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Benefits in Flexibility: Responses to Changing Social and Environmental Conditions in Bumble Bees

A Dissertation submitted in partial satisfaction
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in

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by

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DEDICATION

To all the bumble bees whose lives contributed to this research and whose biology continues to inspire scientific curiosity

ABSTRACT OF THE DISSERTATION

Benefits in Flexibility: Responses to Changing Social and Environmental Conditions in Bumble Bees

by

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Doctor of Philosophy, Graduate Program in Entomology
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Dr. S. Hollis Woodard, Chairperson

Bumble bee colony development is shaped by reproductive and behavioral transitions within the nest, as well as broader ecological conditions that influence colony members across landscapes. Broadly, nests are founded by independent queens, who perform all essential tasks during the earliest stages of nest development, including foraging, egg laying, incubation and feeding. As workers emerge, colonies transition into a eusocial phase, during which workers assume many in-nest and out-of-nest tasks and queens begin to specialize in reproduction. This dissertation examines the early stages of bumble bee colony development and explores how social and environmental variation may shape colony function beyond this initial stage.

In Chapter One, I investigate how social context influences queen reproductive plasticity during early colony development by examining whether brood and workers regulate queen egg-laying, as well as the timing of this regulation. In Chapter Two, I extend this focus on plasticity by examining brood care behaviors after worker emergence. Specifically, I experimentally remove workers from colonies at different sizes

and quantify changes in queen-provided brood care. Finally, in Chapter Three, I examine worker body size variation in a local species (*Bombus vosnesenskii*) to determine whether morphology varies across broad landscape features in California. Collectively, this work demonstrates that bumble bee colony development is shaped by dynamic interactions among social context, queen behavior and environmental conditions. By exploring these three facets, this dissertation provides insights into how bumble bee colonies develop and respond to social and environmental variation.

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Introduction

Bumble bees are critical pollinators in both natural and agricultural landscapes, where they pollinate a wide range of wild plants and commercial crops (Goulson et al., 2008; Cameron et al., 2011; Fisher et al., 2022; Williams, 1998). Their effectiveness as pollinators is often attributed to their relatively large body size, ability to forage at cooler temperatures, and capacity for buzz pollination, a behavior in which high-frequency vibrations release pollen from certain flowers (Heinrich, 2004). With approximately 250 species worldwide, bumble bees are primarily distributed across temperate, alpine, and arctic regions of the Northern Hemisphere. However, many bumble bee populations are experiencing widespread declines, with more than 30% of species estimated to be declining due to habitat loss and degradation, reduced floral resources, pesticide exposure, disease, and rising temperatures (Cameron and Sadd, 2020). Understanding the biological and ecological factors that shape bumble bee reproduction, behavior, and morphology is therefore important for predicting how populations may respond to changing environments.

Bumble bees are primitively eusocial insects in which females are divided into two primary castes: queens and workers. Queens are the reproductive caste and play a central role in colony establishment and maintenance. At the beginning of the colony cycle, queens found nests independently and perform all essential tasks, including foraging, egg laying, thermoregulation, and brood care, without assistance from workers during the solitary or subsocial phase of nest development (Free and Butler, 1959; Cronin et al., 2013). This early nesting stage is especially vulnerable to failure because queens

must balance the energetic demands of reproduction and brood care while navigating environmental stressors such as limited floral resources, predation risk, parasitism, and variable temperatures. Successful rearing of the first worker cohort is therefore a critical transition point in the colony cycle, as it determines whether the colony can progress into the eusocial phase and ultimately produce reproductive offspring.

As colonies develop, they transition into a eusocial phase in which workers begin to assume many in-nest and out-of-nest tasks. During this stage, division of labor becomes more established: workers perform brood care, foraging, nest maintenance, and defense, while queens often specialize more heavily in reproduction (Free and Butler, 1959; Cronin et al., 2013). Cooperative brood care can enhance colony growth by allowing reproductive individuals to allocate more energy toward egg production while workers support offspring development and survival (Gadau and Fewell, 2009). This transition can occur rapidly, with queen brood care declining markedly within days of worker emergence in *Bombus terrestris* (Woodard et al., 2013; Shpigler et al., 2013) and in response to only a few workers in the nest in *B. impatiens* (Sarro Gustilo et al., 2021; Gustilo et al., 2025). However, the extent to which queen egg laying and brood care behavior are fixed versus dynamic remains unclear. In particular, it is not well understood whether the queen's transition away from brood care and toward reproductive specialization represents a fixed life-history shift or a socially contingent response to changing colony conditions.

In addition to social and behavioral flexibility within colonies, bumble bee populations also vary across broader environmental and geographic gradients. Landscape features such as elevation, climate, vegetation, floral resource availability, can all shape the conditions experienced by colonies and may influence traits related to colony development, and ultimately, long-term population persistence (Fitzgerald et al., 2022). One trait that may be especially important across landscapes is body size (Chole et al., 2019). Bumble bees exhibit substantial variation in body size both within and among species, and this variation can be shaped by genetic background, as well as biotic and abiotic factors (Fitzgerald et al., 2022). However, relatively little is known about how body size varies across broad landscape features in many bumble bee species, or how this variation may reflect ecological differences across regions.

In this dissertation, I use bumble bees as a model system to examine how reproductive, behavioral, and morphological variation may shape colony function and population-level responses to environmental conditions. Specifically, I examine queen reproduction in the context of social regulation (Chapter One), brood care plasticity in response to changing social conditions (Chapter Two), and worker body size variation across different California ecoregions (Chapter Three). Together, these chapters explore and advance our understanding of plasticity in social insects, especially in regard to flexibility to both internal nest conditions and broader ecological environments. Rather than having fixed transitions, queen reproductive and brood care behaviors remain responsive to social context, while body size variation across ecoregions suggests that environmental conditions may shape colony-level traits. Combined, these findings

highlight plasticity as a critical mechanism through which bumble bee colonies may maintain development, respond to changing conditions, and persist across variable landscapes.

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CHAPTER 1: SOCIAL CONTROL OF EGG-LAYING IN INDEPENDENTLY NEST-FOUNDING BUMBLE BEE QUEENS

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Abstract

Evolution has shaped diverse reproductive investment strategies, with some organisms integrating environmental cues into their reproductive decisions. In animal societies, social cues can further influence reproductive decisions in ways that might support the survival and success of the social group. Bumble bees are a lineage of eusocial insects wherein queens initiate nests independently. Bumble bee queens enter their eusocial phase only after successfully rearing their first offspring and thereafter exhibit an increased rate of egg-laying. We tested the idea that during bumble bee nest initiation, queen reproduction is socially context-dependent and under the control of social conditions in the nest. Our findings reveal that in the bumble bee *Bombus impatiens*, queen egg-laying follows a dynamic, stereotypical pattern and is also heavily influenced by social group members. During the initial stages of nest initiation, accelerated egg-laying in queens is associated with the presence of workers or older larvae and pupae. Moreover, workers are required for queens to maintain increased levels of egg laying across the nest initiation stage. We also confirmed a previously-described pattern where queens temporarily decelerate egg-laying early in nest-founding, only to increase it again when the first adult workers are soon to emerge. This “pause” in egg-laying was observed in all *B. impatiens* queens as well as in additional species

examined. Our results support the idea that eusocial systems can employ socially context-dependent control of queen egg-laying as a reproductive strategy. In some solitary-founding lineages, including bumble bees, queens may reach their full reproductive potential only after the emergence of the first adult workers, who then take over brood care. This stands in contrast to the hyper-reproductivity observed in some eusocial species. The presence of workers and older brood (who will soon eclose) not only alleviates queen brood care responsibilities but may also provide signals that cause queens to increase their reproductive output. These phenomena may allow queens to adapt their reproductive output to the conditions of the colony. Broadly, these findings highlight the dynamic interplay between social conditions and reproduction in bumble bees.

Introduction

Evolution has shaped reproductive investment strategies in diverse ways. Organisms can integrate information from their surrounding environment (such as food availability and environmental stability) into decisions about whether to invest in the production of offspring or other non-reproductive processes (Heino and Kaitala, 1999; Tokolyi et al., 2012; Brommer et al., 2002; Fischer et al., 2011; McGinley et al., 1987; Kaitala, A. 1991). Environmentally-responsive reproduction is thought to be adaptive. Investing too heavily in offspring production at times when resources are too limited or might be better-directed to other processes, can lead to fitness costs (Brommer et al.,

2002). These fitness costs can come in the form of not producing the maximum number of viable offspring.

For animals that live in social groups, the social environment adds additional dimensions to how offspring investment is mediated. In some eusocial insect systems, where helpers are present at incipient nest stages and are present continuously (honey bees, swarm-founding wasps, non-solitary founding ants), queens can spend much of their lives laying eggs at a high rate. In contrast, the apex of reproductive output is reached more gradually in eusocial insect systems where queens establish nests independently. These queens are the sole caretakers in incipient nests and must provide continuous care for developing offspring until they reach adulthood. Queens that found nests independently (Ross, KG and Matthews, RW, 1991; Cronin et al., 2013; Free, JB. 1959) carry out all care-related tasks alone, including feeding and (in some systems) incubation, which are essential for the early growth and development of their young (Heinrich, B. 1979; Vogt, FD. 1986). Once workers emerge, however, the queen is relieved of providing brood care, as the workers take over the bulk of responsibilities. Consequently, a queen's ability to effectively allocate resources before a worker emerges might significantly impact her personal fitness. The success of a queen and her survival in this critical early nest founding stage directly impacts whether the colony progresses to produce workers and eventually reproductive offspring (Sladen, FWL. 1912; Baron et al., 2017; Leza et al., 2018).

One of the hallmarks of nesting success is the first emergence of offspring, which reflects a successful transition from the subsocial to eusocial stage. From here on, social group members in the nest have myriad opportunities to influence and mediate one another's investment strategies. Social regulation is exemplified in eusocial insects, which live in groups with overlapping generations—where parents coexist with adult offspring—and engage in collective alloparental care. In these groups, reproduction is suppressed in most female offspring (workers) and typically monopolized by one or a few females (queens) within the nest (Michener, CD. 1969; Wilson, EO. 1971; Batra, SW. 1966). Queens inhibit reproduction in workers through mechanisms such as pheromones and dominance interactions (Amsalem et al., 2017; Lopez-Vaamonde et al., 2007; Smith et al., 2019; Reeve et al., 1983).

Queen control of worker reproduction has been studied extensively (Amsalem et al., 2017; Lopez-Vaamonde et al., 2007; Alaux et al., 2004; Cnanni et al., 2000). There is also some limited evidence for the converse: that queen reproduction can be under the control of social group members. Support for this phenomenon has been found thus far in multiple ant lineages, Vespine and *Polistes* wasps, and in bumble bees. Within these systems, the presence and number of workers (Ross, KG and Matthews, RW 1991; Ross, KG., 1988; Woodard et al., 2013; Sarro et al., 2021; Gill, RJ and Hammon, RL, 2011), and even the presence of developing brood (Kwon et al., 2003), can increase queen oviposition rates at the onset of nest initiation. Offspring presence is hypothesized to allow queens to accelerate their egg-laying at a time that coincides with an increasing

number of helpers (workers) in the nest, who will assume and specialize on brood care tasks and allow the queen to allocate resources to reproduction (Sarro et al., 2021). It is currently unclear if this reallocation is permanent, or if queens will revert to earlier, lower egg-laying rates without sustained social conditions. Exploring the under-researched phenomenon of worker control of, and support for, queen reproduction will help illuminate a crucial aspect of eusociality: that in some systems, social group members facilitate reproduction, rather than suppressing it.

