compounds from queens nor interacting with a queen through a mesh screen inhibits worker investment in reproduction, ruling out volatile queen pheromones (though not necessarily contact-based CHC pheromones) in *B. impatiens*. In these experiments, only those nest mates that directly interacted with the queen showed significant reduction of their oocytes (Padilla et al. 2016).

Motivated by the conflicting results of these experiments, here we independently tested the hypothesis that queen-derived cuticular compounds inhibit worker reproduction in *B. impatiens*. We predicted that any effect observed by a single isolated compound (i.e., pentacosane, as in several studies of *B. terrestris*; Van Oystaeyen et al. 2014; Holman 2014) would be even stronger if complemented with the entire natural queen's chemical profile, since chemical context could be important for workers to respond to the queen's signals (Smith et al. 2015). We extracted queen cuticular compounds and presented these compounds to isolated groups of workers, observing ovary activation and oviposition. We assayed compounds extracted from two types of queens: from mid-season (worker-producing) colonies (hereafter MSQs) and late-season (queen-producing) colonies (hereafter LSQs). We predicted that LSQ extracts may have lesser inhibitory effects than MSQ extracts, since worker reproduction increases toward the end of the colony's life in *B. terrestris* (Bourke and Ratnieks 2001), though workers lay few eggs even in late-season colonies of *B. impatiens*, provided they are queenright (Cnaani et al. 2002). We also tested two concentrations of queen compounds from MSQs. We relate our findings to a quite similar experiment performed contemporaneously (Amsalem et al. 2017) in the discussion.

Materials and methods

Experiment 1: mid-season and late-season queen extracts

Colonies

Twelve young *Bombus impatiens* colonies were acquired from Biobest, Inc. on July 12th 2016 (mean number of workers = 44.6, range 31–66). Most colonies were in the worker-producing phase of the colony cycle, although 4 colonies contained 1–2 adult males and 1 colony contained a single adult gyne. In such *B. impatiens* colonies, only ~ 9% of workers have active ovaries (Cnaani et al. 2002). Seventy-two clear, 12 oz plastic cups with ventilated lids were lined with black construction and white filter paper. Each microcolony was created by selecting three workers at random from a single source colony. Microcolonies of three workers are a well-established paradigm for studying reproduction in *B. impatiens* workers (e.g., Cnaani et al. 2002; Gradish et al. 2013; Amsalem et al. 2015b), since workers in such groups will readily activate their ovaries and lay eggs. We created 8 microcolonies from each of nine source colonies, for a total of 72. Microcolonies were provided ad libitum with Biogluc sugar syrup (Biobest, Inc.) and pollen (Brushy Mountain BeeFarm). Using microcolonies instead of full colonies had several advantages: it allowed us to homogenize the social environment each worker experiences, it allowed for treatments to be balanced across source colonies, and it increased the number of social group replicates, which is important given the non-independence of workers in the same social environment.

Treatments

To assay worker responses to mid-season queen cuticular compounds, we extracted the 12 mother queens from the same colonies used to create microcolonies by flash freezing queens at − 80 °C and then placing all queens together in ~ 20 ml of hexane (HPLC grade) for 5 min. The solution was poured off, the queens were rinsed in ~ 5 ml of hexane which was combined with the original extract, and the resulting solution was concentrated under a nitrogen stream. The final volume was 1230 µl, such that we could apply 0.08 queen-equivalents (QE) per day for 8 days to 18 groups, with a solution volume of 8.5 µl per day per group. This amount of queen extract per day was based on a trade-off between the number of microcolonies we could treat and the amount of extract each received, given a finite supply of queens from which to extract. This amount (0.08 QE/day) is ~ 9× the amount per day used in Holman's (2014) experiment, and similar to the 0.1 QE/day used in Amsalem et al. (2017). Aliquots of this solution were made for each day of the experiment and kept at − 80 °C until applied to microcolonies. Using the same protocol, we extracted the cuticular compounds of 9 mother queens from colonies far into the gyne production stage (LSQs). These colonies all had adult gynes (range 6–137, mean 45) when killed by freezing, and were kept at − 80 °C for several months until queens were removed and extracted. These queens were similarly extracted, and the extract similarly concentrated such that 0.08 queen-equivalents were present in 8.5 µl of solution.

