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Original article

Bumble bee queen egg laying is facilitated by both social conditions and nest cavity size

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Abstract – Bumble bees are essential pollinators that have an annual eusocial lifestyle where new cohorts of queens establish colonies each season. The early stages of colony establishment are critical for colony success and are heavily impacted by how readily queens activate their ovaries and initiate egg laying at the start of the season. Understanding the social and nesting factors that influence queen reproduction may provide key insights into early colony establishment. Here, we examine how social conditions and nest cavity size affect the onset of egg laying in early nesting *Bombus impatiens* queens. In the first experiment, queens were assigned to a social condition where they were placed into nesting boxes that either completely restricted contact with nestmates (and were smaller in size) or allowed direct physical contact and then housed either alone or with nestmates (males or workers). We found that contact with either workers or males accelerated egg laying in queens relative to being housed alone. Unexpectedly, queens housed in the restricted cages, regardless of whether alone or with nestmates, also initiated egg laying earlier. This led us to a second experiment, where we examined ovarian activation and hemolymph juvenile hormone (JH-III) concentrations in early queens housed in different nesting cavity sizes. Here, we found that smaller nest cavity sizes lead to a greater degree of ovarian activation in gynes (young, unmated queens), but juvenile hormone concentrations did not differ. Our findings demonstrate that both social conditions and nest cavity size influence reproductive timing in queens in early-stage bumble bee colonies.

bumble bees / rearing / nesting cavity / colony initiation / ovarian activation

1. INTRODUCTION

Bumble bees are a lineage of eusocial insects with an annual colony cycle where new nests are founded and die each year (Sladen 1912). As is true for many lineages within the major eusocial insect groups (Cronin et al. 2013), bumble bee queens found their nests independently at the beginning of each season and must perform all nesting tasks before successfully rearing their first workers, who then take over all brood care, foraging, and nest maintenance tasks (Batra 1966; Michener 1969). During this solitary-to-subsocial-to-eusocial transition, queens are likely more susceptible to stressors, which can synergistically impact survival (*Bombus terrestris:* Fauser-Misslin et al. 2014; Baron et al. 2017; *B. impatiens:* Leza et al. 2018). Success during the solitary and early social stages ultimately is required for colonies to produce hundreds of pollinating workers and new reproductive individuals.

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Elucidating the factors that influence the onset of queen egg laying is important for understanding the conditions that contribute to successful colony initiation. Previous studies have demonstrated that ovarian activation and egg laying can be facilitated by workers within the nest. Specifically, when workers are provided prematurely to early nesting queens, the time until ovarian activation and the onset of queen egg laying is shortened (*B. terrestris:* Woodard et al. 2013; *B. impatiens*: Sarro et al. 2021). Queens appear particularly sensitive to added nestmates of various kinds during early nest development, as similar effects have been observed with the addition of gynes (young, unmated queens) and even worker honey bees (Sladen 1912; *B. terrestris*, *B. pascuorum*, *B. lapidarius:* Ptáček & Drobna 2006; Velthuis & van Doorn 2006; *B. appositus*, *B. bifarious*, *B. centralis*: Strange 2010). This onset of reproduction in queens is associated with an increase in juvenile hormone (JH) levels in the hemolymph. JH is a gonadotropic hormone in most insects, including bumble bees (*B. terrestris:* Bloch et al. 2000; *B. impatiens:* Sarro et al. 2021; Hymenoptera: Shalem et al., 2025). The sensory mechanisms that underlie this socially mediated accelerated reproduction in queens, however, remain unknown. Within the natural colony cycle, queens found nests solitarily. However, understanding the mechanisms through which workers and males influence queen reproduction can inform rearing practices, as it is common to manipulate social conditions to induce queen colony initiation.

Other factors that might impact the onset of queen egg laying are related to the nesting cavity and nesting conditions. Most bumble bees establish nests in pre-existing cavities, often below ground, rather than extensively excavating nests themselves (Sladen 1912; many species: Fussell & Corbet 1992; Liczner & Colla 2019). This means that once queens emerge in spring, they search for pre-existing cavities such as those made by rodents (Sladen 1912), and the size of the nest is largely restricted to the available cavity space. In solitary bees, nesting cavity size can influence sex ratios, offspring body size, and offspring numbers (Longair 1981; in Megachile: O'Neill et al. 2010; in Osmia: Torchio & Tepdino 1980). In honey bees, the size of nesting cavities can impact survivorship, with smaller nesting cavities in the laboratory having more survivorship than larger nesting cavities (Bosua et al. 2018). Little is known, however, about how nest cavity size impacts colony initiation and growth in bumble bees. Some bumble bee rearing practices use small nesting boxes for colony initiation (pre-oviposition) before moving queens to larger foundation boxes (*B. nevadensis*, *B. rufocinctus*, *B. borealis*, *B. fervidus*, *B. terricola*, *B. perplexus*, *B. ternarious*, *B. vagans:* Plowright & Jay 1966; *B. ignitus:* Yoon & Kim 2002; *B. terrestris:* Imran et al. 2017, Ono et al. 1994). This suggests that smaller cavity sizes might have a positive impact on colony initiation, although this has not been empirically shown.