Here, we used the bumble bee *Bombus impatiens* (genus *Bombus*, family Apidae) to test the idea that in this independently-founding eusocial insect lineage, queen reproduction is under the control of multiple signals that are indicative of helpers or the potential for future helpers in the nest. Specifically, we tested whether both adult workers who provide brood care and late-stage brood, which will develop into care-providing workers within days, positively influence queen egg-laying. There is some prior evidence of the positive effects of both workers (Woodard et al., 2013; Sarro et al., 2021) and older brood (Kwon et al., 2003; Kwon et al., 2006; Bucankova et al., 2012; Yoneda, M. 2008) on queen reproduction, but they have not been examined together. The impact of these social group members has also not been examined across the full course of the nest initiation period. Previous natural history observations have noted dynamic egg-laying patterns in early nests of bumble bee queens that can only be partially explained by social regulation. Early observations of bumble bee nests from Alford (Alford, DV. 1971) state that "*once the initial egg clump is completed*, *further eggs are not normally laid until the*

first brood reaches the pupal stage." A similar phenomenon was reported by Sladen (Sladen, FWL. 1912) and Heinrich (Heinrich, B. 1979). Thus, we also sought evidence for this phenomenon that to our knowledge has not been empirically examined. We collected quantitative information about whether queens do temporarily decelerate and then reaccelerate their reproductive rate very early in nest-founding, with the latter in association with social conditions in the nest.

We confirmed that the pattern of temporarily reduced egg laying, previously described by Alford (Alford, DV. 1971) and others (Sladen, FWL. 1912; Heinrich, B. 1979), occurs in *B. impatiens*, and provide some preliminary evidence that it might occur in other bumble bee species. We believe that this biological phenomenon is a consistent behavioral pattern exhibited by bumble bee queens such that the queen can more effectively allocate resources between current brood and investment in her own future reproductive potential. Without this pattern, the queen would potentially face fitness costs by providing time and energy-intensive care to an over-abundance of offspring, which may be beyond her capacity to care for. By temporarily reducing laying eggs, queens can instead allocate more consistent care to existing offspring in the nest, which may increase overall nest fitness. We found that patterns of queen egg laying are heavily influenced by social group members. Queens increased their reproductive output with both the presence of group members that assist with brood care (adult workers) or the artificial addition of group members who will soon do so (older larvae and pupae). Furthermore, we found that the presence of workers is necessary to maintain higher queen reproductive output.

These findings overall support the broad idea that eusocial systems can employ socially context-dependent control of queen egg-laying as a reproductive strategy during early nest establishment and growth.

Materials and Methods

Experiment 1a: egg laying across early nest development

B. impatiens rearing: Fourteen mature *B. impatiens* colonies (consisting of a queen and workers, with queen pupae present) were provided by Koppert Biological Systems (Howell, MI, USA) and maintained within their commercial boxes at the University of California, Riverside's Entomology Building. Colonies were stored at 23 °C and 60–70% relative humidity (r.h.) under constant darkness or occasional red light, which bees cannot see. Individual adult callow queens < 24 h old ($n = 62$), identified by their silvery appearance and inability to fly, were removed from natal colonies. These queens were then placed in individual cups (Hi-Plas plastic container, 7.5 cm in diameter) and fed *ad libitum* with mixed-source honey bee-collected pollen (Koppert Biological Systems) and artificial nectar following the recipe in Boyle et al. (Boyle et al., 2018). At age three days, queens were placed daily in mating cages (BugDorm-6E620 Insect Rearing Cage, 60 × 60 × 120 cm) between 7 and 9 h per day under constant ambient light and allowed to mate for an additional seven days (until age ten days), until they were observed copulating and were presumed to have successfully mated (Treanore et al.,

2021; Djegham et al., 1994; Baer, B. 2003). Adult males (≥ 24 h old) were procured from five natal colonies and were mated with queens from different natal colonies. While in the mating cage, all queens and males received pollen and artificial nectar, as previously described. Queens were singly mated and were not subjected further to mating cages after mating occurred. All nests produced worker offspring, indicating that all queens used in this experiment were successfully mated and were able to produce diploid (female) offspring. A subset of nests ($n = 36$) also produced male offspring (average of 2.97 males per nest). Male offspring were removed from all future offspring analyses, due to low volumes.

Following mating, queens were placed in new individual containers with pollen and artificial nectar and kept in an Invictus Drosophila Incubator maintained at 27 °C and 60% relative humidity. At adult ages 12 and 13 days, all queens were temporarily removed from the incubator and treated with CO_2 gas for 30 min. This treatment causes queens to bypass diapause (Roseler, PF. 1985) but does not otherwise change the course of nest initiation (Roseler, PF. 1985; Gurel, F and Gosterit, A. 2008; Sarro Gustilo et al., 2023; Amsalem, E and Grozinger, CM. 2017). After the second day of CO_2 treatment, queens were placed into plastic nest cages (Biobest Group USA, Inc.; dimensions: 17 cm x 17 cm x 9.5 cm) provided with pollen, a honey-bee wax-covered pollen ball (to provide readily accessible wax), and artificial nectar. These cages were transferred to the University of California, Riverside's Insectary and Quarantine Facility and kept under 24-hour darkness except for brief exposure to light when food was changed and when

photographs were taken. At this stage and for the remainder of the experiment, the nests were maintained at 28 °C and 60% r.h. Across the experiment, pollen was replenished every 5–7 days and the artificial nectar solution was replaced every two weeks to prevent spoilage. A total of 12 queens were removed from this experiment because they either died before initiating a nest ($n = 8$) or the first day of egg-laying was not observed ($n = 4$).

Nest monitoring for eggs

Nests were visually inspected once daily for the presence of eggs and any new eggs observed in nests were assigned to the day they were observed. When queens lay eggs at the earliest stage in nest development, they produce an open waxen cup that eggs are deposited into, then they close it. These egg cups appear as a small, raised mound of wax measuring approximately 6 × 5 mm and standing about 4 mm high (Alford, DV. 1971), typically located either on the pollen provision (Heinrich, B. 1979l; Sladen, FWL. 1912; Rozen et al., 2018), on a central plastic mound within each nest (in the boxes used for this experiment), or on existing brood (as colonies develop), as seen in Supplementary Fig. 1-S2.

Nest imaging and daily egg-laying rates

Beginning on the day the first egg cup was observed in a nest box, we photographed nests nearly every day for the subsequent 44 days. This timeframe encompasses the emergence of the first cohorts of workers and the period when queens likely transition from providing most brood care in the nest to predominantly egg-laying

(Woodard et al., 2013; Sarro et al., 2021; Sarro Gustilo et al., 2023; Shpigler et al., 2013). Photographs were taken between 1000 and 1200 h and individual nests were exposed to light for a limited time (10 min maximum per day) during this process. From the photographs, we counted the total number of egg cups present in the nest and noted their location. This allowed us to calculate the daily increase in egg cups within each nest, accounting for the egg cup rearrangement that bumble bees sometimes do. Occasionally, nests were not photographed on a given day, but these gaps were never longer than three days when this happened (< 10 instances), except for only one nest where no photos were taken during the last four days of the experiment (42–45), but the number of egg cups was still noted visually. When days were missed, we averaged the total new egg cups equally across days to provide an approximate number of new egg cups per day. In some of our early nests, we noted instances of egg cup destruction either through egg pulling or egg eating by the queen. If queens appeared to restart their nests (noted by the disappearances of the first few cups and subsequent appearance of egg cups in a new location) nest initiation was dated as the first new day of egg-laying.

Experiment 1b: patterns of egg-laying in additional bumble bee species

We used a similar methodology to Experiment 1a (but simplified) to perform a small observational study examining egg laying in a set of queens of additional, opportunistically collected species: *Bombus vosnesenskii*, $n = 6$, *Bombus mckayi*, $n = 2$, *Bombus frigidus*, $n = 1$ (see Fig. 1-1; Table 1-1.). This was done to validate (yes/no) whether evidence for the pause (see definition below) can be found in species outside of

B. impatiens. In this observational study, we only tracked queen egg laying until queens resumed laying after the pause. *B. vosnesenskii* queens were hand-netted in the wild from the San Bernardino National Forest in California. After collection, queens were kept cool on ice during transport and maintained at the University of California, Riverside's Insectary and Quarantine Facility using methods similar to those previously described for *B. impatiens*. *B. mckayi* and *B. frigidus* queens were collected from Anchorage, Alaska and reared using similar methods at the University of Alaska Anchorage. Queens were monitored daily until the presence of the first egg cup in the nest was recorded, then egg laying was subsequently monitored only until queens paused, then resumed egg laying.

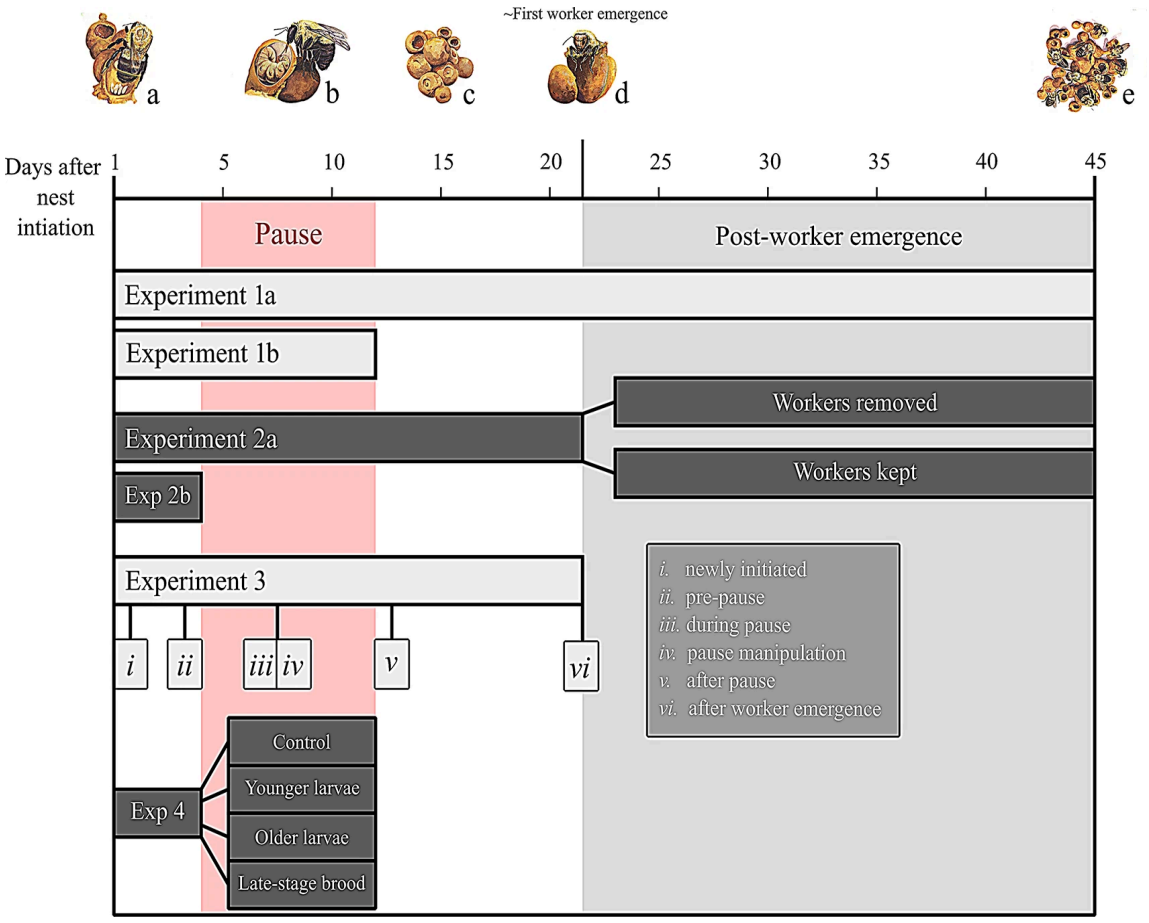

Figure 1-1. Experimental timeline.
Parts a-e of the figure refer to different colony developmental stages: a) nest initiation, b) pause c) reinitiation d) first worker emergence e) late-colony development

Table 1-1. Summary of experiments examining queen egg-laying dynamics and nest development.

