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RESEARCH ARTICLE

Queen–worker aggression in the facultatively eusocial bee *Megalopta genalis*

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Abstract

The establishment of reproductive division of labor in primitively eusocial insects is typically based on dominance interactions from the queen toward her worker daughters. We used a standardized behavioral assay, the circle tube, to observe aggressive and other behaviors between the queen and workers of field-collected social colonies of the sweat bee, *Megalopta genalis*. Queens generally expressed higher levels of aggressive and dominant behaviors than workers. Workers performed two behaviors, 'C-posture' and 'reverse', more frequently than queens. Our data suggest a defensive function for C-posture in workers, as it correlated with the expression of queen aggression. Within queens, the aggressive behaviors 'nudge', 'bite', 'push', and 'chase' correlated with each other in their frequencies of expression; the other two behaviors that were performed more often by queens ('back up' and 'back into') did not. The two behaviors performed more often by workers, 'C-posture' and 'reverse', were not correlated in workers. Queen and worker activity levels were correlated. Body size correlated with increased expression of queen-like behavior in both the queen and worker castes. Queens generally were bigger and had larger ovaries than workers. Queen–worker body size and ovary size differences correlated with behavioral differences. The effects of body size suggest an influence of developmental nutrition on adult behavior.

Keywords Social cooperation · Amines · Circle tube · Sweat bee · Halictidae · Augochlorini

Introduction

Some of the most dramatic elaborations of social cooperation evolved in the eusocial Hymenoptera (Wilson 1971; Bourke 2011). Facultatively eusocial groups comprised of a reproductive queen and one or a few non-reproductive workers permit study of the origins of reproductive division of labor. While these species are not necessarily in the process of evolving toward larger societies, they nevertheless are likely representatives of what the first steps in social evolution may have looked like, and thus offer a proxy for the evolutionary origins of sociality (Schwarz et al. 2007; Kocher and Paxton 2014; Rehan and Toth 2015; Kapheim 2017; Shell and Rehan 2017).

In small-colony eusocial groups, some daughters remain in their natal nest as workers to care for the offspring of their mother, the queen, rather than dispersing to reproduce themselves. Queens aggressively dominate their worker daughters, leading to the subordinate behavior and undeveloped, non-reproductive ovaries characteristic of the worker caste (Michener and Brothers 1974; Michener 1990; Kapheim et al. 2016; Awde and Richards 2018). Aggressive behavior is central to the distinction between queens and workers: queens are aggressively dominant over workers. The preponderance of dominance-based systems in small-colony social insects suggests that aggressively dominant queens that suppress the reproduction of workers represent the ancestral condition in the social Hymenoptera (Michener and Brothers 1974; Michener 1990; Bourke 1999; Jeanne 2003).

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Studies of primitively eusocial sweat bees (Halictidae) in observation nests show that queen dominance behavior and aggression lead to the suppression of worker reproduction (reviewed in Michener 1974, 1990; Dalmazzo and Roig-Alsina 2015, 2018a, 2018b; Kapheim et al. 2016). Experimental queen removal shows that in the absence of a queen, workers of some species can reproduce (Mueller 1991, Smith et al. 2009). Another way to study queen–worker aggression is the use of circle tubes. Circle tubes are a standardized behavioral assay in which two bees are placed into opposite ends of a piece of transparent, flexible tubing, which is then closed into a circle, prompting the bees to interact (Breed et al. 1978). Circle tubes have been used previously to study aggressive interactions in bees (e.g., Lawson et al. 2017; Withee and Rehan 2016; Gonzalez et al. 2018; Smith et al. 2018; reviewed in Pabalan et al. 2000; Packer (2005); Dew et al. 2014), including aggressive interactions between queens and workers (Pabalan et al. 2000; Rehan and Richards 2013). While the arena is obviously artificial, results are consistent with behavioral observations of in-nest behavior for species with both circle tube and in-nest observations (Breed et al. 1978; Smith and Weller 1989; Kapheim et al. 2016; Smith et al. 2018).

Here we use the facultatively eusocial sweat bee *Megalopta genalis* to study queen–worker behavioral interactions in circle tubes. In a previous study on this species, we used a subset of nests to determine queen-like and worker-like behavior (Smith et al. 2018) and showed that it was similar to patterns of queen and worker behaviors in observation nests in the field (Kapheim et al. 2016). Here we expand this analysis to a larger sample size of 65 nestmate queen–worker pairs. We then test four non-exclusive hypotheses for the expression of queen–worker behavioral differences.

First, we test whether aggressive behavior is modulated by the biogenic amine octopamine (OA), which serves as a neurotransmitter, neuromodulator, and neurohormone in insects (Neckameyer and Leal 2017; Kamhi et al. 2017). OA modulates aggression in the solitary insects *Drosophila melanogaster* and crickets (Baier et al. 2002; Hoyer et al. 2008; Stevenson and Rillich 2012; Rillich and Stevenson 2015). OA titers are higher in dominant individuals of small-colony insect societies (Bloch et al. 2000; Cuvillier-Hot and Lenoir 2006; but see Penick et al. 2014). This suggests that modification of the ancestral links between OA and aggression may be involved in the expression of dominant queen and subordinate worker behaviors in social insects. However, no study has experimentally manipulated queen and worker OA levels in a small-colony social insect, or pharmacologically manipulated amines in any bee species other than *Apis mellifera* (Kamhi et al. 2017). To test the hypothesis that OA increases aggression and expression of queen-like behavior, we increased OA levels by treating bees with an OA supplement. We also treated bees with the OA antagonist epinastine (EPN) to study the effect of reducing OA action (Roeder et al. 1998). We predicted that OA supplementation would increase, and EPN treatment decrease, the expression of aggressive, queen-like behaviors.