Here, we used the bumble bee *B. impatiens* (genus *Bombus*, family Apidae) to examine how social conditions and nest cavity sizes mediate the onset of queen reproduction. In a paired set of experiments, we ask if social conditions (or the lack of social conditions) and nest structure impact the onset of reproduction in queens. We first asked whether direct contact with nestmates can prematurely induce egg laying in queens. To prevent direct contact with nestmates, we bisected the available nesting space for queens, which also reduced nest cavity size. In the second follow-up experiment, we more directly asked if limiting nest cavity space impacts queens ovary activation and physiology. Together, these experiments address the broader question of what factors impact the onset of ovarian activation and egg laying in early nest-founding bumble bee queens.

2. MATERIALS AND METHODS

B. impatiens colonies were acquired from Koppert Biological Systems (Howell, MI, USA) and maintained in the University of California, Riverside's Entomology Building. Colonies were maintained at approximately 24–28 °C and 60% relative humidity under red light. All colonies were fed artificial nectar prepared following the

recipe in Boyle et al. (2018) and pollen balls (Koppert Biological Systems, mixed-source, honey bee-collected pollen). Mature colonies were used to source all gynes, males, and workers. Callow gynes (< 24 h old, identified by their silvery appearance) were removed from their natal colonies, placed in individual containers (Hi-Plas crystal clear polystyrene 4 oz cups), and fed ad libitum pollen and artificial nectar (see Boyle et al. 2018). After removal from their natal nests, callow gynes were kept approximately at 27–30 °C and 60% relative humidity in an Invictus Drosophila Incubator until they reached an adult age of 3 days. For experiment 1, after these 3 days, gynes were placed daily into a mating cage (100 × 46 × 46 cm) with males that were sourced from separate colonies from the gynes, based on previous methods (Peto et al. 2025). Gynes were allowed the opportunity to mate for 9 h per day for an additional 8 days (until age 11 days). Gynes and males had access to pollen and artificial nectar (as described above) during their time in the mating cage. Once gynes were observed copulating (and thus referred to as queens thereafter), they were removed from the mating cage, placed in an individual container with pollen and artificial nectar, and were not subsequently subjected to the mating cage (Treanore et al. 2021). All queens used in the experiments were mated by 12 days of age or otherwise not used in the experiment. The sample sizes reported below only reflect queens that successfully mated before day 12.

2.1. Experiment 1: Social effects on queen reproductive status

All queens were treated twice with carbon dioxide gas for 30 min, once at age 12 and once at age 13 days of adulthood. These treatments cause queens to bypass diapause and initiate egg laying (Röseler 1985), but does not otherwise alter the course of colony initiation (Röseler 1985; Gurel & Gosterit 2008; Sarro et al. 2021; Amsalem & Grozinger 2017). Twenty-four hours after carbon dioxide treatments, queens were assigned to one of six experimental groups, which differed in social configuration and whether, if they were housed with nestmates, they were allowed direct contact with nestmates or not. Seventy queens from four natal colonies were distributed into one of the following three social configurations: (1) alone (−), where a single queen was reared with no workers or males added to the nest, (2) with workers (W +), where five callow workers were added, or (3) with males (M +), where five callow males were added. For each queen, the added nestmates (workers or males) were all obtained from the same natal colony but were from a different natal colony than the queen. We were occasionally limited in our ability to pull five callow individuals on the experimental start day because workers and males emerge sporadically and were collected opportunistically. This limitation is reflected in our sample sizes for W + or M + social configurations. Once assigned to a social configuration, queens were further divided into two nest structures, where either a double mesh barrier (Figure S1) was present in the nest, in which approximately ~ 45% of their nesting box was bisected by a double mesh barrier, or the nesting box was unaltered (17 cm × 17 cm × 9.5 cm). The barrier reduced the nesting cavity space available to the queen while still allowing limited airflow and potential transmission of volatile cues across the mesh. Added nestmates were either on the other side of this mesh barrier to restrict contact or allowed direct physical contact with the queen because there was no barrier. One queen died before laying eggs (No barrier (−) = 1) and was excluded from analysis. This resulted in six experimental groups: queen alone/no barrier (hereby referred to as "No barrier (-)", $n = 13$), queen alone/barrier ("Barrier (-)", $n = 13$), with males/no barrier ("No barrier (M +)", $n = 15$), with males/barrier ("Barrier (M +)", $n = 9$), with workers/no barrier ("No barrier (W +)", $n = 11$), with workers/barrier ("Barrier (W +)", $n = 8$).