N refers to the number of queens or nests involved in the experiments. See Fig. 1-1 for more details

Phenomenon	**Experiment**	**Question**	***N*=**	**Response**
Dynamic patterns of queen egg-laying across early nesting period	1a	5-day intervals and patterns of egg-laying	62	number of egg cups
Dynamic patterns of queen egg-laying across early nesting period	3	Proportion of ovaries resorbed in relation to treatment	62	proportion of ovaries resorbed
Influence of brood on queen egg-laying	4	Brood addition and timing of re-initiation	30	number of days until re-initiation
Influence of brood on queen egg-laying	1a	Differences between brood type in nests (pause entry)	62	oldest brood type present in nest
Influence of brood on queen egg-laying	1a	Differences between brood type in nests (pause exit)	62	oldest brood type present in nest
Association between the pause and nest developmental characteristics	1a	Length of the pause and number of days until the first worker emergence	62	number of days until the first brood emergence
Association between the pause and nest developmental characteristics	1a	Length of the pause and number of workers within one week	62	number of workers within one week
Association between the pause and nest developmental characteristics	1a	Length of the pause and the body size of all workers after emergence	62	body size of workers after emerging
Queen egg-laying is also under the control of adult workers in the nest	2a, 2b	Differences in the number of egg clutches after worker emergence	30	total number of egg cups
Queen egg-laying is also under the control of adult workers in the nest	2a	Number of brood in nests and presence/absence of workers in the nest	30	total number of brood

Experiment 2a: impact of adult workers on queen egg laying

Following the same methods as above (for Experiment 1a) for mating, housing, nest imaging/monitoring, and quantifying egg laying, we examined if the presence of workers is necessary for sustained reproductive acceleration in queens, and whether it supports nest development (see Fig. 1-1; Table 1-1). A total of 30 queens from four natal colonies were used for this experiment and separated into two treatment groups: eclosed workers kept in their nest (worker+, $n = 10$) and eclosed workers removed from their nests (worker-, $n = 20$). For the worker- group, all newly-eclosed adult offspring were removed on the day of eclosion and stored at -80 C. In this experiment, we assume that any eggs after worker emergence in the nest were laid by the queen. This is because, at the earliest stages of nest development, which we examined here, queens strongly inhibit worker egg-laying (Duchateau, MJ and Velthuis, HHW, 1998; Starkey et al., 2019). Moreover, we did not see an increase in the number of egg cups in our worker + nests following the emergence of workers beyond that of the observed capacity of queens in the beginning of the experiment, which would be predicted if workers were laying eggs in the nests.

Experiment 2b: eggs per cup before pause

We used an additional separate, smaller set of *B. impatiens* queens ($n = 10$) to determine how many eggs are present within egg cups (which requires destructive sampling) at the onset of nest initiation. Queens were collected from five natal colonies and mated with males from four natal colonies. Otherwise, we used similar rearing

methods as described above but collected queens four days after the first eggs were observed in the nest (see Fig. 1-1; Table 1-1). Queens were frozen for at least 48 h before dissections. We then dissected the nests and counted the number of eggs within each egg cup. These data were used to understand how the number of eggs per cup differs between the earliest and latest stages of nest development (based on a comparison to data from queens in Experiment 2a). Additionally, we used these data to explore whether the number of eggs per cup is influenced by whether workers are left in nests (worker+) or removed on the day of their emergence (worker-).

Nest dissections

For experiments examining nest development and brood (Experiments 2, 3, and 4), the nests were frozen and subsequently dissected on dry ice. The total number of egg cups and brood were counted. We used visual inspections of brood to divide each individual into four different categories corresponding to one or more stages characterized by Rozen et al. (Rozen et al., 2018): (i) eggs; (ii) younger larvae (corresponding to 1st and 2nd instars, housed in communal chambers and fed as a group); (iii) older larvae (corresponding to 3rd and the majority of the 4th instar, larvae begin spinning a cocoon); and (iv) and late-stage brood (which includes the post-defecation stage of the fourth instar, prepupae, and pupae). We followed this same characterization for the brood addition experiment (see below, Experiments 3 and 4) to determine the oldest brood type at re-initiation.

Body size measurements

We measured the body size of all queens and adult worker offspring in all experiments using the Minitool Measuring Scale (in mm). The length of the second marginal cell of both wings was measured and averaged for each bee as a proxy for body size. In bumble bees, the second marginal cell is highly correlated with overall body size (Medler, JT., 1962).

Experiment 3: ovarian development and resorption across nest development

To examine how patterns of ovarian resorption and development change across early nest development, a set of queens ($n = 73$) were collected from five natal colonies and mated with males from six natal colonies, and reared using the same methods as outlined above. Of these original 73 queens, 11 died or failed to initiate within an appropriate timeframe (within a ~ 2-month period) and were removed from the experiment. The remaining 62 queens were then distributed as equally as possible (with respect to their natal colony) among the following six treatment groups. The six treatments included: (i) *newly initiated* (collected on the second day of egg-laying after initiation), (ii) *pre-pause* (collected the day before queens entered the pause period, i.e., the fourth day after nest initiation; timing determined based on data from our primary experiment to determine an appropriate time for this treatment); (iii) *during pause* (collected three days into the pause, also based on data from the primary experiment); (iv) *pause manipulation* (a combination of older larvae and pupae brood were added in clumps of ~ 3–7 brood items four days into the "pause" period) (see Fig. 1; Table 1). The

pause manipulation treatment group was designed to test whether queens develop their ovaries prematurely during the pause if brood are introduced; more information about this methodology and its rationale is provided below; (v) *after pause* (collected five days after queens re-initiating egg-laying) and (vi) *after worker emergence* (collected 10 days after the first worker emerged). Queens were collected on dry ice to preserve tissue integrity for ovary dissections.

Two aspects of ovarian development were considered for all queens ($n = 62$): levels of resorption and lengths of the terminal oocytes. Ovary resorption status (resorbed or unresorbed) was qualitatively scored for each of the eight terminal oocytes. Resorbed oocytes are identifiable by their yellow coloring and misshapen appearance (Duchateau, MJ and Velthuis, HHW. 1989) and were removed from oocyte length analyses because resorption can result in misshapen oocytes with unreliable length measurements. We then measured the lengths of the eight terminal, unresorbed oocytes using methods such as Duchateau and Velthuis (Duchateau, MJ and Velthuis, HHW. 1989) and Medler (Medler, JT., 1962).

Experiment 4: impact of artificial brood addition on queen egg-laying

A set of 48 queens (from two natal colonies, mated with males from seven colonies) was used to test whether the addition of brood to nests influences queen egg-laying during the pause. These queens were mated similarly as described above and were allowed to initiate egg laying and lay eggs until they entered the pause. Cessation of

egg laying was visually confirmed for each queen, and they all entered the pause ~ 4–5 days later, similar to the timeframe observed in our primary experiment. Three days after entering the pause, brood was artificially added apart from the control group (see Fig. 1-1; Table 1-1). Of the initial 48 queens, 13 were removed from the dataset due to mortality before entering the pause, resulting in a final sample size of 35 queens for this experiment. These 35 queens were distributed into one of four treatment groups: (i) *control* (unmanipulated nests, no brood added; $n = 9$); (ii) *younger larvae added* ($n = 5$); (iii) *older larvae added* ($n = 10$); (iv) *late-stage brood added* ($n = 11$). For the brood addition treatment groups, clusters of worker brood, consisting of an estimated five individuals (range: 2–8), were carefully removed from a separate set of worker-producing colonies ($n = 6$) and placed in queen nests. After the manipulation, queens were monitored daily, noting the date of egg-laying reinitiation. Queens and their nests were collected after queens re-initiated egg laying. We dissected all nests at the end of the experiment to retroactively determine how much brood was added to each nest. Here, we used nest dissection methods similar to those described above for Experiment 2B.

Statistical analysis

All statistical analyses were performed in R version 4.3.1 (R Core Team, 2023) and only p-values < 0.05 were considered significant. All data were non-normally distributed based on Shapiro-Wilk tests. For most of our analyses, we used generalized linear mixed models (GLMMs) to explore how different factors contributed to our experimental responses. Otherwise, chi-squared tests (to explore the influence of brood

on queen egg laying) or Fisher's Exact tests (to explore the influence of worker presence/absence on the number of eggs per cup and the total eggs at the end of the experiment) were used. We included the timing of certain nest development events (e.g., number of days before new egg cups are present), colony developmental stage, various treatments (e.g., worker presence/absence), brood type, and the duration of pause as possible fixed effects. Queen natal colony was always included in all models as a random effect. When biologically relevant (e.g., for analyses exploring ovarian resorption), body size and queen ID (nested random effect) were also included as random effects in models. GLMMs were performed with the glmmTMB functions in the R package glmmTMB (Brooks et al., 2017). We tested all possible models for each response variable based on all additive and interactive combinations of fixed effects while holding random effects constant. The best-fit model for our data was selected based on Akaike's Information Criterion (AIC) using the "model.sel" command within the MUMIn package (Barton, K. 2024). Following model selection, a one-way analysis of variance (ANOVA) was used to compare the data, and an Estimated Marginal Means (emmeans) post-hoc was performed to create pairwise comparisons. See Supplementary materials for more information regarding model selection (Table 1-S1.), best-fit model descriptions, and details regarding data selection.

For Experiment 1a, we calculated an average number of new egg cups per day across 45 consecutive days for all queens in the experiment ($n = 62$). To examine queen egg laying patterns across time, and to look for a period of cessation of egg laying, total

new egg clutches were concatenated for individual queens in 5-day intervals (days 1–5, 6–10, 11–15, 16–20, and 21–25). We also related the duration of each queen's pause to the following nest developmental characteristics: (i) the number of days until the first adult worker emerged in the nest (total range observed: 18–29 days); (ii) the total number of workers to emerge over a 7-day period, which likely represents the first cohort to emerge (Costa et al., 2021) (total range observed: 1–7 days); and (iii) the body sizes of all adult workers to emerge from the nest (total range observed: 1.45–3.0 mm; size was based on average of wing marginal cells as described above).