The established microcolonies were randomly assigned to one of the three treatments as follows: (1) 18 cups were daily given 8.5 µl of mid-season queen extract pipetted into the bottom of the container, (2) 14 were daily given 8.5 µl of the late-season queen extract, and (3) 40 cups received 8.5 µl of hexane a day, the control. These assignments were balanced across source colonies, such that each source colony yielded 2 groups in the MSQ treatment, no more than 2 groups in the LSQ treatment, and 4 groups in the control treatment.

Data collection

The microcolonies were maintained for 8 days in an insectary with a temperature that ranged 27–28 °C, and relative humidity that ranged 31–43%, balancing cup placement on shelves across treatments. The insectary was retained in continual darkness, and observations were conducted under red light. We checked microcolonies every 24 h for egg cups, with the observer blind to treatment group. After these checks, 8.5 µl of treatment solution (or hexane for controls) was applied to the bottom of each cup using disposable pipette tips.

Twenty-four hours after the eighth treatment (i.e., 8 days after the start of the experiment), microcolonies were killed with dry ice and adults were stored at − 80 °C. The egg cups in all of the microcolonies were carefully opened using fine forceps to count all of the eggs and larvae present. While egg eating commonly occurs in bumblebees, it is rare in small queenless groups (Amsalem et al. 2015b). We found no evidence of oophagy (such as open egg cells, etc.).

Worker's head width, a proxy for body size, was measured under a stereomicroscope using a scaled ocular. Each worker was then dissected under a stereomicroscope in distilled water. All measurements were made blind to the treatment received. The length of the terminal oocyte in the three most-developed ovarioles was quantified under a dissecting scope using a hand-held microscope ruler (5 mm with 0.1 mm increments). There was a total of four ovarioles in each of the subordinate's ovaries. Mean terminal oocyte length for each individual served as an index of ovarian activation. We also scored workers for the presence of resorbed oocytes (yellowish or gray deformed oocytes).

Experiment II: higher dose of mid-season queen extract

Colonies

In the second experiment, we repeated the assay with a higher dose of queen compounds. We received 14 source colonies on November 8th 2016 also from Biobest, Inc. The number of workers from these colonies was similar to that in Experiment I (mean = 61, range 51–71). Two of those colonies were not used as a source for worker groups since they only contained 1 or 2 males.

Treatments and data collection

Queens from all 14 colonies were killed and extracted using the same procedure as described above. The hexane extract was concentrated to approximately 795 µl, which was similarly aliquoted into 8 vials and stored at − 80 °C.

We created a total of 22 three-worker microcolonies. 11 cups received 9 µl of hexane as the control, while the rest received the same volume of queen extract. This reduction in the number of microcolonies allowed us to use a higher amount of queen cuticular extract: 0.16 queen-equivalents per day to 11 groups (double the amount of queen extract used in Experiment I.). The microcolonies were kept at a temperature that ranged 19–26 °C and relative humidity between 16–37%. We collected the same data as described in Experiment I.

Statistical analyses

Generalized linear mixed models (GLMMs) were used to analyze data using the glmer function in the package

lme4 (Bates et al. 2014). Effects (slopes) are reported with ± 1 standard error. All statistical tests were performed in R (v3.3.0, R Core Team 2017). All tests were performed separately for Experiments I and II. For each individual, we calculated the average terminal oocyte length (mm), which was used as a response variable, and, treatment, and individual head size were fixed effects, with source colony as a random effect. We also ran a binomial GLMM with a binary response variable of "reproductive" (defined as at least one oocyte > 2 mm) vs "not reproductive" with the same predictors (Holman et al. 2017). We also assessed how head size varied across treatments using *t* tests, to detect inadvertent confounds between body size and treatment. We only observed 7 individuals who exhibited evidence of oocyte resorption (5 in control microcolonies, 2 in treatment microcolonies, all in Experiment 1), and thus did not analyze ovary regression statistically. Individuals with oocyte resorption were removed from statistical analyses of ovary development.