All queens were then relocated to the University of California, Riverside's Insectary and Quarantine and kept under low red light at 27–30 °C and 60% relative humidity. All individuals (queens, workers, and males) were provided ad libitum nectar and pollen as described

above, either together or separately (based on barrier structure). Once transferred, nests were left undisturbed except when food or nectar was supplemented, and nests were monitored daily for the appearance of the first eggs. Within early nests, these egg cups are easily visually identifiable as small raised mounds of wax, typically located on pollen provisions (Figure S2; Alford 1971; Heinrich 1979; Sladen 1912; Rozen et al. 2018). Nests were collected after the first eggs were laid and stored at −80 °C. Pollen balls were dissected to confirm that there were no hidden egg cups within the whole pollen ball and that the date eggs were observed was, in fact, the date of first eggs. One queen died, as reported above, and was removed from our study sample. Reported data refers to the remaining 69 queens.

2.2. Experiment 2: Nest cavity size

Callow queens were pulled from source colonies and treated twice with carbon dioxide to bypass diapause as described above, with the exception that these queens were not mated. Unmated queens are easier to prepare for experiments and will develop their ovaries along a similar timeframe as mated queens (Sarro et al. 2021) but produce male-destined (unfertilized) eggs rather than female-destined (fertilized) eggs. Furthermore, not mating queens minimizes variation introduced by the mating process (Baer & Schmid-Hempel 2005). Mating does influence oogenesis and egg laying in some insects, but not bumble bees (Amsalem et al. 2015b), but can change some physiological traits related to nutritional physiology (Kocher et al. 2008; Amsalem et al. 2015a). From here on, we continue to refer to these queens as gynes to signal their unmated status within this experiment. After the second carbon dioxide treatment, gynes were assigned to one of three experimental groups that varied in nest cavity size (not social conditions, as all gynes were alone for the duration of the experiment in experiment 2). Seventy-five gynes from seven colonies were distributed among three nesting box sizes (dimensions reported as L × W × H): large (16 × 16 × 12 cm, $n = 26$), medium (16 × 16 × 8 cm, $n = 24$), and small (11 × 11 × 7.5 cm, $n = 25$). Nesting boxes were made from modified Rubbermaid storage containers, where the largest nesting box size approximated the size of the original nesting box in experiment 1 (Exp 1: 17 cm × 17 cm × 9.5 cm, 2.75 L vs Exp 2: 16 × 16 × 12 cm, 3.07 L). Our medium nesting box group reflected a one-third reduction in volume from the large box, whereas our smallest nesting box was approximately a two-thirds (~ 70%) reduction in volume from the largest nesting box. Gynes were then moved to the Insectary and Quarantine Facility and maintained as described above. After 4 days, gynes were collected and frozen at 20 °C for ovarian analyses (Sarro et al. 2021). This metric for ovarian activation has been well-characterized in bumble bees (Duchateau & Velthuis 1989) and can be detected on a timeframe of days (4 days: Sarro et al. 2021; Peto et al. 2025) rather than weeks, as is the case for egg laying. The ovaries were dissected and measured as described below. We examined ovarian activation in this experiment (rather than the time until egg laying, as in experiment 1), specifically, the lengths of each of the eight terminal oocytes from individual gynes. A subset of gynes from each nesting box structure (large nesting box, $n = 10$, medium nesting box, $n = 12$, small nesting box, $n = 13$) was used for JH quantification before being stored for later ovary dissection. Four gynes used to quantify JH were removed from the analysis due to unequal colony representation, and thus, the final sample size for JH analysis is as follows: large nesting box, $n = 8$; medium nesting box, $n = 12$; small nesting box, $n = 11$.

2.3. Hemolymph collection and JH analysis

We quantified JH-III titers in hemolymph to determine whether levels of this hormone were affected by nest cavity size and related to queen ovarian activation. For hemolymph collection, gynes were restrained using plastic marking tubes (Betterbee, Greenwich, CT, USA). Heads were swiftly removed to expose the open neck