To analyze if worker presence is necessary for sustained and accelerated egg-laying (Experiment 2), we compared the accumulation of eggs for fifteen days post-worker emergence between the two treatments: (worker presence, worker+, $n = 10$) and worker absence (worker-, $n = 20$). For this analysis, queens were aligned such that day one was the first day workers were noted per individual nest, as the actual day of actual first worker emergence (range day 19–27 out of 45) varied per individual queen. The fifteen-day period post-worker emergence was determined based on this alignment and egg accumulation was determined in three five-day sequential segments across this period. According to the AIC score, the best-fit model included the additive interaction between the presence/absence of workers and the number of days after the first worker emergence. The second-best model (an interactive effect with an AIC score different by two) was chosen for this analysis because there is not likely to be a biologically relevant

additive interaction between presence/absence of workers and the number of days after the first worker emergence.

For Experiment 3, we examined the terminal oocyte length (using only non-resorbed ovaries) and resorption status for all queens used in the experiment. There was no difference in terminal oocyte length and thus was not included in the remaining analyses (see Supplementary, Fig. 1-S3).

For Experiment 4, all queens within the younger larvae group rejected the brood added to their nests, thus the five *younger larvae added* nests were excluded from the subsequent analyses. With the remaining queens, we used the number of days until egg-laying was re-initiated as our response variable. In our statistical analysis, we considered the amount of brood added to nests as a factor in our model selection, but the best-fit model excluded this factor, and thus only treatment was included in the analysis; see Supplementary Material.

For more information about the analyses completed for the manuscript, see Supplementary material, Table 1-S2.

Results

Dynamic patterns of queen egg laying across the early nesting period

All *B. impatiens* queens in Experiment 1a ($n = 62$) began laying eggs within an average of 28.18 ± 1.92 standard error of the mean (s.e.m) days. In all queens, the onset of egg laying was followed by a complete cessation in egg laying that began, on average, 4.21 ± 0.14 s.e.m. days after their first eggs were observed in the nest (Fig. 1-2). This cessation in egg laying lasted, on average, 7.74 ± 0.19 s.e.m. days, with a minimum length of four days and a maximum of 11 days, and always occurred between days 2–13 of the experiment. Hereafter, we refer to this cessation as a "pause", defined as a complete cessation of egg-laying for two or more consecutive days. We define exiting the pause as then re-initiating egg laying for two or more consecutive days. When egg laying was compared across discrete five-day intervals before worker emergence, the lowest amount of egg laying occurred during the pause (which fell within the 6–10 day period; Fig. 1-3; GLMM $\chi^2 = 178.78$, d.f. = 4, $p < 0.001$). During the interval that we refer to in this experiment as the pause period, as it best coincided with when most queens were in the pause, queens produced an average of 0.44 +/- 0.10 s.e.m. egg cups. This small number of eggs is likely due to the natural variation in the start and cessation of the pause, where the timing of the egg-laying period is variable across queens. There were also secondary declines in egg laying that occurred in the periods of days 11–15 (4.42 +/- 0.30 s.e.m. egg cups) and 21–25 (2.52 +/- 0.31 s.e.m. egg cups), but there was no complete cessation of egg laying observed in any queen other than during the pause period. During the first five-day interval (days 1–5), there was an average of 6.79 +/-0.26

s.e.m. new egg cups present, and during days 16–20, there was an average of 5.53 +/- 0.31 s.e.m. new egg cups. 100% of queens from the three other species that we observed (*B. vosnesenskii*, *B. mckayi*, *B. frigidus*) also exhibited the pause.

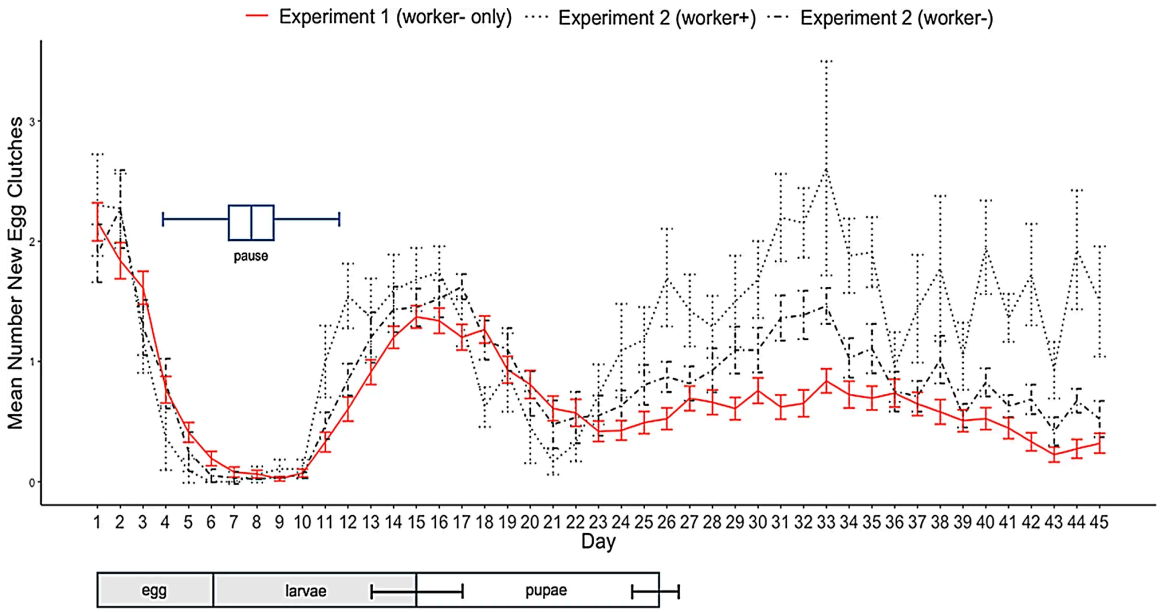

Figure 1-2. Differences in queen egg laying across five-day sequential periods in Experiment 1a.

Figure shows, across the 45-day experiment (X-axis), the daily mean (+/- s.e.m.) number of new egg cups present in nests (Y-axis) for all queens. Queens are separated into two lines according to the experiments: Experiment 1a data are shown in red, $n = 62$; Experiment 2 worker + is shown with the small, dotted line, $n = 10$, and Experiment 2 worker- is shown with the dot-dashed line, $n = 20$. Regardless of the experiment, all queens had a pause period, although the timing varied. A boxplot showing the duration of the pause for Experiment 1a is shown in blue, with the minimum = 4 days (left whisker), the median = 8 days, and the maximum = 12 days (right whisker). Below, the typical development times of *Bombus impatiens* workers (re-created and modified from Cnaani et al., 2002) are shown to demonstrate how the development of the first brood in the nest relates to our queen egg-laying data

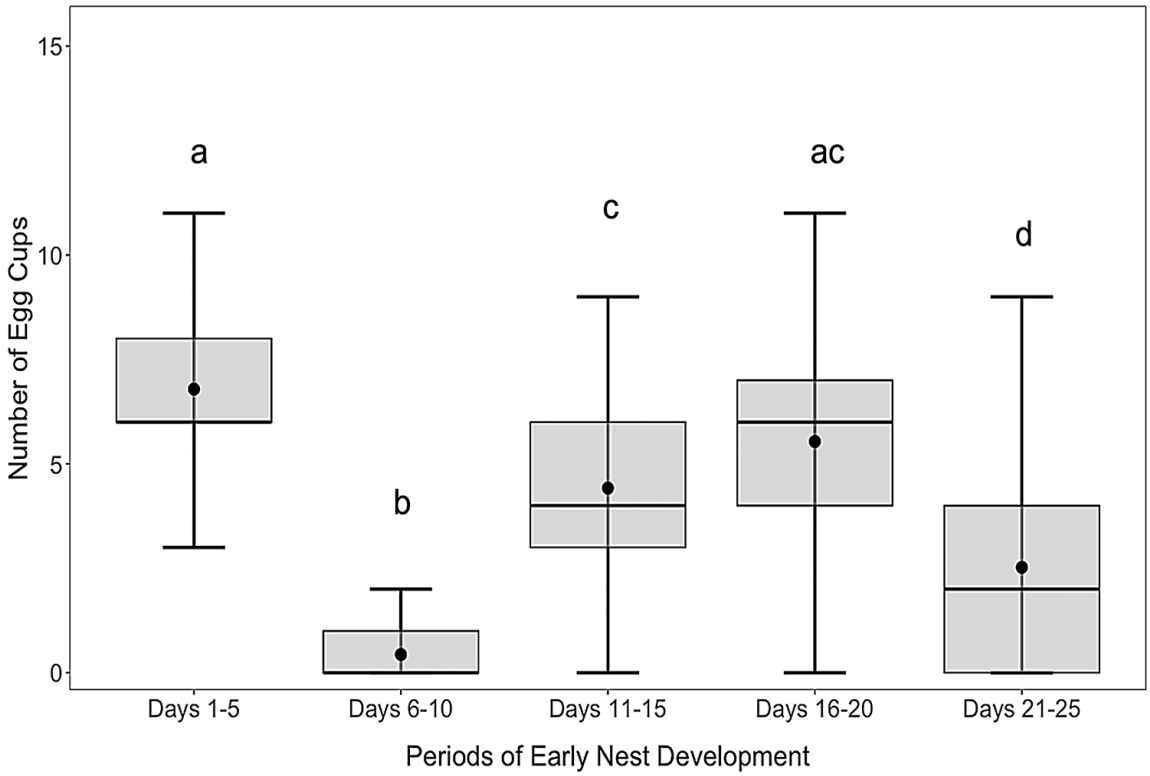

Figure 1-3. Patterns of queen egg laying across the early nesting stage (Experiments 1a and 2).

The X-axis represents five sequential, five-day periods in the experiment. The Y-axis shows the number of new egg cups observed in nests during each period. Box plots display median values (horizontal lines), interquartile ranges (boxes), and data variability (whiskers). Central dots represent means, and error bars indicate standard errors. Different letters above box plots denote statistically significant differences between periods based on an emmeans post-hoc pairwise test ($p < 0.05$)

The pattern of queen reproductive development noted in Experiment 1a (encompassing both the pause and post-worker emergence) is further reflected in our analyses for Experiments 2 and 3. We found that the number of eggs in individual egg cups, as well as queen ovarian development and ovarian resorption across these timeframes, are dynamic according to social context. Pre-pause, queens produced an average of 1.48 +/- 0.07 s.e.m. (range of 1–4; $n = 10$ queens) eggs per egg cup. At the end of the experiment, worker- queens in Experiment 2 ($n = 20$) had an average of 5.15 +/- 0.26 s.e.m eggs in each cup, whereas queens with workers in the nest ($n = 10$) had an average of 5.66 +/- 0.22 s.e.m eggs in each cup; Fisher's test to compare between treatments after 45 days, $p = 0.4$. At the end of the experiment (day 45), all queens with workers (worker+) had more egg cups (range: 1–9 cups, average = 5.6+/- 0.73 s.e.m.) than queens without any workers (worker-) (range: 1–7 cups, average = 2.45 +/- 0.49 s.e.m).

The highest ovarian resorption levels were observed immediately before and during the pause (Fig. 1-4.; GLMM $\chi^2 = 32.54$, d.f. =5, $p < 0.0001$; model included colony developmental stage). The greatest differences in the proportion of oocytes resorbed were in the *newly initiated* vs. *during pause* ($Z = -3.21$, $p = 0.0169$); *pause manipulation* vs. *during pause* ($Z = -5.18$, $p < 0.0001$), and *during pause* vs. *after pause* ($Z = 3.11$, $p = 0.0231$) (Fig. 1-4.). No other groups differed significantly from each other (*pre-pause* vs. *pause manipulation*, $Z = 2.81$, $p = 0.06$).