At the level of the microcolony, we used negative binomial GLMMs to assess how cumulative number of eggs laid varied by treatment and source colony. The anova() function was used to obtain χ^2 statistics for predictor variables in GLMM models by comparing to a reduced model without the predictor in question. Similarly, we used survival models to analyze the time until the first egg appeared, using the survreg() function in the survival package (Therneau 2015).

Results

Ovary development

In Experiment I, average terminal oocyte length was similar among workers treated with compounds of queens from both mid- and late-season colonies and workers not treated with any queen compounds at all (Fig. 1). A GLMM revealed a significant positive effect of head size (effect = 0.75 ± 0.21 s.e., $\chi^2 = 11.3$, *d.f.* = 1, $p = 0.0007$), but no effect of treatment ($\chi^2 = 1.48$, *d.f.* = 2, $p = 0.48$), on individual bee mean terminal oocyte length (Fig. 2). Variance explained by source colony was small (0.026, vs 0.40 residual variance). Similarly, in experiment II, when workers were treated with a higher concentration of queen extract, head size but not treatment had a significant effect on ovary development (Figs. 1, 2: $\text{effect}_{\text{head size}} = 1.59 \pm 0.68$ s.e., $\chi^2 = 7.54$, *d.f.* = 1, $p = 0.006$; $\text{effect}_{\text{treatment}} = -0.28 \pm 0.21$, $\chi^2 = 1.79$, *d.f.* = 1, $p = 0.18$). Variance explained by source colony was 0.19, relative to 0.73 residual variance.

In Experiment I, the majority of the workers possessed mature ovaries, regardless of which treatment they were exposed to: 89% (184/207) had at least one terminal oocyte larger than 2 mm, indicating the presence of eggs

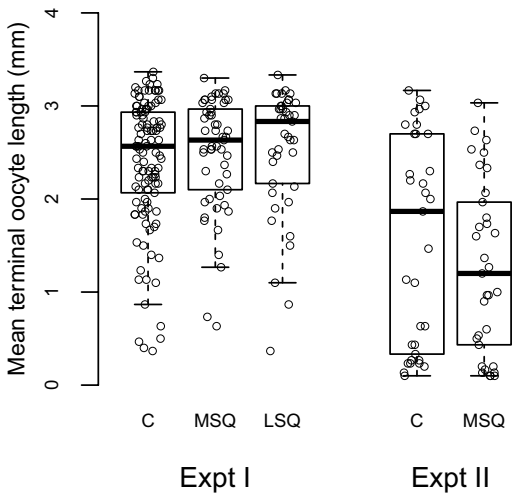

Fig. 1 Average terminal oocyte size (mm) in workers assigned to three different treatments. Workers were maintained in groups of three for 8 days during which they were exposed daily to either hexane (*C* control solvent) or queen extract (*MSQ* mid-season queen extract, *LSQ* late-season queen extract). Experiments I and II doses were 0.08 and 0.16 queen-equivalents per day, respectively. In neither experiment did treatment have a significant effect on oocyte size (see main text). Bars indicate medians, boxes show 1st and 3rd quartiles, and whiskers indicate ×1.5 the interquartile range (or the extreme value, if it is more distant from the median)