cavity. Using 10-µl glass capillary tubes, hemolymph was immediately collected from the cavity and transferred into a mixture of 50 µl acetonitrile (Fisher Scientific A998-4, Waltham, MA, USA) and 50 µl of 0.9% sodium chloride solution (Fisher Scientific S271-500), followed by rapid freezing on dry ice. The samples were stored in an autosampler vial (Fisher Scientific c5000-1W) with 9-mm autosampler inserts (Fisher Scientific C4010-630) and Teflon-lined vial caps (Fisher Scientific C5000-54B) at −80 °C until extraction. JH extraction and quantification were performed based on Kai et al. (2018) and Sarro et al. (2021), with modifications described in Goldberg et al. (2025). Four microliters of 5 ng/µl citronellol was added to each sample as an internal standard. After adding 100 µl of hexane, samples were vortexed for 30 s and allowed to stand for 5 min, followed by centrifugation at 1000 × g for 5 min. The organic phase was transferred to a new vial using a Hamilton syringe (Hamilton, #80600, Reno, NV, USA). An additional 100 µl of hexane was added to the original vial, and the extraction was repeated to ensure complete recovery of JH. The hexane extracts from both extractions were combined into one vial and stored at −80 °C until analysis. JH titers were determined by gas chromatography-mass spectrometry according to Sarro et al. (2021), using a Thermo Scientific Trace 1310 GC-MS equipped with an AI 1310 autosampler and a TSQ Duo triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). A 2 µl aliquot of the hexane extract was injected into the GC inlet maintained at 230 °C. Helium was used as the carrier gas at a constant flow rate of 1.2 ml/min. The injector was operated in splitless mode with a splitless time of 1 min, followed by a split flow of 25 ml/min. The purge flow rate was set to 50 ml/min, and the gas saver flow rate was maintained at 25 ml/min for 2 min. Analytes were separated on a Thermo Scientific TG-5MS column (0.25 mm i.d. × 28.33 m, 0.25 µm film thickness). The column oven was initially held at 60 °C for 1 min, increased to 160 °C at 25 °C/min, and then ramped to 280 °C at 12 °C/min. The transfer line and ion source temperatures were both maintained at 280 °C. The mass spectrometer was operated in electron ionization mode. Citronellol and JH-III were detected using selected reaction monitoring. Citronellol was detected at a retention time (RT) of 5.75 ± 0.1 min using the transition *m/z* 81.0 to 79.1 with a collision energy of 10 V. JH-III was detected at an RT of 10.85 ± 0.1 min using the transition *m/z* 85.9 to 59.1 with a collision energy of 10 V. A dilution series of JH-III (Millipore-Sigma, #J2000, Burlington, MA, USA) containing 0.1 ng/µl citronellol was used to generate standard curves. Queen bodies were stored at − 80 °C until they were processed for ovary dissection.

2.4. Ovary dissections

All queen bodies were stored at −80 °C until they were processed for dissection. Ovaries were removed from the abdomen using fine-point dissection forceps. The lengths of all eight terminal oocytes were measured with an ocular micrometer and recorded (Duchateau & Velthuis 1989).

2.5. Statistical analysis

All analyses were performed using R version 4.2.1 (R Core Team 2023). For both experiments, GLMMs were run using the glmmtmb() function in the glmmtmb package (Brooks et al. 2017). Data distributions were evaluated for normality using the Shapiro-Wilk normality test with the shapiro.test() function in the dplyr package (Wickham et al. 2022). Data were further visualized using ggdensity() function in the ggplot package (Wickham 2016). Generalized linear mixed models were fitted using a negative binomial distribution for experiment 1 and Gamma distribution for experiments 2 and 3. Models were compared using model.sel() function in the MuMIn package (Barton 2022), and the best-fit model was selected based on Akaike's information criterion (AIC). Model assumptions for all GLMMs were assessed using simulation-based residual diagnostics through the DHARMa package (Hartig 2022). For all models, there was

no evidence of overdispersion, excess outliers, or systematic deviations among groups. One model, ovarian activation by box group, showed a minor but significant deviation from residual uniformity (Kolmogorov-Smirnov test $D = 0.066$, $p = 0.016$). However, visual inspection did not indicate substantial model misfit, and this deviation may reflect the increased sensitivity of the test associated with the relatively large sample size. The remaining models showed no evidence of deviation from expected distributions. Thus, the assumptions underlying the selected models are reasonably met. Following selection of the best-fit model, a one-way analysis of variance (ANOVA) was performed, and an estimated marginal means post hoc using the emmeans() function in the emmeans package (Lenth 2023) was used to create pairwise comparisons. Queen natal colony was included as a random effect in all models to account for potential non-independence among individuals originating from the same colony. For the ovarian analysis, where queens contribute multiple data points, Queen ID was additionally included as a nested random effect with natal colony. Fixed effects included box configuration (Exp 1: Barrier or No Barrier; Exp 2,3: Large, Medium, Small), social configuration (Exp 1: Alone, M+, W+), and their interactions, depending on the specific hypothesis being tested. Additional information, including model selections, best-fit descriptions, and data selection, can be found in the supplement.