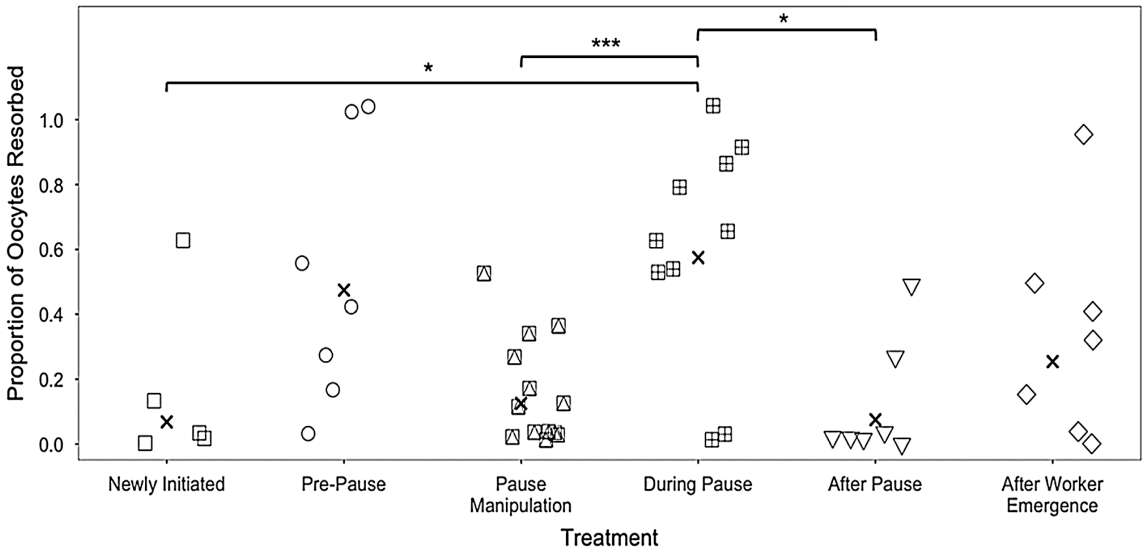

Figure 1-4. Resorption status of queen ovaries at different stages of colony development, or with brood artificially added to the nest (pause manipulation treatment) in Experiment 3 ($n = 62$).

Shaped points represent the proportions of resorbed oocytes for individual queens, jittered to better visualize overlapping points. Each column represents a treatment with the X symbol amongst the jittered points representing averages for a given treatment group. An emmeans post-hoc pairwise test was used to determine differences between treatments. Brackets indicate statistically different means, with a black star (*) above them indicating a level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Influence of brood on queen egg laying

In Experiment 1a, queens always entered the pause after they had, on average 6.40 (+/- 0.19 s.e.m.) egg cups in their nests ($n = 62$), and were more likely to enter the pause when they only had eggs in the nest (71% of queens; $n = 44$) rather than young larvae in addition to eggs (29%; $n = 18$) (pairwise chi-square test, $p < 0.001$). Exiting the pause was also associated with social conditions in the nest. When queens exited the pause, they were more likely to have late-stage brood present as the oldest developed brood in the nest (63% of queens; $n = 39$), as opposed to having older larvae (34%; $n = 21$) or younger larvae (3%; $n = 2$), and no queens had only eggs when they exited the pause (Fig. 1-5A).

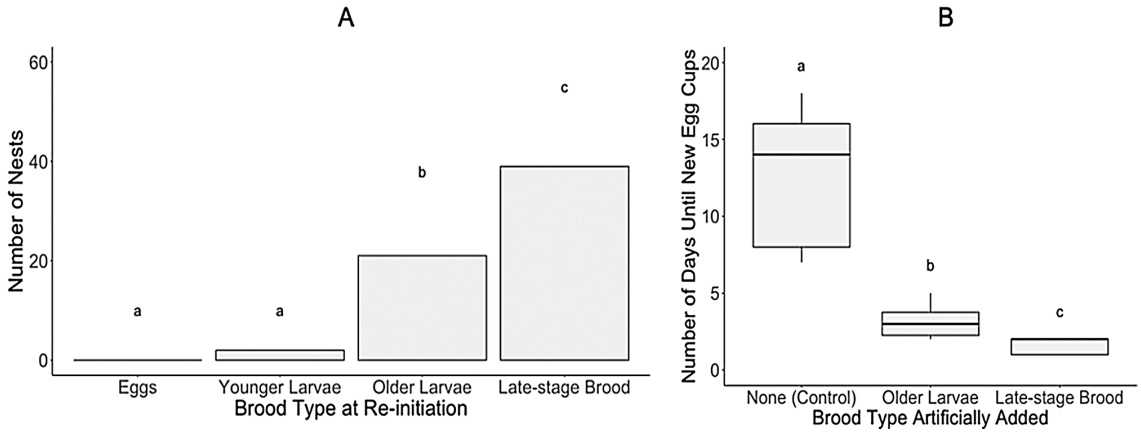

Figure 1-5. Influence of brood on queen egg-laying behavior. Part A (Experiment 1a).

The oldest brood in the nest when queens exited the pause (i.e., re-initiated egg-laying), based on our first experiment ($n = 62$ queens). X-axis, the oldest brood type present in the nest at re-initiation; Y-axis, the number of nests. Part B (Experiment 4): Number of days until re-initiation of egg-laying in brood addition experiment ($n = 30$ queens). X-axis, brood type artificially added; Y-axis, the number of days between brood addition and the first day new eggs were observed in the nest. Means and standard error are shown. The letters represent significant differences between treatment groups based on emmeans pairwise comparison for both parts A and B of the figure.

In our brood addition experiment (Experiment 4), queens took less time to re-initiate egg laying when we artificially added either *older larvae* ($n = 10$) or *late-stage brood* ($n = 11$) to the nests during the pause, relative to the *control* queens where no brood were added ($n = 9$) (GLMM $\chi^2 = 96.516$, d.f. = 2, $p < 0.001$). Queens re-initiated within an average of 1.55 +/- 0.16 s.e.m days when late-stage brood were added, and within an average of 3.1 +/- 0.31 s.e.m days when older larvae were added, as compared to unmanipulated (control) queens, which took 12.5 +/- 1.46 s.e.m. days to reinitiate (Fig. 1-5B). Post-hoc pairwise comparisons using emmeans revealed significant differences among all groups: *control* versus *older larvae* ($Z = 6.70$, $p < 0.001$), *control* versus *late-stage* brood ($Z = 7.96$, $p < 0.001$), and *older larvae* versus *late-stage brood* ($Z = 4.36$, $p < 0.001$).) Regardless of what stage brood was added to nests, the number of days until re-initiation was four times faster for manipulated queens than queens in the control treatment. In all cases where older larvae (third instar and/or pre-defecation fourth instar) were added to the nests, these larvae had developed into late-stage brood (post-defecation fourth instar, prepupae, and/or pupae) by the time the queens re-initiated brood care.

Association between the pause and nest developmental characteristics

All queens invariably paused in our study (see Fig. 1-3), so we examined whether the pause duration (which did vary; range of 4–11 days across all queens) was associated with any nest developmental characteristics using queens from Experiment 1a. There was a strong positive relationship between the length of the pause and the duration of time until the first worker emergence (GLMM $\chi^2 = 16.12$, d.f. =1, $p < 0.001$). We did not find

any association between the duration of the pause and either the number of workers to emerge within the first week (GLMM $\chi^2 = 0.9273$, d.f. = 1, $p = 0.3356$) or the body size of all workers produced by nests (GLMM $\chi^2 = 0.0024$, d.f. =1, $p = 0.9609$).

Queen egg-laying is sustained by adult workers in the nest

Lastly, in Experiment 2, we asked whether workers that emerge in young nests are required for queens to maintain high levels of egg laying by allowing workers to remain in some nests ($n = 10$; worker+) or removing them on the day each worker emerged ($n = 20$; worker-). On average, workers emerged 22.23 +/ 0.38 s.e.m. days after the first eggs were noted in the nest. Queens with workers present in the nest (worker+) had more egg cups compared with those that had no workers (workers-) at later intervals, with the greatest differences occurring 6–10 days (worker + average = 8.92 +/-0.96 s.e.m., worker- average = 4.19 +/-0.59 s.e.m.; emmeans pairwise comparison, Z = 3.80, $p = 0.001$) and 11–15 (worker + average = 9.38 +/-1.51 s.e.m., worker- average = 3.81 +/-0.44 s.e.m.; emmeans pairwise comparison, Z = 4.78, $p < 0.001$) days after the first worker emergence (Fig. 1-6). During the first five days (1–5 days) after worker emergence, the number of egg cups did not differ between the two groups (worker + average = 2.78+/-0.63 s.e.m., worker- average = 2.17 +/-0.39 s.e.m.; emmeans pairwise comparison, Z = 0.91, $p = 0.361$). Independently, the timing ($\chi^2 = 25.25$, df = 2, p-value < 0.001) and treatment ($\chi^2 = 34.40$, df = 1, p-value < 0.0001) were significant factors in the GLMM, but the interaction between the two factors was not.

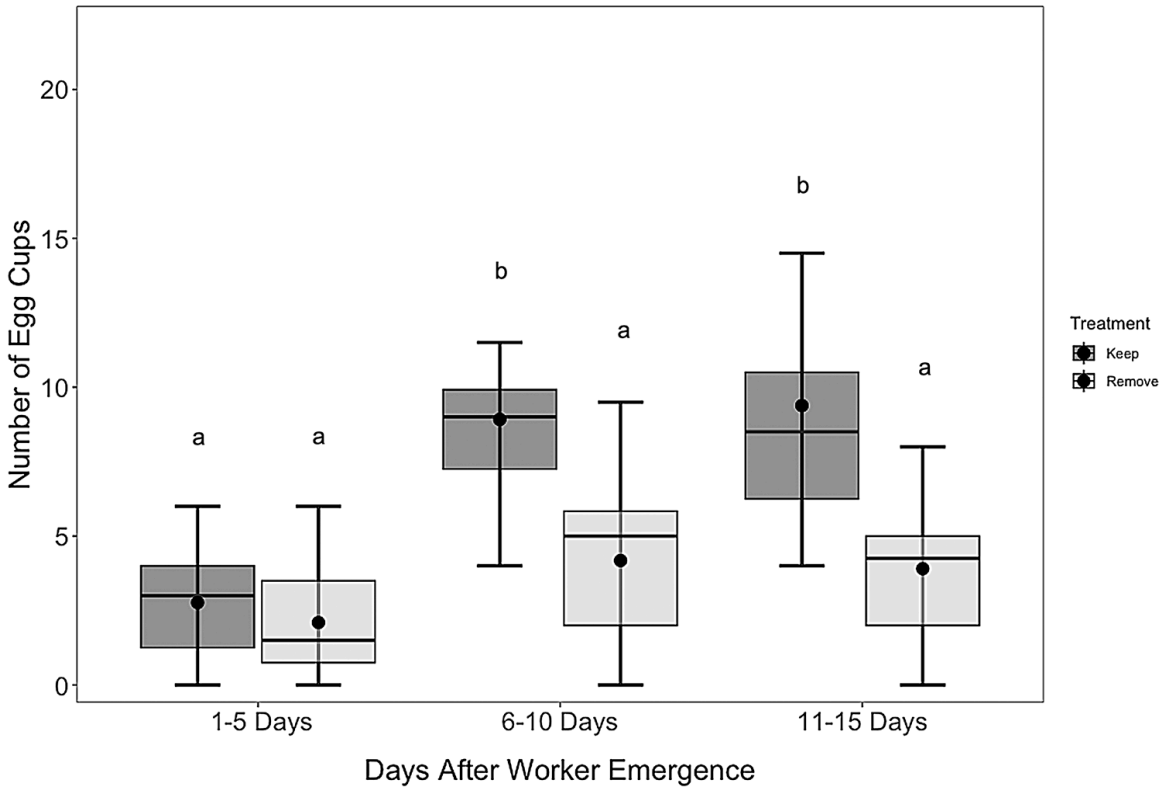

Figure 1-6. Differences in the number of egg cups between three distinct periods after worker emergence (Experiment 2).