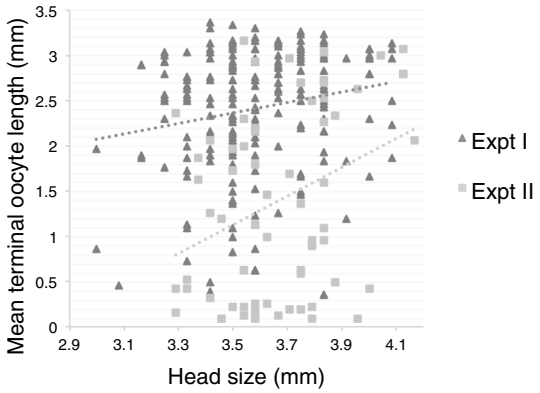

Fig. 2 Ovary development as a function of head size in all workers used in our experiments. The lines depicted are linear regressions, but tests for significance were performed using GLMMs (see main text). A significantly positive relationship was found in both experiments

that are 'ready-to-lay' (Table 1; Amsalem et al. 2015b). In contrast, in Experiment II only 50% (33/66) of workers had a ready-to-lay egg (Table 1). Scoring workers categorically as either reproductive or not yielded results similar to those when treating ovary development as a continuous variable (binomial GLMM: Expt I: $\text{effect}_{\text{MSQ}} = 0.58 \pm 0.60$; $z = 0.97$, $p = 0.33$; $\text{effect}_{\text{LSQ}} = 0.44 \pm 0.62$, $z = 0.71$, $p = 0.48$; Expt II: $\text{effect}_{\text{MSQ}} = -0.41 \pm 0.54$, $z = -0.76$, $p = 0.45$).

Table 1 Workers with "ready-to-lay" eggs (> 2 mm) by treatment

	Group	# Workers reproductive	# Workers non-reproductive	Total
Expt I (low dose)	MSQ	49	4	53
	LSQ	37	4	41
	Control	98	15	113
Expt II (high dose)	MSQ	15	18	33
	Control	18	15	33

Egg laying

Many more eggs were observed after 8 days in Experiment I than in Experiment II (Fig. 3). However, an effect of treatment was not significant for either experiment (Expt I: negative binomial GLMM: $\text{effect}_{\text{MSQ}} = 0.16 \pm 0.15$; $\text{effect}_{\text{LSQ}} = 0.17 \pm 0.15$; overall treatment $\chi^2 = 1.57$, $d.f. = 2$, $p = 0.46$, Expt II: negative binomial GLMM: effect $= -1.21 \pm 1.24$, $z = -1.12$, $p = 0.26$). In Expt I, source colony as a random effect was associated with negligible variance (2.3×10^{-6}) in egg production. In Expt II, source colony accounted for 0 variance.

In Experiments I and II, the first egg cell was observed after 2 days (Fig. 4). There was only one larva observed, consistent with previous reports that it takes 5 days for eggs to hatch into larvae (Van Oystaeyen et al. 2014). In Experiment I, there was a significant negative correlation between latency to egg laying and the cumulative number of eggs found in the cage by the end of the experiment ($F_{1,57} = 7.95$, $p = 0.007$). A mixed effect survival model indicated no difference in the latency to reproduce between our treatments ($\text{effect}_{\text{MSQ}} = -0.02 \pm 0.04$; $z = -0.39$, $p = 0.69$; $\text{effect}_{\text{LSQ}} = 0.06 \pm 0.04$, $z = 1.31$, $p = 0.19$). A survival model for Expt II found no significant effect of treatment on latency to reproduce (effect $= 0.23 \pm 0.29$, $z = 0.82$ $p = 0.42$). Only 5 microcolonies reproduced in Experiment II, and 4 of them were in the control set (Fig. 3b).