3. RESULTS

3.1. Nesting configuration on queen reproductive status

Nesting configuration and social configuration both significantly influenced the time until egg laying ($\chi^2 = 29.1137$, df = 2, $p < 0.0001$; Figure. 1). Social configuration had a significant impact on the time until eggs were laid in the overall model ($\chi^2 = 77.2559$, df = 2, $p < 0.0001$). Direct physical contact with workers (6.8 ± 1.1 days standard error of the mean (S.E.M)) and males (9.2 ± 0.87 days S.E.M) both reduced the time until queens initiated egg laying, relative to when queens were held alone with no barrier in the nest (22.3 ± 1.6 days S.E.M., No Barrier (−) vs No Barrier (W+): $Z = 8.440$, $p < 0.0001$; No Barrier (−) vs No Barrier (M+): $Z = 7.705$, $p < 0.0001$). Workers and males did not significantly differ in their influence on egg laying ($Z = 1.961$, $p = 0.3650$). There was no reduction in the time until eggs were detected in the nest when social group members were present across from the barrier (Barrier (M+): 11.55 ± 1.32 days; Barrier (W+): 8.25 ± 1.18 days S.E.M; Barrier (M+) vs No Barrier (M+): $Z = -1.613$, $p = 0.59$; Barrier (W+) vs No Barrier (W+): $Z = 2.237$, $p = 0.2209$). There was a significant overall effect of the barrier on the model ($\chi^2 = 6.8537$, df = 1, $p < 0.01$). Queens held alone in a nesting box with a barrier (~45% reduction in space) had a significantly reduced time until egg laying compared to our control queens (average 11.77 ± 0.7 days; Barrier (−) vs No Barrier (−): $Z = 5.678$, $p < 0.0001$).

3.2. Nest cavity size

Nesting cavity size had a significant effect on ovarian activation ($\chi^2 = 32.219$, df = 2, $p < 0.0001$; Figure. 2A). Gynes held in the smallest nest cavity size had more developed ovaries (1.13 ± 0.04 mm S.E.M) than gynes in the medium (0.72 ± 0.03 mm S.E.M; $Z = -4.100$, $p = 0.0001$) and the largest (0.62 ± 0.03 S.E.M; $Z = -5.447$, $p < 0.0001$) nest sizes. Gynes held in the medium and the largest nesting cavity did not differ in their ovarian activation ($Z = -1.633$, $p = 0.232$). JH-III concentrations did not differ between groups ($\chi^2 = 2.2466$, df = 2, $p = 0.33$; Figure 2B).

4. DISCUSSION

The ability of bumble bee queens to develop their ovaries and initiate egg laying during the initiation stage of the colony cycle is