Data are presented for queens with workers present (worker+, $n = 10$) and nests with workers removed (worker-, $n = 20$). X-axis, time periods in five-day intervals; Y-axis, the number of new egg cups in the nest. Box plots display median values (horizontal lines), interquartile ranges (boxes), and data variability (whiskers). Central dots represent means, and error bars indicate standard errors. An emmeans post-hoc pairwise test was used to determine differences between the number of egg cups between treatment types (worker + vs. worker-) and between the five-day intervals. Different letters above box plots denote statistically significant differences between periods based on an emmeans post-hoc pairwise test ($p < 0.05$)

The total number of brood (regardless of stage, including eggs, younger larvae, older larvae, and late-stage brood) at the end of Experiment 2 was also positively associated with the presence of workers. The average number of total brood, regardless of stage, in the nests of queens with workers was 126.9 +/- 12.92 s.e.m., relative to an average of 35.26 +/- 4.75 s.e.m. for nests with workers removed (GLMM: $\chi^2 = 470.55$, d = 1 $p < 0.001$).

Discussion

We demonstrated that in bumble bees, queen reproduction is flexible such that egg-laying accelerates and decelerates with changes in social conditions across nest development. Thus, social conditions prove to be a powerful influence on the pace of queen egg laying and overall nest growth. We demonstrated that both developing brood and adult workers socially control queen egg laying in *B. impatiens*, building on previous work (Woodard et al., 2013; Sarro et al., 2021; Shpigler et al., 2013; Cnanni et al., 2002; Robinson, GE. 1992; Ratnieks et al., 2006).

In contrast to the hyper-reproductivity observed in some eusocial systems, including fire ants (*Solenopsis invicta* (Tschinkel, WR. 1988a; Tschinkel,WR. 1988b), western honey bees (*Apis mellifera* (Jackson et al., 2011)) and driver ants (*Dorylus wilverthi* (Raignier, A and Boven, JKA., 1955), solitary-founding queen egg laying occurs at a slower pace (Cronin et al., 2013). In solitary-founding systems, queens are non-reproductive for much of their adult life and only transition to egg-laying around the

time that nest sites are located Cronin et al., 2013, but see Sarro et al. (Sarro et al., 2022). In bumble bees, most of which follow such an annually social lifestyle, queens only reach their full reproductive potential (i.e., maximum rate of egg-laying) after the first adult workers emerge in the nest and assume brood care (Woodard et al., 2013). We found strong evidence that in *B. impatiens*, and suggestive evidence in additional bumble bee species (albeit with limited data), queens exhibit a stereotypical pattern where they reduce their egg-laying rate for several days soon after nest initiation, then accelerate it around the time they have late-instar larvae or pupae in the nest. This pattern, which we refer to as the "pause," has been reported by previous bumble bee researchers (Heinrich, B., 1979; Sladen, FWL., 1912; Alford, DV., 1971) but has not been fully formally characterized or studied. The function and significance of the pause for queens and their nests are not currently known, but we propose that it allows queens to time their accelerated egg laying so that it coincides with the emergence of the first worker brood, who assume brood care duties in nests. If true, then the pause is a life history strategy that allows queens to match their reproductive timing to social conditions in the nest. This strategy might allow queens to minimize their performance of brood care and ultimately maximize egg-laying and nesting success.

We also found novel evidence that workers are required for queens to sustain higher rates of egg-laying beyond the earliest stages of nest initiation. Egg-laying rates in bumble bee queens never reach the same magnitude as egg-laying as in the aforementioned hyper-reproductive systems, but at the height of colony development

queens can lay several cohorts of brood day and ultimately produce up to several hundred offspring in their nests (Sladen, FWL., 1912; Husband, RW., 1977; Bourke, AF., 1997). In many animal systems, the number of subordinate individuals is positively associated with the number of offspring that the dominant or primary reproductive can produce (e.g., honey bees (Smith et al., 2017), *Myrmica* ants (Brian, MV., 1957); fire ants (Tschinkel, WR., 1988); bumble bees (Sladen, FWL., 1912); meerkats (Russell et al., 2003)). With an increasing number of individuals available to perform care-related duties and foraging, the dominant or primarily reproductive individual is hypothesized to be able to specialize more fully in reproduction. Our work demonstrates that the switch to hyper-reproductivity in bumble bee queens is not permanent but instead is plastic, based on the presence of workers in the nest, or as seen previously, depending on the number of workers in the nest (Shpigler et al., 2013).

Two types of social group members positively influenced queen egg laying in our study: late-stage brood (older larvae and pupae) and adult workers. We note that younger brood may also positively impact queen egg laying, as we were not able to include them in our brood manipulation experiment; yet we found in our unmanipulated queens that only older brood were associated with resumed egg laying following the pause, suggesting that younger brood do not have an influence. Older brood and adult workers both reflect helpers in the nest, although they differ in whether they indirectly predict help in the nest (late-stage brood, which will eclose on the order of days and soon take on brood care tasks) or are a more direct signal of help (adult workers). Prior studies have

shown that bumble bee queen egg-laying can be induced prematurely by adding a variety of group members to the nest. This includes adult workers (Woodard et al., 2013; Sarro et al., 2021), pupae (Kwon et al., 2003; Kwon et al., 2006; Bucankova, A and Ptacek, V., 2012; Yoneda, M. (2008), conspecific queens (Plowright, RC and Jay, SC. 1966; Alford, DV. 1975; Duchateau, MJ., 1985; Ptacek, V. 1985; Ono et al., 1994), workers of different bumble bee species (Ptacek, V. 1985; Ono et al., 1994), and even honey bees (Stange, JP. 2010). The wide range of individuals and associated stimuli previously described suggests that queens are highly sensitive to a multitude of cues, some of which may be indicative of help or the potential of help in the nest.

Queens may be particularly sensitive to social cues from brood in the subsocial stage, as they interact closely with brood and bear the initial burden of carrying out care for brood in the nest. There are precedents for brood control over social dynamics in social insects, including queen bumble bees. In social Hymenoptera, brood pheromones regulate and elicit a broad range of behaviors related to reproduction and brood care (Orlova, M. and Amsalem, E. 2019). In honey bees, larvae produce brood pheromone, which has a broad range of physiological and behavioral effects on workers. These effects include inducing worker foraging by reducing juvenile hormone production (Le Conte at al., 2001; Pankiw, T. 2004) and reducing egg laying by inhibiting ovarian development (Arnold et al., 1994). In bumble bees, larvae can emit hunger signals that indicate their nutritional status and release brood-feeding behavior by workers in the nest (Smeets, P. and Duchateau, MJ., 2001; de Boer, SPA and Ducheateau, MJHM., (2006)).

Brood in young bumble bee nests can also suppress circadian rhythmicity in nest-founding queens, resulting in queens performing brood care across a 24-hour cycle (Eban-Rothschild et al., 2011). A recurring theme from these studies is that brood can alter traits in caregivers in context-dependent ways that support social cohesion and the growth of social colonies.

We also found evidence that queens reduce their egg-laying rate and begin to resorb their oocytes, right at the time that their first brood requires the most intensive and consistent need for care (i.e., when their first brood have reached the larval stage). The pause has been previously noted by Alford (Alford, DV., 1971), who stated that after the first cohort of eggs are laid in bumble bee nests, additional egg cups are not typically observed until the first offspring have reached the pupal stage. Yet, neither Alford, nor other early bumble bee researchers who also noted this pattern (Heinrich, B., 1979; Sladen, FWL., 1912), articulated its significance for queens and their young nests. The pause seems to involve both a cessation of egg laying and ovarian resorption, and we did not see similar resorption rates to the same degree outside of the pause timeframe. The proximate mechanisms causing queens to temporarily halt (and even reverse) their ovarian development and egg-laying are unclear. Egg laying is limited by food availability and nutrition in many animal systems, including birds (Nilsson, J-A and Svensson, E., 1993; Clifford, LD and Anderson, DJ., 2001; Martin, TE., 1987), fish (Brooks et al., 1997; Torsabo et al., 2002; Volkoff, H., 2018), amphibians (Girish, S. and Saidapur, SK., 2000; Scott, DE and Fore, MR., 1995), ants (Kipyatkov, VE and Lopatina,

EB., 1997), and also in other bee systems (Mattila et al., 2001), including solitary (Cane, JH., 2016) and social (stingless bees) (Roubik, DW., 1982) species. Queens were well-fed in our experiments, so some non-nutritional cue is likely driving this stereotypical pattern. The driver or cue may be either internal to queens (for example, age-related changes in reproductive hormone production) or a social cue derived from very young brood (eggs or early instar larvae), as these are the only other social group members at this earliest stage in nest development. Ovarian resorption was highest during the pause in our study, which suggests that queens are reallocating resources away from ovarian development and toward other processes during this period (Ohgushi, 1996; Koch and Meunier, 2014; Nozaki and Matsuura,, 2021; Williams, 2005). Perhaps, as egg production can be costly, bumble bee queens may need to redirect resources to other processes during the pause, such as brood care (Monaghan et al., 1998). Under natural conditions, the pause may also function to allow queens to direct more resources towards stressors, such as nutritional stress, parasites and/or pathogens, and pesticide exposure, rather than in egg production. These factors are known to play a crucial role in early nesting development (Watrous et al., 2019; Mullins et al., 2020; Gustilo et al., 2025).

All *B. impatiens* queens in our experiment performed the pause, with only the duration of the pause varying across the queens. The longer that *B. impatiens* queens paused in our study, the later their first workers emerged. The reason for this relationship is unclear, but it is generally consistent with a previous study in *B. impatiens* showing that queen brood care can, counterintuitively, delay offspring development times (Costa

et al., 2021), although an opposite pattern has been observed in *B. terrestris* (Shpigler et al., 2013). We also observed the pause in a limited data set for the closely related *Bombus vosnesenskii* ($n = 6$) and an even smaller set of additional species (*Bombus mckayi*, $n = 2$; *Bombus frigidus*, $n = 1$), and it has been mentioned more broadly in the literature and by bumble bee biologists (see Supplementary Material). We propose, therefore, that the pause does serve some purpose for young nests, which may be specifically related to how bumble bee queens balance brood care and egg laying during nest establishment.