Second, we tested whether individual behavioral variation correlates with the behavior of the other nestmate bee in the circle tube. We predicted that overall activity would correlate between queens and workers, because many behaviors are reactions to nestmates (e.g., Buckle 1982; Pabalan et al. 2000; Dalmazzo and Roig-Alsina 2018a). Likewise, we predicted that the expression of defensive or subordinate behaviors in workers would correlate with the expression of aggressive behaviors from the queen. Previous studies on *M. genalis*, as well as *Lasioglossum zephyrum*, suggest that the behavior 'C-posture' is defensive (Buckle 1982; Smith et al. 2003, 2018; Kapheim et al. 2016), although it is an aggressive act in other species (e.g., Pabalan et al. 2000; Packer 2005). C-posture is when a bee curls its abdomen ventrally so that the sting is directly under the mandibles, and both are directed toward the interacting individual (see ethogram, Table 1). We predicted that worker expression of C-posture would correlate with aggression from the queen.

Third, we tested whether body size differences between the queen and worker, as well as differences in ovarian development, affect behavior. Previous studies of bees in circle tubes have shown that larger bees with larger ovaries are often, but not always, more aggressive than smaller bees (Wcislo 1997; McConnell-Garner and Kukuk 1997; Pabalan et al. 2000; Arneson and Wcislo 2003; Richards and Packer 2010; Rehan and Richards 2013; Lawson et al. 2017). In *M. genalis* social groups, queens have well-developed ovaries, while workers have non-reproductive slender ovaries, and queens are typically larger than workers (Smith et al. 2008, 2009; Kapheim et al. 2011, 2012). In a previous study on this species comparing caste-matched individuals from different nests, we showed that larger bees were more aggressive, but ovary development did not influence behavior (Smith et al. 2018). However, in the current study, the bees are nestmates that share a developmental history, and both body size and ovary size are a product of social interactions: body size because queens determine worker larval nutrition during provisioning (Kapheim et al. 2011), and ovary size because queens suppress ovary development of workers through social dominance (Smith et al. 2009; Kapheim et al. 2012, 2016). We predicted that queen–worker pairs with greater ovary and body size differences would also have greater behavioral differences, because all three aspects of the phenotype are influenced by queen manipulation and dominance.

Fourth, we tested the hypothesis that workers' worker-like behaviors are expressed independently of queen presence, but that queen presence suppresses the expression of aggressive queen-like behaviors in workers, by comparing the

Table 1 Ethogram of behaviors included in the study (from Smith et al. 2018)

Behavior	Definition
C-posture	Abdomen curled anteriorly under head to present sting and mandibles to the other bee
Nudge	Quick contact with the other bee; forward movement toward the other bee and backward again without pause in between
Push	One bee applies force to another with its head
Back into	One bee backs up, pushing the other with its abdomen
Nip	One bee closes mandibles < 1 cm from the other bee
Bite	One bee closes mandibles around a body part of another bee
Back up	One bee walks backwards from an interaction without turning around
Reverse	One bee turns around so that its abdomen is facing the other bee before walking away while facing forwards
Follow	One bee follows another at a walking pace after they either back or reverse out of an interaction
Chase	One bee quickly pursues another that is moving away. Distinguished from following by moving at faster than normal walking speed
Antennate	One bee touches its antennae to the head of the other bee
Head–head	Both bees have heads touching each other without pushing
Pass	Bees walk past each other in the tube

results of this study to our previous study of non-nestmate bees (Smith et al. 2018). In the previous study, we found that when matched with other workers, from different nests, workers still expressed higher levels of worker-like behavior than queens or solitary females that were matched with other queens or solitary females, respectively. Workers' levels of queen-like behaviors in caste-matched circle tubes, on the other hand, were similar to queens and solitary females (Smith et al. 2018). Thus, we predicted that workers in circle tubes with their queen (this study) would show dramatically less queen-like behavior than workers in the previous caste-matched non-nestmate trials (Smith et al. 2018).

Methods

Research site and study organism

We collected all bees for this study from the forest of Barro Colorado Island, Panama (BCI; 9°09′N, 79°51′W). BCI is a lowland tropical semi-deciduous moist forest (Leigh 1999). We have previously described the biology of *Megalopta genalis* (Augochlorini, Halictidae) (Smith et al. 2003; Wcislo et al. 2004; Kapheim et al. 2013). In summary, a foundress female constructs a tunnel nest in a dead stick suspended in the vegetation. Bees forage during the 60–90 min before sunrise and after sunset, they are inside their nest during the day (Kelber et al. 2006; Smith et al. 2017). *M. genalis* is facultatively solitary or eusocial: in some nests, the first daughter remains in the nest as a sterile, subordinate worker, and the original foundress now becomes a social queen. Eusocial nests typically have 1–3 workers (mode = 1). In other nests, all offspring disperse, and the foundress remains a solitary reproductive. In social nests, the workers perform all foraging trips, and the queen rarely leaves the nest. Reproductive division of labor is maintained by queen dominance; workers can enlarge their ovaries and reproduce if the queen is removed (Smith et al. 2009). Workers are typically smaller than queens and solitary reproductives, reflecting reduced pollen provisioning by the foundress (Kapheim et al. 2011, 2012).

Collections and experimental trials

We collected nests the morning of the circle tube trial, during daylight when all bees were inside. We plugged the nest entrance with cotton wool, wrapped the stick containing the nest in a plastic bag, and brought it back to the BCI lab facilities. We refrigerated collected stick nests for ~ 15 min, and then opened the nests. Nests were designated solitary or social based on the number of adult females found inside. We assumed that the largest female was the queen. If more than one worker was present, we picked the smallest one to use for the trials. We later confirmed worker and queen status by ovarian dissection (see below). Following ovary dissection, if the putative queen did not have the largest ovaries or if one of the workers appeared to be a newly emerged female rather than a mature worker, that pair was excluded from the study. If the size difference was not obvious, we marked both individuals on the wing with Decocolor paint pens to distinguish them. We marked both individuals in case marking influenced behavior (Packer 2005).