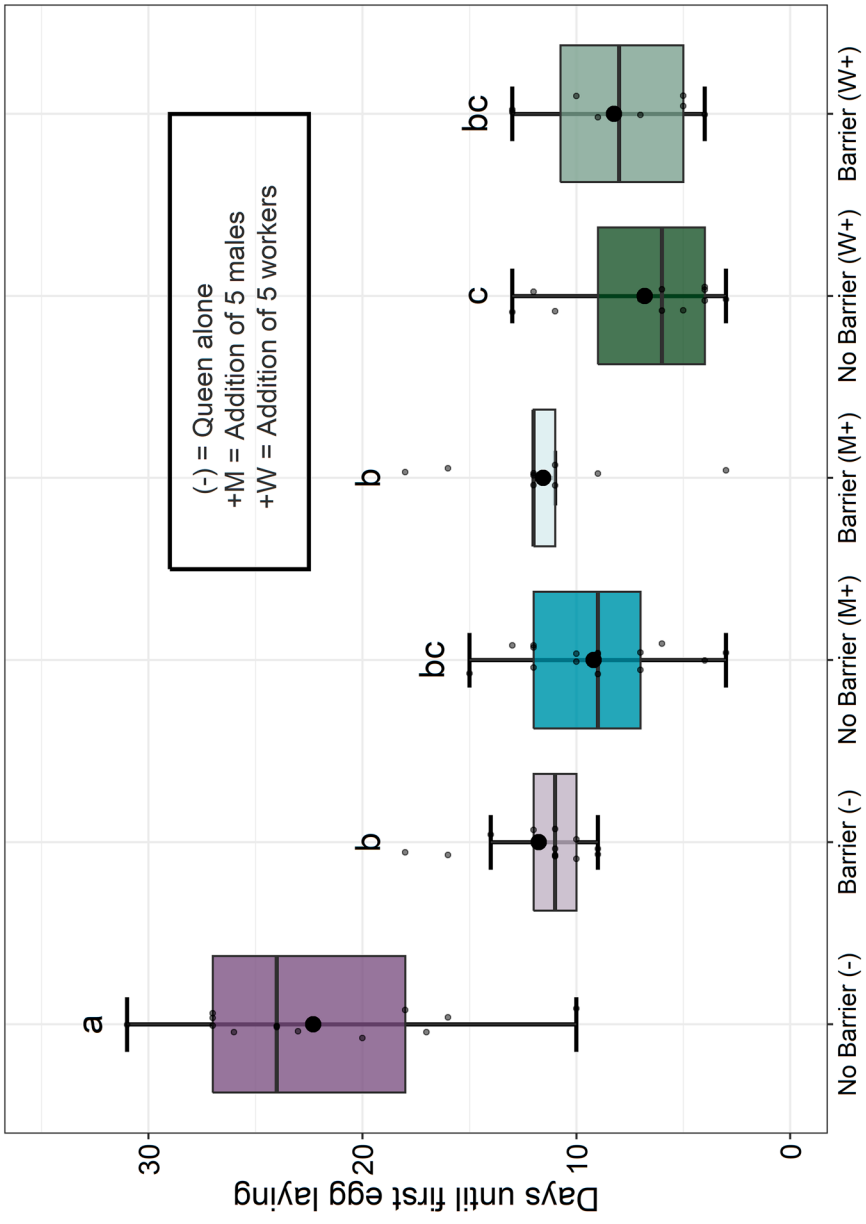

Fig 1 Influence of workers, males, and nest structure on the timing of queen egg laying. Bar graph showing the number of days until queens laid their first eggs as a function of nest structure and social configuration. Middle bar represents the median of the data, the upper box limit represents the 75th percentile of the data, and the lower box limit represents the 25th percentile. Black dots represent the mean of the data, and smaller, jittered dots represent individual data points. Different letters indicate significant differences between plots revealed by post hoc pairwise (Tukey) tests

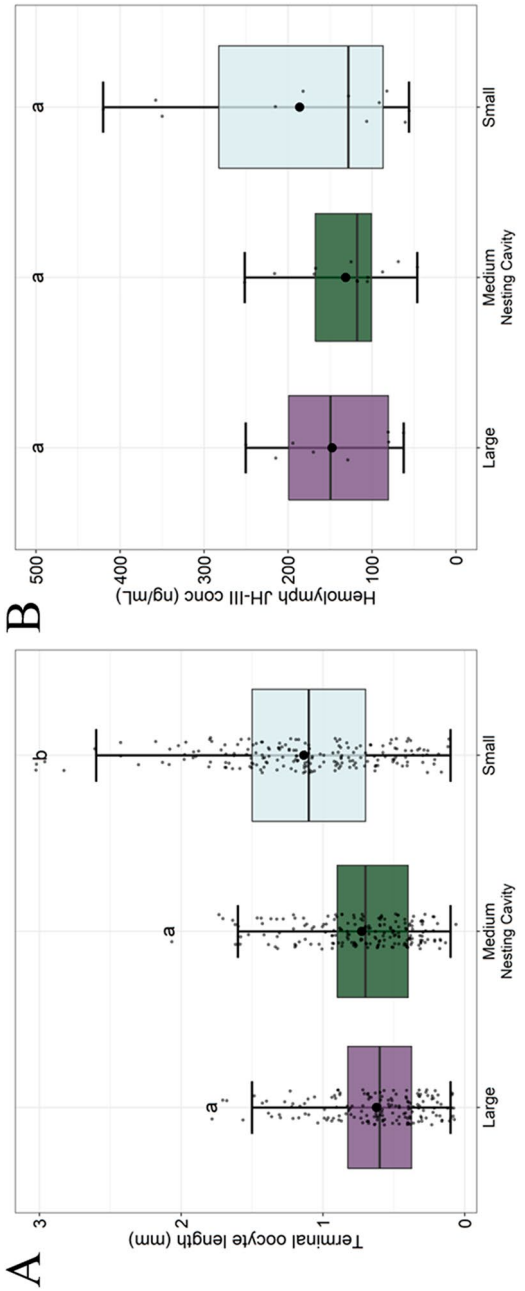

Fig 2 Influence of nest cavity size on ovarian activation and circulating JH-III levels. **A** Influence of nest cavity size on ovarian activation. **B** Influence of nest cavity size on JH-III hemolymph concentration. Box and whisker plot showing terminal oocyte lengths (in millimeters) or JH-III hemolymph concentrations after 4 days of treatment. Different letters indicate significant differences between plots revealed by post hoc pairwise (Tukey) tests.

foundational to successful nest establishment. Here, we demonstrate that ovarian activation and the onset of bumble bee queen egg laying is influenced by both social conditions and nest cavity size. Both workers and males in the nest reduced the time until egg laying when they were allowed direct contact with queens, with workers having a stronger positive impact. These results are consistent with previous studies showing that the presence of adult bumble bee workers, additional young queens, or honey bee workers accelerates the onset of egg laying in bumble bee queens (*B. terrestris:* Woodard et al. 2013; *B. impatiens:* Sarro et al. 2021; *B. appositus*, *B. bifarious*, *B. centralis*: Strange 2010; *B. terrestris:* Ono et al. 1994). Our findings extend these studies by demonstrating that male contact with queens can also accelerate egg laying in early-stage queens.