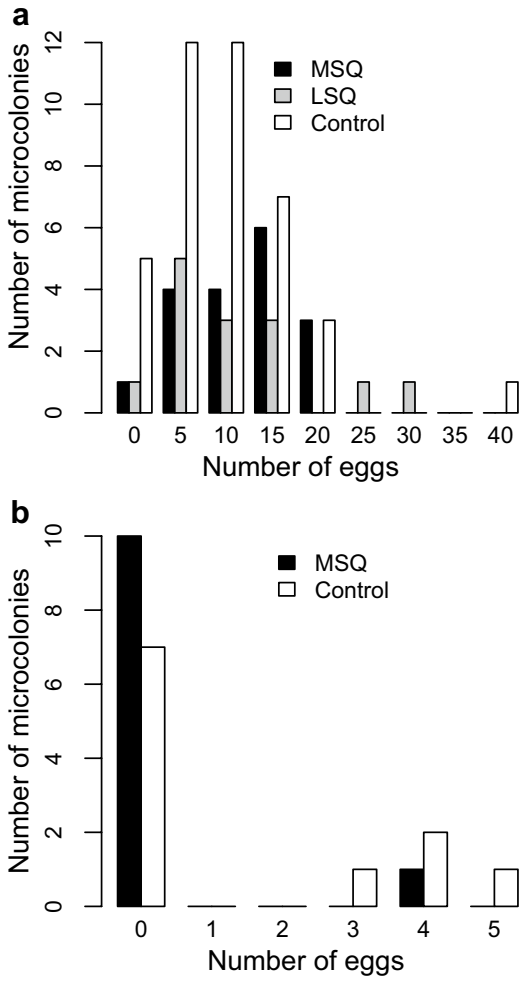

Fig. 3 Histograms of number of eggs per microcolony after 8 days in **a** Experiment I and **b** Experiment II. *MSQ* mid-season queen extract, *LSQ* late-season queen extract. Sample sizes (number of microcolonies) for Expt I: Control: $n = 40$, MSQ: $n = 18$, LSQ: $n = 14$, and for Expt II: Control: $n = 11$, MSQ: $n = 11$. Experiments I and II doses were 0.08 and 0.16 queen-equivalents per day, respectively. In neither experiment did treatment have a significant effect on number of eggs produced (see text)

Resorption

Resorption refers to deformations or change of coloration in mature oocytes, and is thought to be an indication of a reduction in reproductive potential. In their 2014 study, Van Oystaeyen et al. found increased resorption to be the major effect of exposing *B. terrestris* workers to pentacosane, and this effect was replicated in *B. impatiens* (Amsalem et al. 2015b). In our study, resorption occurred in only 2.5% (7 of 280) of workers analyzed (5 workers in control colonies and 2 workers in LSQ colonies in Experiment I) and thus was not analyzed statistically.

Body size

Head width measurements ranged from 3.0 to 4.1 mm. Head width did not differ between treatments (Welch's t tests: Expt I: control vs MSQ: $t_{114.98} = -0.92$, $p = 0.36$; control vs LSQ: $t_{72.5} = -1.12$; $p = 0.26$. Expt II control vs MSQ: $t_{61.6} = 1.19$, $p = 0.24$), thus body size was not a confounding factor. As reported above, body size was found to be positively associated with workers' oocyte length in both experiments (Fig. 2).