We randomly assigned pairs to one of three groups: supplemental octopamine (OA) treatment, epinastine (EPN) treatment, or solvent control. We treated bees with 1 µl of dimethylformamide (DMF) applied to the thorax following Barron et al. (2007) who demonstrated that OA applied in this manner raised brain titers. The DMF contained either 2 µg OA (octopamine hydrochloride, Sigma-Aldrich St. Louis MO, USA), 2 µg EPN (epinastine hydrochloride, Sigma-Aldrich St. Louis MO, USA), or no additional chemicals (solvent control). For the worker treatment trials, the worker was treated with OA ($n=13$), EPN ($n=14$), or solvent control ($n=15$), and the queen was always treated with the solvent only. For the queen treatment trials, the queen was treated with OA ($n=8$), EPN ($n=9$), or solvent control ($n=6$), and the worker treated with solvent only.

All worker treatment trials were conducted in June and July of 2015, and all queen treatment trials from February to May of 2016. After treatment, we placed each bee in a 1.5 ml centrifuge tube with the end cut off and plugged with cotton wool, for 15 min at ambient temperature. To begin the trial, we removed the cotton from each tube, and placed the open end into the end of a 30 cm tube of clear flexible PVC with an inner diameter 8 mm. One bee was inserted at each end, and the ends of the flexible tube were joined with a length of wider tubing to secure the tube in a circle. We recorded each pair of bees for 15 min using a Logitech c920 camera. Trials were run in ambient temperature under natural daylight between 14:00 and 17:00 local time.

We freeze killed bees after each trial. We measured head width with calipers as a measure of body size, and dissected the abdomen to measure ovary size by photographing the ovaries dorsally at 10× magnification through a dissecting microscope and measuring their total area using ImageJ following Smith et al. (2008, 2009). Some individuals were lost after ovarian measurements, but before head size measurements and some bees' ovaries were destroyed during dissection, leading to uneven sample sizes for these variables.

We scored videos using the ethogram of behaviors from Smith et al. (2018) which was modified from Kapheim et al. (2016) and Dew et al. (2014; Table 1). Videos were scored blind to treatment type (OA, EPN, or control). However, the size difference between queens and workers made it impossible to score blind to caste.

Comparisons with non-nestmate circle tubes

To compare queens and workers from this study to the queens and workers from a previous study that were in circle tubes with non-nestmates of the same caste (Smith et al. 2018), we calculated the ratio of queen-like to worker-like behavior for each individual. To do this, we added one to the total of queen-like and to the total of worker-like behaviors to avoid dividing by zero. The use of ratios was to standardize for any differences in activity between the two studies.

Statistics

Ovary area and head width were normally distributed continuous variables, and analyzed with standard parametric statistics. All of the recorded behavioral variables were count variables with non-normal distribution and many zeros. Thus, we used non-parametric statistics for comparisons between groups. For comparisons between nestmates, we used Wilcoxon pair rank test to account for the non-independence of nestmates. We used Kruskal–Wallis test to compare across amine treatment groups. We use Spearman's rank correlation and rank-based partial correlation (ρ) for correlational analyses of behaviors (Conover 1999). We use partial correlation controlling for activity to account for the effects of different activity levels between circle tubes. All statistics were performed in SPSS.

Data availability

The datasets analyzed during the current study are available at https://doi.org/10.6084/m9.figshare.8161649.

Results

Effects of amine treatments

There were no effects of worker amine treatment on worker behavior ($N=15$ control workers, 13 OA-treated workers, 14 EPN-treated workers; Kruskal–Wallis test $p>0.05$ across amine treatment groups; see ESM for summary statistics, test statistics and p values), with the exception of 'antennate' (control mean $=2.08\pm2.57$ SD, OA $=0.85\pm1.2$, EPN $=0.23\pm0.44$; Kruskal–Wallis $H=11.14$, $df=2$ $p=0.004$) where the EPN-treated workers antennated less than control workers (Bonferroni pairwise post hoc $p=0.003$) but not OA-treated workers (post hoc $p=0.22$; there was no difference between OA and control workers, $p=0.44$). There were no differences in total activity between the three treatment groups (Kruskal–Wallis test $H=0.51$, $df=2$, $p=0.78$). There were no effects of queen amine treatment on queen behavior between treatment groups ($N=6$ control, 8 OA-treated queens, 9 EPN-treated queens; Kruskal–Wallis test $p>0.05$ for all behaviors tested; see ESM for summary statistics, test statistics and p values). There were no differences in total activity between the three queen treatment groups (Kruskal–Wallis test $H=0.87$, $df=2$, $p=0.65$).

Behavioral differences between castes

Because there were no effects of amine treatments, we pooled all individuals ($N=65$ queen–worker pairs) for analysis of queen–worker caste differences. There were behavioral differences between queens and workers. Queens performed the behaviors 'push', 'back into', 'back up', 'bite', 'chase', and 'nudge' more frequently than workers (Fig. 1, see Table 2 for statistical tests). We designate these behaviors as "queen-like". Workers performed the behaviors 'C-posture'