Conclusion

We provide further evidence that bumble bee nests, even at their most nascent developmental stage, contain multiple mechanisms of social regulation that coordinate the reproductive status and activities of social group members. We also present novel empirical data that queen egg laying does not follow a steadily increasing pattern, and instead, there are periods wherein queens reduce their egg laying that may relate to conditions within the colony. This is counter to the prevailing framework that social insect queens are continuously hyper-reproductive, which is true in some systems and at some colony developmental stages, but is not all-encompassing. Our work on the pause was inspired by early natural history descriptions of bumble bee nesting biology (Heinrich, B., 1979; Sladen, FWL., 1912; Alford, DV., 1971), demonstrating the value of detailed organismal observations as the foundation for more comprehensive studies. Our findings shed light on a life stage (solitary nest-founding) that is extremely under-studied in bumble bees and other eusocial insect systems. This early nesting stage may be

especially important for population dynamics in solitary-founding species, as it is compulsory to the overall success of the colony. Social control of queen egg laying may allow queens to more fully realize both personal and nest fitness. We propose that complex mechanisms have evolved that support nesting success by placing queen reproduction at least partially under the control of social group members.

Supporting information

Best-fitting models:

Table 1-S1. Patterns of egg-laying across 5-day intervals			
Model	TM	df	AIC
Null	NA	4	1244.4
eKRE1	+	**8**	**1465.1**

Included TM, i.e., timing of nest development and source colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 1-S2. Resorption of ovaries			
Model	**T**	**df**	**AIC**
Null	NA	6	95.8
OVQ1	+	**11**	**78.2**

Included T, i.e., treatment of queen nests (when artificial brood addition occurred) as fixed factors and source and queen body size as random factors. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 1-S3. Length of non-resorbed terminal oocytes			
Model	**T**	**df**	**AIC**
Null	**NA**	**4**	**1037.5**
OVresorbN1	+	9	1041.7

Included T, i.e., treatment of queen nests (when artificial brood addition occurred) and source and queen body size as random factors. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 1-S4. Artificial brood manipulation					
Model	T	B	T:B	df	AIC
Null	NA	NA	NA	3	166.3
MRR1s	**+**	**NA**	**NA**	**5**	**106.4**
MRR2s	NA	NA	+	5	124.5
MRR3s	NA	NA	*	6	127.5
MRR4s	NA	+	NA	4	147.2

Included T i.e. Treatment type (type of brood added), B i.e. Number of brood added as fixed factors and source colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 1-S5. Length of pause and first worker emergence			
Model	**L**	**df**	**AIC**
Null	NA	4	224.4
F1	**+**	**5**	**235.6**

Included L i.e. Length of the pause and source colony for queens as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 1-S6. Total # of workers within first week			
Model	**L**	**df**	**AIC**
Null	**NA**	**4**	**204.6**
KWQ1	+	5	207.0

Included L i.e length of the pause and source colony for queens as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 1-S7. Worker body size and length of pause			
Model	**L**	**df**	**AIC**
Null	**NA**	**4**	**24.8**
WB1	+	5	26.9

Included L i.e length of the pause and source colony for queens as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 1-S8. Number of egg clutches post-worker emergence					
Model	**T**	**D**	**T:D**	**df**	**AIC**
Null	NA	NA	NA	4	464.5
MegKRETime1	+	NA	NA	5	445.3
MegKRETime2	NA	+	NA	6	446.5
MegKRETime3	**NA**	**NA**	**+**	**7**	**421.1**
MegKRETime4	NA	NA	*	9	423.1

Included T i.e. treatment (worker-/worker+) in nest, D, timing of nest development (# days after the first worker emergence) and source colony for queens as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.
Note: As an additive interaction may not be biologically relevant, the next best fit model (MegKRETime4) was used for the analysis.

Table 1-S9. Total # brood and worker presence/absence			
Model	**T**	**df**	**AIC**
Null	NA	2	1069.5
TotBrood1	+	**3**	**484.7**

Included T i.e. treatment (worker-/worker+) in nest and source colony for queens as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Pause in other species:

We observed three other species (*B. vosnesenskii*, *B. mckayi*, *B. frigidus*) to determine if the pause occurred. See methods of Experiment 1b for more details.

For *Bombus vosnesenskii*, we collected 12 queens in total. Out of the 12, 8 queens initiated normally, but 2 were removed due to lack of consistent photo data collection. Of the 8 total queens, 6 initiated normally, and thus data was collected. For *B. mckayi* and *frigidus*, we had limited specimens, and only three nests were available for observation. Additionally, we spoke with Omar Arguello from El Colegio de la Frontera Sur, Chiapas, Mexico, who confirmed that this pattern occurred in the tropical bumble bee species *Bombus ephippium* (personal communication).

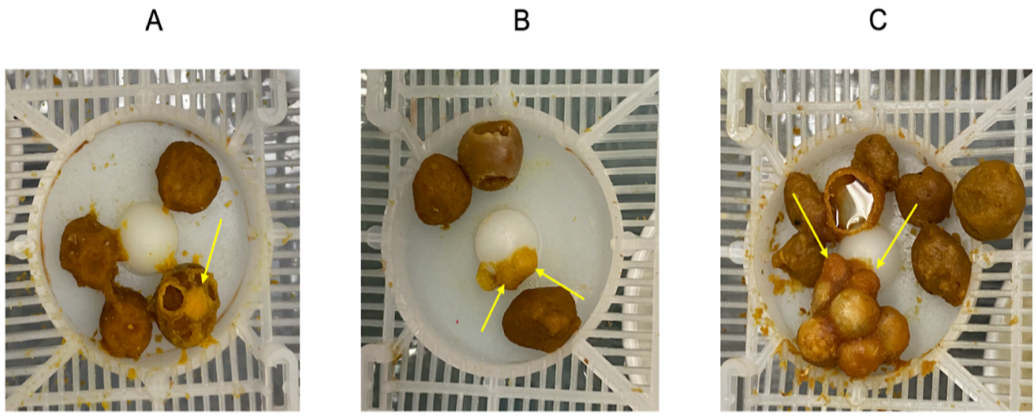

Figure 1-S1. Example images of egg cups.

Photos show examples of when the first egg cups in the nest are laid directly on a pollen ball (A), directly onto the central plastic mound that is part of the nest box (B), or after pause, re-initiation on brood present (C). Individual egg cups are denoted with an arrow in photos. Other items in nests include pollen balls provided to nests (seen in all photos) and honey pots (in C only).

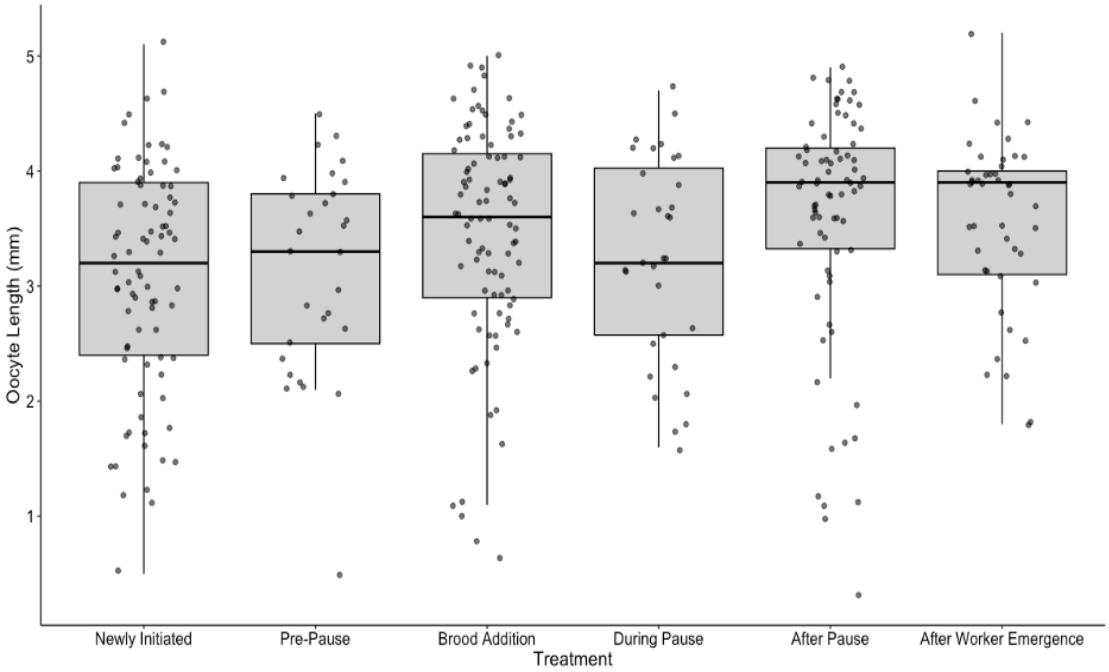

Figure 1-S2. Terminal Oocyte Length.

Length of terminal oocytes measured per treatment type. Of the 62 queens used in the experiment, those without all 8 terminal oocytes present were excluded from the analysis. Thus, a subset of queens used in the experiment (n = 56) were analyzed. Treatment (when artificial brood addition occurred) was not a significant factor in the GLMM model selection and thus was not included.

Table 1- S10a. Summary of the analyses of related experiments examining queen egg-laying dynamics and nest development.

Phenomenon	Question	Exp.	N	Result	Type of Analyses	Family/ Distribution	Fixed Effects	Response Variable	Random Factors
Dynamic patterns of queen egg-laying across early nesting period	5-day intervals and patterns of egg-laying (e5kre)	1a	62	Sig	GLMM	Zero-inflated negative binomial	Timing	# of egg clutches	Natal colony
Dynamic patterns of queen egg-laying across early nesting period	Proportion of ovaries resorbed in relation to treatment	3	62	Sig	GLMM	ordbeta	Treatment	Proportion of ovaries resorbed	Natal colony & body size
Influence of brood on queen egg-laying	Brood addition and timing of re-initiation	4	30	Sig	GLMM	Gamma	Treatment	# days until re-initiation	Natal colony
Influence of brood on queen egg-laying	Differences between brood type in nests (pause entry)	1a	62	Sig	Pairwise chi-squared test	n/a	n/a	Oldest brood type in the nest	n/a
Influence of brood on queen egg-laying	Differences between brood type in nests (pause exit)	1a	62	Sig	Pairwise chi-squared test	n/a	n/a	Oldest brood type in the nest	n/a

Table 1- S10b. Summary of the analyses of related experiments examining queen egg-laying dynamics and nest development (continued).

Phenomenon	Question	Exp.	N	Result	Type of Analyses	Family/ Distribution	Fixed Effects	Response Variable	Random Factors
Association between the pause and nest developmental characteristics	Length of the pause and # days until first worker emergence	1a	62	Sig	GLMM	Gaussian	Length of pause	# days until first brood emergence	Natal colony
Association between the pause and nest developmental characteristics	Length of the pause and # of workers within one week	1a	62	Null	GLMM	n/a	n/a	# of workers within one week	n/a
Association between the pause and nest developmental characteristics	Length of the pause and the body size of all workers after emergence	1a	62	Null	GLMM	n/a	n/a	Body size of workers	n/a
Queen egg-laying is also under the control of adult workers in the nest	Differences in the number of egg clutches after worker emergence	2	30	Model overall not sig.	GLMM	Zero-inflated negative binomial	Treatment + timing	Total # egg clutches	Natal colony
Queen egg-laying is also under the control of adult workers in the nest	# of brood in nests and presence/ absence of workers in nest	2	30	Sig	GLMM	Poisson	Treatment	Total # brood	Natal colony

Table 1- S10ab. Summary of the analyses of related experiments examining queen egg-laying dynamics and nest development. N refers to the number of queens or nests involved in the experiments. n/a refers to not being included either because of the analysis in question (chi-square tests do not include the same information as GLMMs) or because the results were not significant in the model and thus not included.