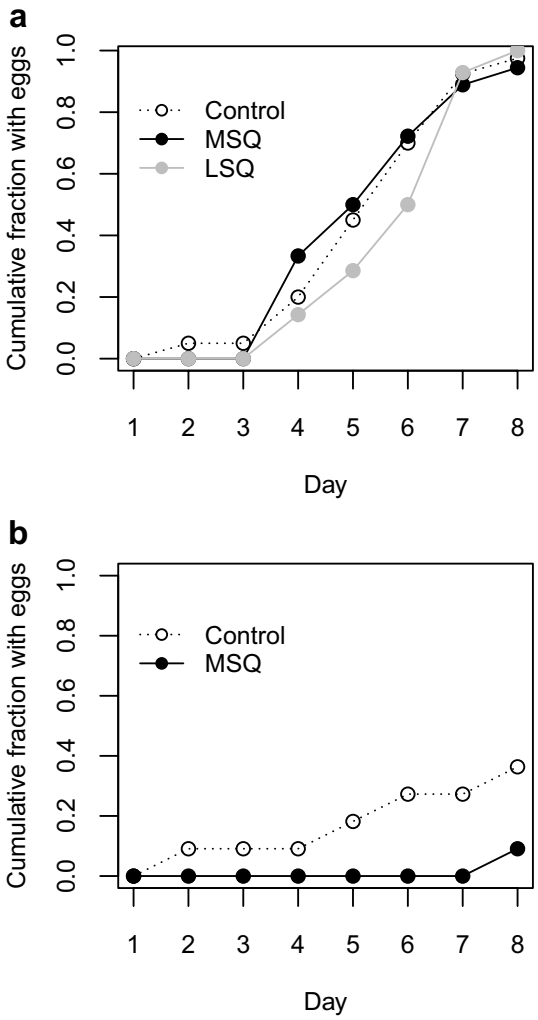

Fig. 4 Latency to egg laying by three-worker microcolonies over 8 days for **a** Experiment I, and **b** Experiment II. Checks for egg presence made at the end of each day. *MSQ* mid-season queen extract, *LSQ* late-season queen extract. Sample sizes (number of microcolonies) for Expt I: Control: $n=40$, MSQ: $n=18$, LSQ: $n=14$, and for Expt II: Control: $n=11$, MSQ: $n=11$. Experiments I and II doses were 0.08 and 0.16 queen-equivalents per day, respectively. In neither experiment did treatment have a significant effect on latency to egg laying (see text)

Discussion

Given the recent controversy and conflicting results on the effects of queen-derived CHCs on worker's reproduction in *Bombus* (Bloch and Hefetz 1999; Van Oystaeyen et al. 2014; Holman 2014; Amsalem et al. 2015b, 2017; Padilla et al. 2016; Holman et al. 2017), we set out to provide an independent test of the hypothesis that queen CHCs inhibit worker reproduction. Analogous to Holman's (2014) replication of Van Oystaeyen et al.'s (2014) result showing an effect of pentacosane on worker reproduction in *B. terrestris*, we view our experiment as a "conceptual replication" (Kelly 2006) of Amsalem et al.'s (2015b) study, as we used queen extracts instead of pure synthetic hydrocarbons as our treatment. We carried out our study independently but contemporaneously with a recently reported experiment that similarly applied queen extracts to three-worker microcolonies (Amsalem et al. 2017); thus, our study could be viewed as a "partial replication" of that study. In agreement with their findings, the prediction that cuticular compounds extracted from queens would have an effect on worker's reproduction was not supported by our observations. Based on the ovary development of individuals and the cumulative number of eggs present in microcolonies at the end of the experiment, the application of queen compounds showed no significant effect on *B. impatiens* workers. Our sample size was high in Experiment I (53 MSQ-treated workers, 41 LSQ-treated workers, 113 control workers), and the positive, small effect sizes suggested that additional samples would not have changed our conclusions. In Experiment II, with a larger dose of MSQ queen extract, our sample size (33 workers per treatment) was moderate. In this case, the effect was large and in the predicted direction (Fig. 1) but was not significant when worker's ovary development was scored continuously or categorically. Similarly, more microcolonies treated with queen extract had eggs after 8 days, relative to controls (4 vs 1; Fig. 3), but this difference was not significant. Given the low sample size at the individual level, and especially at the microcolony level, it is possible that a larger sample size would have revealed a significant effect in Experiment II. A very similar recent experiment using an only slightly lower dose (0.1 queen-equivalents/day instead of our 0.16) similarly found no effect in *B. impatiens* microcolonies (Amsalem et al. 2017). In that experiment, workers in 6 microcolonies exposed to mated queen extract laid an average of 24.7 eggs in 10 days, while 6 control groups laid an average of 21.3 eggs in the same period. Although sample sizes were not exhaustive in either of the two prior studies on *B. impatiens* CHCs (Amsalem et al. 2015b, 2017), the combined results of those papers and ours suggest that queen CHCs do not affect worker's reproduction in this North-American species. However, given the uncertainty in our large (non-significant) negative effect of queen compound in Experiment II, and the relatively low sample sizes in the studies of Amsalem et al. (2015, 2017), we cannot conclusively rule out a CHC queen pheromone in this species.