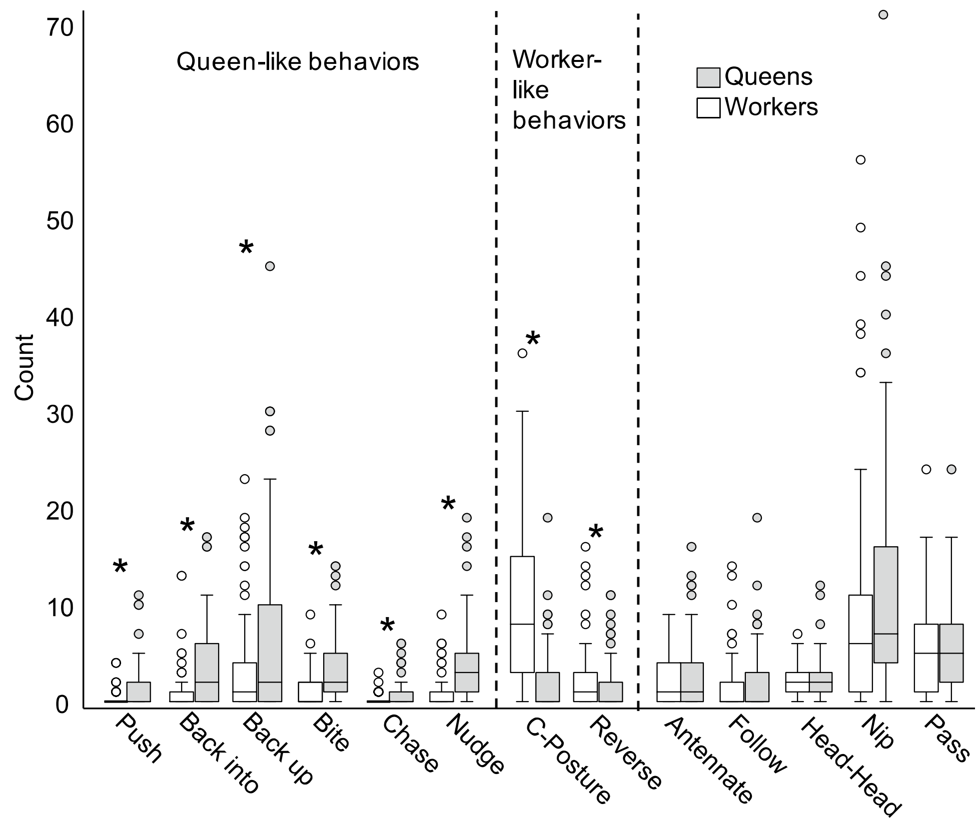

Fig. 1 Behaviors of nestmate queens and workers. The *Y* axis shows frequency of each behavior observed. Asterisks mark behaviors that significantly differed between castes (see Table 2 for statistics). Dashed lines separate queen-like behaviors, worker-like behaviors, and other behaviors that did not significantly differ between castes. For all box plots, horizontal lines show the median, boxes the interquartile range (IQR), and whiskers up to 1.5*(IQR). Dots represent data points > 1.5*(IQR) from the median

Table 2 Behaviors by caste

	Wilcoxon paired rank test results		
	Z	*p*	*N*
Queen-like behaviors			
Push	− 3.85	< 0.001	65
Back into	− 3.92	< 0.001	65
Back up	− 2.88	< 0.004	65
Bite	− 4.59	< 0.001	65
Chase	− 3.65	< 0.001	65
Nudge	− 4.53	< 0.001	65
Worker-like behaviors			
C-posture	− 5.78	< 0.001	65
Reverse	− 2.91	0.004	65
Other behaviors			
Antennate	NA		65
Follow	− 0.82	0.41	65
Head–Head	NA		65
Mandible touch	NA		65
Nip	− 1.66	0.10	65
Pass	NA		65

Results of Wilcoxon pair rank test between nestmate queens and workers (N = 65 nests)

"NA" indicates behaviors with all or many tied rank scores preventing calculation of the test

and 'reverse' more often than queens (Fig. 1, Table 2). We designate these behaviors as "worker-like". The behaviors 'antennate', 'head–head', and 'pass' often were performed by both bees at the same time, leading to numerous tied ranks that precluded tests of caste differences. The behaviors 'follow' and 'nip' did not differ between castes (Table 2).

We summed the instances of all behaviors for each individual to measure 'activity'. Queens' activity was greater than workers (Queen mean = 49.22 ± 35.30 behaviors, worker mean = 41.38 ± 30.58 behaviors; Wilcoxon signed rank test $z = -2.53$, $p = 0.01$). Nestmate queen and worker activity were correlated ($\rho = 0.77$, N = 65, $p < 0.001$; Fig. 2).

Individual bees did not express high levels of both queen-like and worker-like behaviors. Queens that were more queen like were less worker like (partial correlation controlling for activity = − 0.42, $N = 65$, $p = 0.001$). A similar relationship was seen in workers: workers that expressed more worker-like behavior expressed less queen-like behavior (partial correlation controlling for activity = − 0.28, $N = 65$, $p = 0.03$). The two worker-like behaviors, C-posture and 'reverse', did not correlate with each other in workers (partial correlation controlling for activity = − 0.19, $n = 65$, $p = 0.14$). Of the queen-like behaviors, bite, push, and nudge all correlated positively with each other in queens, and chase correlated positively with nudge (see Table 3 for statistics). However, 'back up' correlated negatively with push, bite, and nudge,

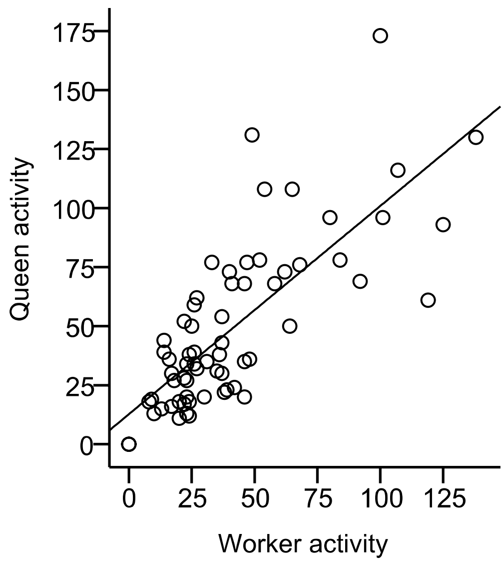

Fig. 2 Queen and worker activity were correlated ($\rho = 0.77$, $p < 0.001$). Each point represents the sum of all observed behaviors of the worker and of the queen from the same nest. The trend line represents a linear regression

Table 3 Rank-based partial correlations of queen behaviors, controlling for activity, are shown for each pair of behaviors

	Push	Bite	Chase	Nudge	Back into
Back up	**– 0.33****	**– 0.50****	– 0.19	**– 0.45****	**0.28***
Push		**0.67****	0.13	**0.42****	0.04
Bite			0.24	**0.43****	– 0.13
Chase				**0.38****	0.07
Nudge					0.12

*$p < 0.05$, **$p < 0.01$, $N = 65$ queens, for all partial correlations

Bold-type values are statistically significant;

and positively with 'back into'. 'Back into' did not correlate with any of the other queen-like behaviors (Table 3).