Our findings support the possibility that tactile and/or contact pheromones (i.e., requiring direct contact to be transmitted) may regulate queen ovarian activation in *B. impatiens* queens, although more work should explore this phenomenon more directly. There is evidence that cuticular hydrocarbons, which are contact pheromones, from gyne (unmated queens), worker, and male bumble bees, and also honey bees, can stimulate ovarian activation in bumble bee (*B. terrestris*) workers (*B. terrestris:* Ge et al. 2025). This is consistent with our result that both workers and males had a positive impact on queen ovarian activation when they could directly contact the queen. It is unclear whether more long-range pheromones might also play a role in this, due to our design in experiment 1. Specifically, given that the barrier on its own had an impact on queen egg laying in our first experiment, we are prevented from making any conclusions about whether pheromones transmitted across the barrier also had an impact on bumble bee queen reproduction. Previous studies, however, have demonstrated that long-distance pheromonal cues alone do not appear to impact ovarian activation (*B. impatiens*: Padilla et al. 2016; Amsalem et al. 2015c; Melgarejo et al. 2018; *B. terrestris*: Bloch & Hefetz 1999). Still, long-range pheromonal communication plays a major role in communication between bumble bee castes (Leonhardt et al. 2016; *B. terrestris:* den Boer & Duchateau 2006). Thus, it is entirely possible that multiple mechanisms, including contact and long-distance chemical cues, and also non-pheromonal cues (*B. terrestris:* Ge et al. 2025), contribute to the socially mediated onset of queen reproduction.

We additionally found multiple lines of evidence that a smaller nest cavity size induces ovarian activation and egg laying in bumble bee queens, regardless of mating status. In our first experiment, our barrier nesting boxes (which were smaller in size) alone reduced the time until egg laying compared to queens in unaltered nesting boxes. In our second experiment, we investigated this phenomenon further and found again that a smaller nest cavity can accelerate ovarian activation. Specifically, gynes housed in our smallest nesting cavity size had larger terminal oocytes than queens in our medium (~ 33% reduction) or largest (~ 70% reduction) boxes. Bumble bees are cavity nesters who often occupy pre-existing cavities produced by rodents and other small vertebrates (Sladen 1912). However, bumble bee nests can be difficult to find, and most reports describe entrance tunnels rather than nesting cavity dimensions. Thus, data on the exact dimensions of natural nesting cavities are largely lacking and might vary greatly in size depending on the original creators of these nests. Some observational reports exist describing natural nesting cavities (summarized in Table I; but see Sakagami & Katayama 1977); these chambers do vary in size, but volumetrically mirror our smallest nesting boxes (see *B. hypocrita hypocrita*). In light of this, our data suggests that the size of available nesting cavities might also influence queen ovarian activation and the timing of nest establishment in wild queens. As bumble bees re-use existing cavities and do not do much excavation, this creates a potential trade-off in wild queens: a smaller nesting cavity may promote accelerated ovarian activation during early nest development, but may also limit the overall size and reproductive capacity of the colony. Some evidence for this is in *Megachile rotundata*, where nest diameter impacted the size and

Table I Summary of published descriptions of natural nest cavity dimensions for *Bombus* species. Species name and cavity descriptions are reported as provided in the original sources. Entries describing only the cavity floor do not contain sufficient information to estimate cavity volume and are marked as only referring to floor dimensions. Three-dimensional shapes, such as spherical/ovoid descriptions, are additionally marked when noted by the source. Dimensions are reported in centimeters

Species	Nest cavity description	Source
B. ardens ardens	13 × 14 cm, cavity floor	Sakagami and Katayama (1977)
B. ardens sakagamii	12 × 12 × 9(h) cm	Sakagami and Katayama (1977)
B. diversas tersatus	30 × 25 cm, cavity floor 10 × 15 cm, cavity floor	Sakagami and Katayama (1977)
B. diversus diversus	3 × 16 cm, cavity floor	Sakagami and Katayama (1977)
B. excellens	25 × 17 × 15 cm	Hoffmann et al. (2024)
B. hypocrita hypocrita	11 × 13 × 14(h) cm, ovoid 10 × 12 × 8 (h) cm	Sakagami and Katayama (1977)
B. hypocrita sapporoensis	20 × 25 × 16(h) cm	Sakagami and Katayama (1977)
B. lapidarius	Up to 18 cm diameter	Wagner (1907)
B. lapponicus	4–6 cm diameter, spheroid	Martinet et al. (2022)
B. melaleucus	30 × 20 × 15 cm, cavity floor	Hoffmann et al. (2004)
B. nevadensis	5–15 cm deep, 5–10 cm wide	Rao and Skyrm (2013)
B. shaposhnikovi	10–12 cm diameter, spherical	De Meulemeester et al. (2011)

the number of offspring produced (O'Neill et al. 2010). Other factors, such as food availability (*B. vosnesenskii*: Williams et al. 2012, *B. terrestris*: Rotheray et al. 2017), are also known to restrict colony growth in bumble bees and may play a more important role in influencing colony size than nest cavity size under ecologically relevant conditions.