Our doses of 0.08 and 0.16 QE/day in Experiments I and II, respectively, were in line with other experiments on bumblebees. We applied ~ 9× and 18× QE/day used in the experiment of Holman (2014), which found a significant effect of pentacosane in *B. terrestris* worker's ovary development, while Van Oystaeyen et al. (2014) used 2 QE/day and reported a significant effect on worker's ovary resorption. In *B. impatiens*, Amsalem et al. (2015) used 0.00006–0.009 QE/day of synthetic pentacosane, and

Amsalem et al. (2017) used 0.1 QE/day of queen extract, in neither case finding an effect on worker's reproduction. See Holman et al. (2017) for a summary of doses used in a variety of similar experiments on other social insects. While the two studies reporting significant effects in bumblebees used synthetic pheromones (Van Oystaeyen et al. 2014; Holman 2014), we tested the complete set of compounds extracted from queens (which could have included non-CHCs in addition to CHCs, because we did not fractionate our extractions). We reasoned that the full complement of queen compounds would have, if anything, a stronger effect than isolated synthetic compounds, given the possible importance of chemical context in worker's response to the queen's signals (Smith et al. 2015). However, we only applied a fraction of the compounds a queen possesses, and full queen-equivalents have yet to be tested in *B. impatiens* (Holman et al. 2017). Because we applied our treatment as a small splatter of droplets that quickly dried, it is possible that the small surface area was not as readily detected by workers, which could have biased our results. Applying compounds to a queen dummy (cf Bloch and Hefetz 1999; Amsalem et al. 2017), and in full queen-equivalent amounts, would be worthwhile in future studies.

The most puzzling result from our study is the dramatic difference we observed between worker's reproduction in Experiments I and II. Given how quickly workers began reproducing in Experiment I, it is possible that some workers may have come from colonies in which workers were reproducing already. We observed eggs as soon as day 3, though it typically takes longer than this for *B. impatiens* workers to activate their ovaries (e.g.. Amsalem et al. 2015b; our Experiment II). However, worker reproduction in our young source colonies would be surprising, given low rates of worker ovary development in queenright colonies of *B. impatiens* (Cnaani et al. 2002). We can think of two possible explanations for the difference we observed. First, the rearing room temperature in Experiment II was 19–26 °C, while in Experiment I it was 27–28 °C. Lower temperatures reduce worker's ovary development in honeybees (Lin and Winston 1998), which could explain the observed difference. Alternatively, although our colonies were ordered from the same source and were received in the same developmental stage (mean of 44 workers in Experiment I, and 61 workers in Experiment II), it is possible that there was something different about the health or condition of the bees themselves, since they were ordered in two separate batches roughly 4 months apart. Despite this difference in baseline reproduction, within each experiment our treatment bees were compared to control bees from the same source kept under the same conditions, so we believe our results are still meaningful.

Our findings are consistent with the recent work on *B. impatiens* indicating the absence of a classical queen pheromone in this species, suggesting the presence of the queen is essential to inhibiting worker reproduction (Amsalem et al. 2015b, 2017; Padilla et al. 2016), though future experiments should involve higher amounts of queen compound with larger sample sizes, ideally with analyses at the group, rather than individual, level. This contrasts with work on *B. terrestris* that found meaningful effects of queen-derived compounds on worker's ovary status (Van Oystaeyen et al. 2014; Holman 2014). It will be useful to see if similar experiments can reproduce effects of queen CHCs on worker's reproduction in other species of the genus *Bombus*, given its diversity (Cameron et al. 2007), just as similar projects have begun to do in *Lasius* ants and vespine wasps (Holman et al. 2013, 2016; Oi et al. 2016).

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