Effect of each caste's behavior on the other caste

Queens' expression of queen-like behavior correlated with workers' expression of worker-like behavior (partial correlation controlling for activity = 0.30, $N = 65$, $p = 0.02$). This was driven by a correlation between queen aggressive behaviors (chase, bite, nudge, and push) and worker C-posture expression (partial correlation controlling for activity = 0.32, $p = 0.01$). Worker expression of C-posture was negatively associated with queen expression of two behaviors generally considered tolerant (Dew et al. 2014), antennate (partial correlation controlling for activity = – 0.25, $p = 0.047$) and pass (partial correlation controlling for activity = – 0.33, $p = 0.008$).

Effect of body size and ovary development

Queens were significantly larger than workers (head width: queen mean = 4.08 ± 0.50 SD mm, worker mean = 3.38 ± 0.39 mm; ANOVA $F_{120, 1} = 69.67$, $p < 0.001$) and had larger ovaries (queen mean = 2.08 ± 0.79 mm^2, worker mean = 0.48 ± 0.42 mm^2; ANOVA $F_{117, 1} = 191.35$, $p < 0.001$). Larger queens with larger ovaries had larger workers, also with larger ovaries (relative to other workers). Both head width and ovary size were correlated among nestmates (head width: $r = 0.69$, $N = 61$, $p < 0.001$; ovary area: $r = 0.43$, $N = 58$, $p = 0.001$). Because body size and ovary size are confounded with caste, we looked within castes for effects on behaviors.

Body size and ovary size were correlated within castes (queens: $r = 0.53$, $N = 57$, $p < 0.001$; workers: $r = 0.34$, $N = 59$, $p = 0.008$). Ovary size also correlated with overall activity (queens: $\rho = 0.38$, $N = 59$, $p = 0.003$; workers: $\rho = 0.35$, $N = 62$, $p = 0.006$), but body size did not (queens: $\rho = 0.18$, $N = 61$, $\rho = 0.16$; workers: $\rho = 0.17$, $N = 61$, $p = 0.20$). Thus, we use partial correlation to analyze the effects of ovary and body size.

Larger queens were more queen like and less worker like than smaller queens (partial correlation controlling for ovary size and activity, queen like = 0.47, $df = 54$, $p < 0.001$; worker like = – 0.35, $df = 54$, $p = 0.009$). There was no correlation between ovary size and queen-like or worker-like behavior after controlling for activity and body size, among queens (queen like = 0.05, $df = 54$, $p = 0.69$; worker like = – 0.12, $df = 54$, $p = 0.38$).

Larger workers were more queen like than smaller workers, but there was no effect of worker size on worker-like behavior (partial correlation controlling for ovary size and activity, queen like = 0.46, $df = 56$, $p < 0.001$; worker like = – 0.13, $df = 56$, $p = 0.35$). There was no correlation between ovary size and queen-like or worker-like behavior, after controlling for body size and activity, among workers (queen like = – 0.47, $df = 56$, $p = 0.53$; worker like = – 0.01, $df = 56$, $p = 0.99$).

Effect of ovary and body size variation on the opposite caste

We correlated the ratios of head width and ovary size with the ratios of queen-like and worker-like behavior. This showed that when differences between queen and worker body size were relatively large, the differences in ovarian development were too. The ratio of queen:worker ovary size correlated with the ratio of queen:worker body size ($\rho = 0.47$, $N = 57$, $p < 0.001$; Fig. 3). The ratio of queen to worker body size and ovary size also affected behavior. In pairs where the differences between queen body size and ovary size were relatively large, the queens were also more

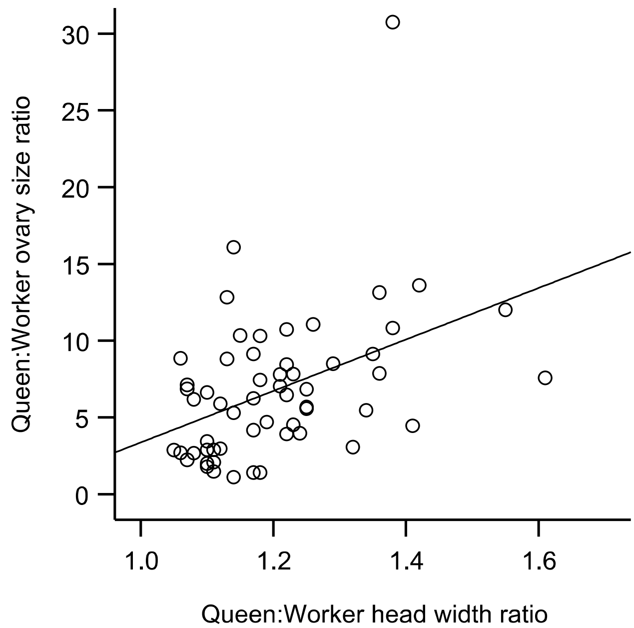

Fig. 3 Queen–worker differences in body size (expressed as the ratio of queen head with to worker head width on the *X* axis) correlate with queen–worker differences in ovary development (expressed as the ratio of queen ovary size to worker ovary size). The trendline represents a linear regression

queen like relative to workers. The ratio of queen:worker body size and ovary size correlated with the ratio of queen:worker expression of queen-like behavior (body size ratio: $\rho = 0.29$, $N = 61$, $p = 0.03$; ovary size ratio: $\rho = 0.31$, $N = 58$, $p = 0.02$, Fig. 4a). The correlation between body and ovary size ratios and worker-like behavior was negative: in pairs where the differences between queen body and ovary size were relatively large, the queens were less worker like (body size ratio: $\rho = -0.29$, $N = 61$, $p = 0.03$; ovary size ratio: $\rho = -0.34$, $N = 58$, $p = 0.01$, Fig. 4b). Although queens typically expressed low levels of worker-like behavior, there were five pairs in which the queens did more worker-like behaviors than the workers, and these were also pairs in which the queens and workers had small body size and ovary size differences (Fig. 4b).