It is also unclear what mechanism underlies the positive effect of smaller nesting cavities on queen ovarian activation. Conditions within the nest, such as the relative humidity and temperature within a cavity, are known to impact the timing of initial colony initiation and impact overall nest development in bumble bees (*B. ignitus*: Yoon et al. 2002). Smaller nesting cavities may alter several characteristics of the nest environment, including thermogenic and respiratory properties, which may impact development (*B. terrestris*: Weidenmüller et al. 2002; *B. hypnorum*, *B. hortorum*, *B. argillaceus*, *B. pascuorum*, *B. humilis*, *B. sylvarum*: Gradišek et al. 2023). There is evidence that elevated abdominal temperatures accelerate ovary development in bumble bees (*B. flavifrons*, *B. sylvicola*, *B. frigidus*, *B. balteatus*, *B. lucorum*, *B. hyperboreous*, *B. polaris:* Vogt et al. 1994; *B. impatiens:* Vogt et al. 1998). Smaller nesting cavities might more easily maintain higher nesting temperatures and overall queen body temperature, potentially accelerating reproductive timelines. Thus, one possible explanation is that smaller nesting cavities facilitate ovarian activation through thermogenic mechanisms. A second potential explanation could be an increase in carbon dioxide levels within smaller nests. Queens diapause in small underground cavities, in which carbon dioxide levels increase over winter. The overwintering process can be bypassed in the lab through two treatments of carbon dioxide narcosis, which have been shown to stimulate ovarian activation within a few days of treatment (*B. impatiens*: Amsalem & Grozinger 2017). The effect of carbon dioxide narcosis on bumble bee ovarian activation appears to be dose-dependent in mated queens, but does not differ in non-mated queens (*B. impatiens*: Cressman & Amsalem 2023).

Counter to the idea that thermogenic or carbon dioxide–related mechanisms induce ovarian

activation in queens, we also observed differences in reproduction within queens housed in the barrier boxes of experiment 1. Although these nesting boxes did reduce queen nesting cavity space, the barrier wall was constructed completely from metal mesh, which may have allowed for more heat and gas exchange than the smallest nesting cavity in our second experiment. Additionally, we saw an effect of reduced nesting cavity size on the onset of ovarian activation or egg laying in both experiments. Thus, it seems unlikely that heat retention and gas exchange alone, or together, fully explain the differences in ovarian activation that we observed.

Finally, we did not detect differences in JH-III concentration in gynes across nesting cavity sizes, despite detecting differences in their ovarian activation. Although JH is a known gonadotropic hormone, and its levels have been shown to increase around the time of ovarian activation in bumble bee gynes and with worker presence (*B. impatiens:* Sarro et al. 2021; *B. terrestris:* Bloch et al. 2000; Bloch et al. 1996), gynes were housed alone for the duration of the second experiment. When housed alone, queens do not exhibit the same spike in JH after 4 days as gynes with workers (*B. impatiens*: Sarro et al. 2021). This would suggest that although nesting cavity space can help facilitate the accelerated onset of reproduction, nest cavity size alone is insufficient to produce the whole suite of physiological and behavioral changes associated with the onset of reproduction.

5. CONCLUSION

Together, our experiments show that queen egg production and laying during the early nesting stage are sensitive to both social conditions and nest cavity size. We thus add to the current evidence that queen egg laying is socially regulated (Sladen 1912; Ptáček & Drobna 2006; Velthuis & van Doorn 2006; Strange 2010; Sarro et al. 2021; Woodard et al. 2013) by demonstrating that direct contact with both workers and males can accelerate the onset of egg laying. Further, we present evidence that the onset of egg laying in queens is sensitive to nest size. This presents a uniquely counterintuitive situation in which queen egg laying is accelerated in smaller nesting cavities, but ultimately, the nest growth capacity is restricted due to size limitations. These findings highlight the importance of both social and nest conditions in shaping reproductive strategies during early colony establishment. Further work is needed to clarify the mechanisms underlying these patterns.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

MEM led the experimental design, carried out the experiments and statistical analysis, and led authorship of the manuscript. CPC contributed to experimental design and statistical analysis, and provided authorship of the manuscript. KEM contributed to the experimental design and quantification of juvenile hormone. DF contributed to the quantification and analysis of juvenile hormone. NY contributed to the experimental design and quantification of juvenile hormone. GB helped secure funding to support this research. SHW helped secure funding to support this research, co-designed the experiments, and provided authorship of the manuscript. All authors read and approved the final manuscript.

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DATA AVAILABILITY

All data and associated code are available through Dryad and will become available upon publication: https://doi.org/10.5061/dryad.2280gb673.

DECLARATIONS

Ethics approval and consent to participate Not applicable.

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