Comparison with non-nestmate circle tubes

The queen-like:worker-like behavior ratio did not significantly differ between queens in nestmate (this study) and queens in non-nestmate, queen–queen circle tubes from Smith et al. (2018) (Mann–Whitney $U = 652.00$, $N = 84$, $p = 0.90$; Fig. 5a). However, workers in circle tubes with nestmate queens behaved differently than workers in circle tubes with non-nestmate workers. Workers in circle tubes with their nestmate queen (this study) showed a lower ratio of queen-like to worker-like behaviors than did workers in circle tubes with workers from other nests (Smith et al. 2018) ($U = 28.50$, $N = 88$, $p < 0.001$; Fig. 5b). This result was driven by increased queen-like behavior by workers in matched-caste, non-nestmate trials relative to those in circle tubes with their nestmate queen ($U = 73.00$, $N = 75$, $p < 0.001$; Fig. 5c), but not by changes in expression of worker-like behavior ($U = 427.50$, $N = 75$, $p = 0.47$; Fig. 5c).

Discussion

Amine treatments

Here we show that queens and workers differ in their rates of expression of several behaviors in circle tubes. These behaviors were not affected by topical treatment with a single dose (2 μg) of OA or EPN, contrary to our predictions. However, responses to manipulation might have been seen had we used higher or lower doses (e.g., Okada et al. 2015). Similarly, pharmacological treatments at earlier stages in development may also have influenced adult expression of queen- and worker-like behaviors (e.g., Schulz et al. 2002). Understanding the role, if any, of OA in the expression of *M. genalis* caste expression requires further study.

Behavioral differences between castes

Our observations of queen–worker circle tubes are consistent with previous observations of in-nest behavior, suggesting that the queen–worker differences we observed represent caste-specific behavior patterns, rather than artifacts of the circle tube assay (Kapheim et al. 2016). Studies of other sweat bees that compare in-nest and circle tube behaviors also found that the two corresponded (Smith and Weller 1989; Kukuk 1992). Our designation of queen- and worker-like behaviors is the same as that of Smith et al. (2018). That study used only the control nests (no amine treatment) that we present here. Here we show that the larger sample size of all 65 nests shows similar results.

While we did not record exactly the same behaviors as Kapheim et al. (2016), the expression of ‘C-posture’ by workers and the preponderance of apparently aggressive behaviors like pushing and biting by queens towards workers, rather than vice versa, are consistent with previous in-nest observations. The tendency of queens, rather than workers, to engage in pushing and biting behaviors is similar to other sweat bee species in which both castes have been examined (reviewed by Pabalan et al. 2000) as well as other small-colony bees (Lawson et al. 2017). ‘C-posture’, the curling of the abdomen anteriorly under the head to present both the mandibles and sting in the same direction, has previously been reported as an aggressive behavior (Arneson and Wcislo 2003; Packer 2005), but our results and Kapheim

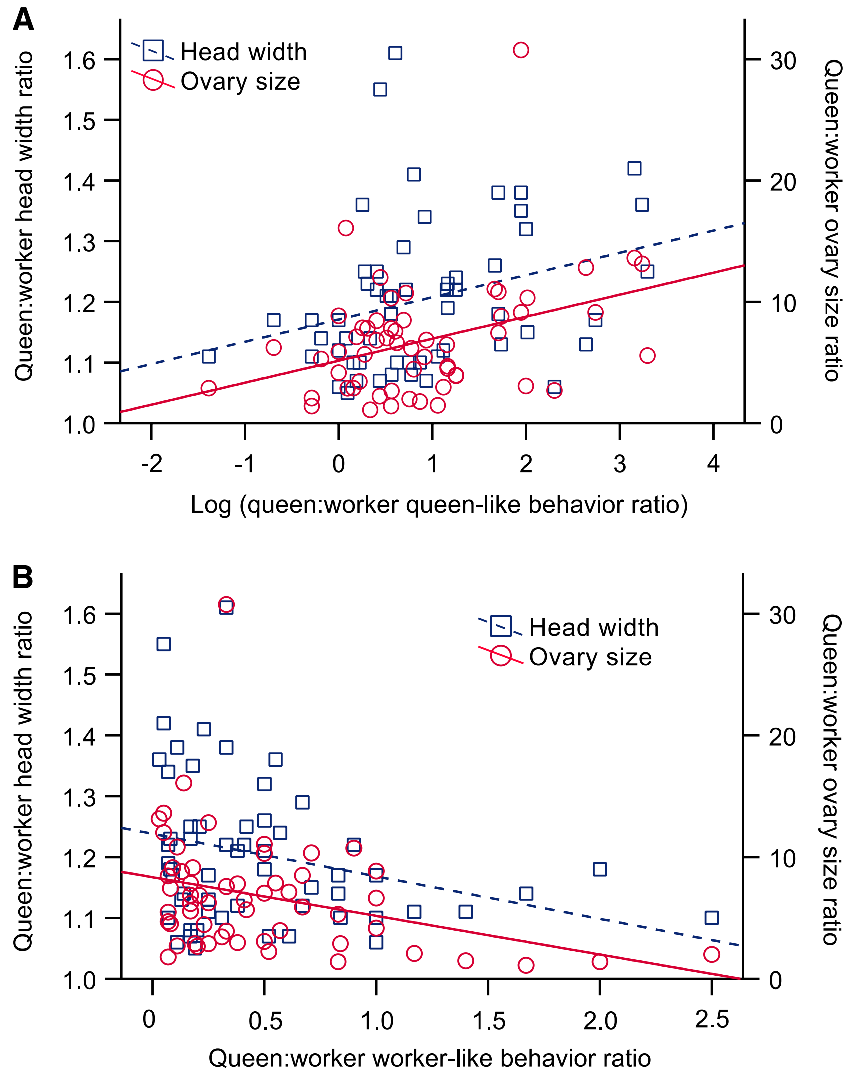

Fig. 4 The ratios of body size and ovary size in queen–worker pairs correlate with the ratios of queen-like and worker-like behaviors. In all panels, blue squares show the queen:worker head width ratio, plotted on the left vertical axis, and red circles show the queen:worker ovary size ratio, plotted on the right vertical axis. In **a**, the horizontal axis shows the queen:worker ratio of 'queen-like' behavior log transformed to distinguish between the many clustered data points. In **b**, the horizontal axis shows the ratio of 'worker-like' behavior. Dashed blue lines are a linear regression of head width and behavior ratios, while solid red lines are a linear regression of ovary area and behavior ratios

et al. (2016) suggest that in *M. genalis* it is characteristic of subordinate workers and therefore defensive. In our other circle tube study on *M. genalis* in which bees were placed in circle tubes with other bees of the same caste from other nests, we showed that individuals' expression of C-posture correlated negatively with aggressive behaviors, again suggesting that it is a defensive behavior (Smith et al. 2018). Relative C-posture frequency was the single best predictor of caste among the behaviors we recorded.

The two worker-like behaviors ('C-posture' and 'reverse') were not correlated in workers, perhaps because they represent alternative reactions: stay and defend (C-posture) or turn and flee (reverse). Among queens, there appeared to be one suite of aggressive behaviors correlated with each other (chase, bite, push, nudge). However, these were either not associated, or negatively correlated with the other two queen-like behaviors, 'back up' and 'back into'. The association of chase, bite, push and nudge is not surprising, as these are typically associated with dominance behavior of queens toward workers (Michener 1990; Pabalan et al. 2000). In previous studies of *Lasioglossum zephyrum* in laboratory observation nests, 'backing', which is equivalent to what we call 'back up', was characteristic of queens (Michener 1974, 1990; Breed and Gamboa 1977). Breed and Gamboa (1977) showed that when pollen foragers returned to the nest, queens backed down the tunnel, leading the forager, who followed, to the open cell to be provisioned with pollen. It is difficult to interpret queen backing/back up behavior in circle tubes, but the *L. zephyrum* observations are consistent with our observations that 'back up' is characteristic of queens, but unrelated to queen-like aggressive behaviors.

Effects of nestmate behaviors

What explained variation in behavior? The behavior of the other individual in the circle tube, individual body size, and the interaction between those factors affected the expression of queen-like and worker-like behavior. Overall activity

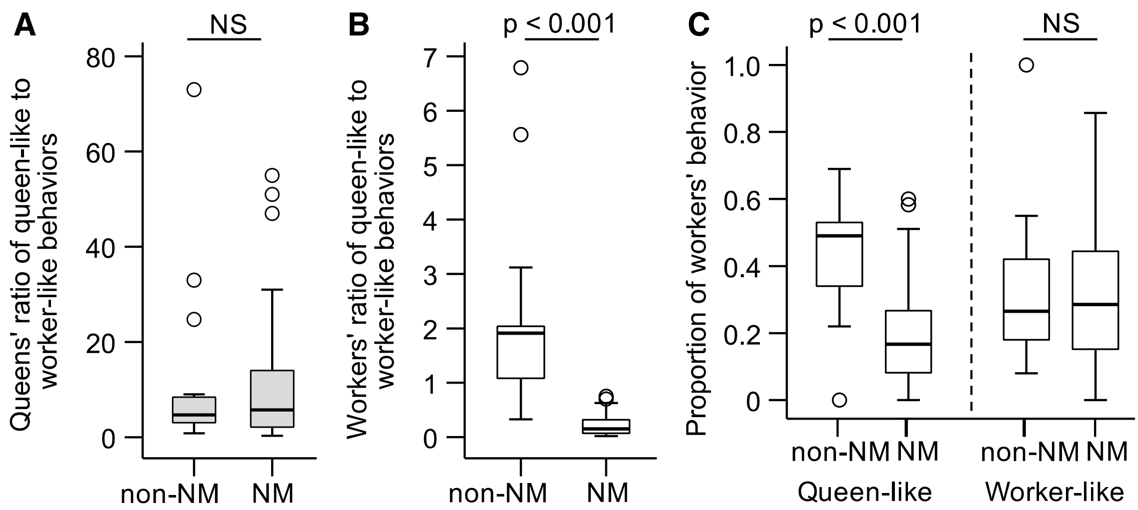

Fig. 5 Comparisons of queen (**a**) and worker (**b**) behavior in circle tubes with nestmates (NM) of the opposite caste (this study) and in circle tubes with non-nestmates (non-NM) of the same caste (Smith et al. 2018). Panel C shows that the relationship in (**b**) is driven by differences in queen-like, but not worker-like, behavior. In **a**, **b**, boxplots show the distribution of the ratio of queen-like to worker-like behaviors for each individual. In **c**, boxplots show the distribution of the proportion of all behaviors that were queen-like or worker-like for each individual. For all boxplots, horizontal lines show the median, boxes the interquartile range (IQR), and whiskers up to 1.5*(IQR). Dots represent data points > 1.5*(IQR) from the median

was strongly correlated between both castes (Fig. 2), suggesting that many behaviors were in response to the other nestmate in the circle tube. Queens that were more queen like were associated with workers that were more worker like, as we predicted, but this pattern was driven by worker C-posture in response to queen aggression, as discussed above, rather than broader associations between queen-like and worker-like behaviors. The other worker-like behavior, 'reverse', showed no such association, suggesting that workers' response to aggression was to defend themselves (C-posture) rather than to turn and run away (reverse). Conversely, queen expression of the tolerant behaviors antennate and pass correlated negatively with worker expression of C-posture, suggesting that less aggressive queens elicited fewer C-postures from workers.

Effect of body size and ovary development on behavior

Body size had a dramatic effect on behavior. We know that body size influences caste in *M. genalis*, probably because queens manipulate offspring pollen provisions to create smaller, more easily dominated worker daughters (Kapheim et al. 2011, 2012). Our data here show that larger queens performed more queen-like behaviors and fewer worker-like behaviors than smaller queens, when controlling for ovary size and activity levels, which matches our predictions. Larger workers performed more queen-like behaviors than smaller workers, but there was no effect of size on worker-like behaviors. These results are consistent with our previous study of *M. genalis* in circle tubes, where we showed that when matched with non-nestmates of their own caste (queens with queens, workers with workers, and solitary females with other solitary females), larger bees were more queen like and less worker like than smaller ones (Smith et al. 2018). Together, the data from this study and Smith et al. (2018) suggest that size plays an important role in the expression of caste-specific behavior.

Ovary size dramatically differed between castes, consistent with the queens' monopolization of reproduction and also consistent with several previous studies on this species (Kapheim et al. 2012; Smith et al. 2008, 2009, 2018). Within each caste, ovary size correlated with body size, which is not consistent with previous studies of *M. genalis* (Kapheim et al. 2012, Smith et al. 2018). However in partial correlations controlling for body size and activity levels, ovary size did not correlate with queen-like or worker-like behavior. This is consistent with Smith et al. (2018) who also found little effect of within-caste ovary size variation, especially relative to body size variation. This suggests that while ovary development is obviously central to reproductive division of labor between reproductive queens and non-reproductive workers, variation in ovary development within castes is not linked to variation in behavior within castes. In a previous study on *Megalopta* bees, ovary size influenced behavior in circle tubes, but that study did not control for caste, so the effect was likely that of caste, rather than ovary size per se (Arneson and Wcislo 2003). Ovary size influenced behavior in one (Wcislo 1997), but not in other sweat bee species studied in circle tubes (McConnell-Garner and Kukuk 1997; Richards and Packer 2010).

Given the developmental and physiological links between body size, ovary development, and behavior (e.g., Kapheim et al. 2012; Smith et al. 2013; Lawson et al. 2017; reviewed in Kapheim 2017, Hamilton et al. 2017), it is not surprising that all three variables correlated with each other so frequently in this study. However, despite the difficulty of dealing with multiple correlations, the most consistent result from the partial correlations is the influence of size on caste-typical behaviors. A recent study on a bee in a different family (*Ceratina calcarata*, Apidae) showed that experimental reduction of larval pollen provisions reduced both body size and aggressive behavior (Lawson et al. 2017). This suggests that the effect of body size on caste-typical behavior that we report here may be indicative of a broader effect of developmental nutrition on behavior (Kapheim 2017).

In general, pairs with larger body size and ovary size differences were also pairs in which the queen did relatively little, and the workers did relatively more, worker-like behavior. A previous study on observation nests showed that the few cases where workers superseded their queen were all in nests where the worker was similar sized or larger than her queen (Kapheim et al. 2013). This suggests that despite the obvious artificiality of a circle tube relative to a natural nest, the behavioral differences we observe reflect natural differences in social development between nests. Future studies quantifying conflict, productivity, and queen tenure in observation nests with different queen:worker size ratios would be productive.

This effect of relative size on aggressive behavior has been seen in other circle tube studies of sweat bees (e.g., Smith and Weller 1989; Pabalan et al. 2000). However, in our previous study comparing non-nestmates matched for caste (including solitary females, as well as queens and workers), we found no effect of relative body size or ovary size (Smith et al. 2018). We hypothesize that this is because the current study used nestmates that shared a developmental history, and relative size, ovary, and behavior differences were thus intrinsically linked, as described above. In our previous non-nestmate study, on the other hand, bees were interacting with unknown individuals for the first time (Smith et al. 2018).

Nestmate and non-nestmate comparisons

In a previous study of *M. genalis* using circle tubes to compare non-nestmate bees matched by caste, we showed that workers in circle tubes with other workers apparently increased queen-like behavior when released from queen control (Smith et al. 2018). They performed a similar amount of queen-like behaviors as did queens in circle tubes with other queens. Worker-like behavior, however, was not so flexible: even in caste-matched pairs, workers still did more worker-like behavior than did queens in caste-matched pairs (Smith et al. 2018). The data from the current study show that queens express similar ratios of queen-like to worker-like behavior in both nestmate and non-nestmate circle tube assays (Fig. 5a), but workers do not (Fig. 5b), as we predicted. The difference in worker behavior comes from decreased queen-like behavior when in circle tubes with their nestmate queen, relative to non-nestmate, caste-matched pairs. There was no difference in the relative expression of worker-like behaviors (Fig. 5c). These data suggest that queen presence directly suppresses worker's expression of queen-like behaviors. Worker-like behavior, though, continues without a queen (Smith et al. 2018) perhaps because it is the product of longer-term developmental manipulation (Michener and Brothers 1974; Kapheim et al.2016; Lawson et al. 2017).

Conclusions

Our data show that queens generally express higher levels of aggressive and dominant behaviors than workers, as would be expected from previous studies of observation nests (Michener 1990; Kapheim et al. 2016; Dalmazzo and Roig-Alsina 2015, 2018a, b). Our study is consistent with previous literature on primitively eusocial sweat bees highlighting the role of queen dominance in the development of the worker caste (Michener and Brothers 1974; Michener 1990; Kapheim et al. 2016), and suggests that the effects of queen manipulation of workers are seen in the correlations of between-caste divergence in body size, ovary development, and behavior.

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