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CHAPTER 2: QUEEN BROOD CARE BEHAVIOR IS PLASTIC AND SOCIALLY REGULATED IN AN ANNUALLY EUSOCIAL INSECT

Abstract

In primitively eusocial insect systems, the transition from subsocial to eusocial is associated with a transition in the division of labor, as queens shift from performing brood care during the nest founding state to specializing primarily in reproduction after workers emerge. The dynamics of this transition are not very well understood, especially if it is a fixed behavioral shift, or is reversible. In this study, we examined plasticity in queen-provided brood care in the bumble system, by quantifying feeding and incubation across different colony developmental stages (small, medium, large, and mature), by experimentally removing workers from colonies. We tested the capacity at which queens retain their capacity to perform brood cares after workers are removed from the colony and whether these responses change as the colonies grow. We discovered that even before worker removal queens performed brood care at all stages of colony development. We elucidate that feeding and incubation are regulated independently in the nest after worker removal and that queens showed variation in brood care allocation to different staged brood. Collectively, our work shows that the dynamic between reproductive specialization and brood care behaviors are not strictly irreversible. Instead, queens retain incredible flexibility when social conditions change within the nest. More broadly, this work highlights how social regulation can influence flexibility during life history transitions in primitively eusocial insects.

Introduction

Phenotypic plasticity is the ability of a single genotype to produce different phenotypes, including in response to changing environmental conditions (Sommer, R., 2020; Schlichting and Pigliucci, 1998; Houston and McNamara, 1992). Plasticity can be particularly important in the context of parental care, where environmentally responsive shifts in brood care and reproductive investment can enhance fitness (Westrick et al., 2022; West-Eberhard, 2003; Stein and Bell, 2019). Across many taxa, plasticity in parental care behavior is organized in association with life-history transitions. During these transitions, parents undergo stereotypical changes in behavior and physiology, often in response to environmental cues, offspring development, and the physical condition of the parents. These shifts allow parents to integrate the costs of care with other fitness-related demands, including self-maintenance, future reproduction, and survival. For example, in earwigs, female earwigs adjust the quantity and frequency of feeding based on their physical condition, with females in poorer condition providing reduced and shorter-duration care (Kölliker and Vancassel, 2007; Wong and Kölliker, 2012).

For animals that live in social groups, such as social insects, plasticity in brood care behavior is often shaped not only by environmental cues but also by social conditions (presence, number, and identity of group members, and their interactions (Woodard et al., 2013; Sarro et al 2021; Johnson, 2003; Huang et al., 1994; Robinson, 1992; Fisher et al., 2022; Holldobler and Wilson, 1990; Torres et al., 2012)). Some of the most extreme examples of plasticity in brood care behavior are found in queens of the

eusocial insect systems that have solitary colony founding. In these systems, queens must first function as the sole caregiver and primary reproductive, as queens begin the colony cycle alone and perform all tasks required for early colony establishment, including nest construction or maintenance, brood care, and reproduction (Cronin et al., 2013). Within these solitary-founding systems, once daughter workers emerge, the social environment of the nest changes dramatically (Oster and Wilson, 1978; Rubenstein and Abbot, 2017). When workers take over the nest-maintenance and brood-care tasks previously performed by the queen, this allows the queen to reduce direct care and shift toward a primarily reproductive role (Woodard et al., 2013; Cronin et al., 2013). This transition in the nest, from maternal care to worker-mediated sibling care, represents a major social developmental transition, in which the organization of brood care changes in response to developing social conditions within the colony.

Most bumble bees (genus *Bombus*, family Apidae) are solitary-founding eusocial insects that are an excellent model for examining changes in queen brood care behavior across life-history transitions. Bumble bees have a primitively eusocial lifestyle in which queen brood care behavior, as well as ovarian activation and egg laying, undergo dramatic changes in association with nest founding. After overwintering solitarily, new queens begin developing their ovaries (Gustilo et al.) and searching in spring for suitable nesting sites to lay their eggs and found their colonies (Alford, 1971; Heinrich, 2004). Bumble bee queens perform all essential tasks within the nest during this early stage, with the two primary brood care behaviors being incubation and progressive provisioning

(feeding) of larvae. Once workers begin to emerge, however, queens reduce brood care behaviors and shift primarily toward egg laying, as workers assume most non-reproductive tasks (Sarro et al., 2021; Fisher et al., 2022; Shpigler et al., 2013; Woodard et al., 2013; Yadav et al., 2016; Peto et al., 2025). This transition can occur rapidly, with queen brood care declining markedly within days of worker emergence (*B. terrestris*; Woodard et al. 2013; Shpigler et al., 2013) and in response to only a few workers in the nest (in *B. impatiens*; Gustilo et al. 2021; Gustilo et al., 2025). Whether these changes in queen egg laying and brood care behavior are fixed or flexible, however, remains unclear. In particular, it is unknown whether the queen's transition away from brood care and towards reproductive specialization represents a fixed life history shift or is dynamic and socially contingent.

Here, we asked if queen brood care behaviors are plastic in response to worker presence or absence within the nest. We first quantified queen brood care behavior, including the performance of both feeding and incubation, across and beyond early colony development in queens of the bumble bee *Bombus impatiens*. This was done to establish a baseline trajectory of queen behavioral change over time. We then experimentally removed workers across colonies at different developmental stages to test whether queens adjust their brood care behavior and egg laying in response to such abrupt changes in the social environment. We hypothesized that queen behavioral plasticity declines as colonies mature. Specifically, we predicted that queens in early-stage colonies would retain a greater capacity for increasing brood care following worker removal, whereas queens in larger, more mature colonies would be less likely to

do so. Our study sheds light on the role of social regulation of reproduction in social insects, highlighting how queens can modify their behavioral roles in response to shifting social conditions.

Materials and Methods

Bumble Bee Rearing and Colony Establishment:

Nine mature *Bombus impatiens* colonies were obtained from Koppert Biological Systems (Howell, MI, USA) and maintained within their commercial boxes at the University of California, Riverside Entomology Building. Colonies were stored at 23 °C and 60–70% relative humidity under constant darkness or dim red light, which bumble bees cannot perceive. Individual adult callow queens (< 24 h old), identified by their silvery appearance and inability to fly, were removed from natal colonies. Queens were placed individually in plastic containers (Hi-Plas plastic containers, 7.5 cm diameter) and provided ad libitum mixed-source honey bee–collected pollen (Koppert Biological Systems) and artificial nectar prepared following the recipe in Boyle et al. 2018.

At three days of adult age, queens were placed daily into mating cages (BugDorm-6E620 insect rearing cages; 60 × 60 × 120 cm) between 7 and 9 hours per day under ambient light conditions and allowed the opportunity to mate until age ten days. Queens remained in mating cages until the first time copulation was observed (Treanore et al., 2021; Peto et al., 2025; Watrous et al., 2019) were singly mated and were not returned to mating cages after mating occurred. Adult males were obtained from three natal colonies and were paired with queens from different colonies to avoid

within-colony mating. While in the mating cage, both queens and males were provided pollen and artificial nectar ad libitum (as described above). Queens were singly mated and were not returned to mating cages after mating occurred.

Following mating, queens were housed individually with pollen and artificial nectar in an Invictus Drosophila incubator maintained at 27 °C, constant darkness, and 60% relative humidity. At adult ages 12 and 13 days, queens were treated with CO_2 gas for 30 minutes over two days to induce them to bypass diapause (Röseler and van Honk, 1990; Amsalem and Grozinger, 2017). Following the second CO_2 treatment, queens were placed into plastic nest cages (Biobest Group USA, Inc.; 17 cm × 17 cm × 9.5 cm) containing pollen, artificial nectar, and a honey bee wax-covered pollen ball. Nest cages were then transferred to the University of California, Riverside Insectary and Quarantine Facility and maintained under similar temperature and humidity conditions and under constant darkness except for brief light exposure during feeding or nest inspections. Pollen was replenished every 5–7 days, and artificial nectar was replaced every one to two weeks to prevent spoilage. Nests were inspected daily for new worker emergence, and the dates of adult worker emergence were noted. Newly-eclosed workers (adult age < 24 hr) are identified by their silvery appearance. Once the first worker emerged, nests were transferred to a secondary room within the Insectary and Quarantine Facility with similar environmental conditions. Infrared security cameras (VIGICA Peashooter QD520) were placed above all nests to generate continuous video recordings used to quantify brood care behaviors (described below, with additional detail in Gustilo et al. 2023 and Fisher et al, 2022).

Colonies were maintained until they reached worker numbers of approximately 5, 15, or 50 workers, which we refer to as "small (S)," "medium or intermediate (M)," or "large (L)" colonies, respectively. These group sizes were chosen to represent different stages of early nest development, across which queens progressively reduce their brood care behavior and increase their egg laying (Shpigler et al 2013; Sarro et al., 2021). By later stages of colony development (~30 days post-first worker emergence, when there may be ~50-70 workers in the nest (Peto et al., 2025; Rotheray et al., 2017), queen-provided brood care is typically minimal (<5%, Shpigler et al., 2013; Leza et al., 2018; Sarro et al., 2021). As such, our largest worker group (~50 workers) represents a developmental stage in which queen brood care is expected to be rare or nearly absent.

Worker removal manipulation

Workers were manually removed from a subset of nests using long forceps while avoiding disturbing the queen and brood. After worker removal, nests were monitored for an additional five minutes to allow for the detection and removal of any additional workers that were not originally detected because they were hidden within the nest structure. In the control nests, workers were briefly (2-3 seconds) handled with forceps to mimic the disturbance associated with worker removal and were returned to the nest. All treatment and control nests were undisturbed after this period.

Removing workers from the nest (termed "sociotomy") is an established, powerful tool for studying social regulation and other social effects by examining responses in remaining individuals and nesting conditions (Gardener et al. 2007; Kolmes and Winston, 1988; Backen et al., 2000).

Video collection, selection, and scoring:

Behavioral recordings were collected for 48 hours, consisting of a 24-hour pre-manipulation baseline period (referred to as "pre-manipulation" period) and a 24-hour post-manipulation period ("post-manipulation"). In manipulated colonies, all workers were removed from the nest (W-), whereas in control colonies, workers remained present (W+). "Pre-manipulation" period thus refers to the 24-hour time segment where videos were recorded for a baseline of behaviors, regardless of whether workers were removed or not. "Post-manipulation" refers to the 24-hour time segment of videos recorded after the treatment (worker removal, W-, or worker presence remains W+). We looked at the 24-hours after manipulation to investigate the queens' behavior immediately after this treatment. When discussing both time periods together, we call this the "entire experimental phase." These recordings allowed us to compare individual queen behavior before and after worker removal, differences in queen behavior by treatment at a given colony size, and group-level differences in queen behavior across colony sizes. Recordings and manipulations were conducted at approximately 10:00 AM, the same time for each colony, such that pre- and post-manipulation periods corresponded to equivalent times of day across colonies.

Video analysis and nest dissections were conducted on 50 nests total, with the following numbers of nests in each treatment group: S/W+ (n = 7), S/W- (n = 9), M/W+ (n = 8), M/W- (n = 8), L/W+ (n = 9), and L/W- (n = 9). No queens died during the experiment; thus, these queen/nest sample sizes were used for all analyses.

Of the total ten hours observed per nest, two hours were observed before treatment (pre-), and eight hours after treatment or sham (post-treatment) (see Supplementary Figure 1). We selected these ten hours as follows: the first and final hours of the 24-hour recording, for both pre- and post-manipulation, were excluded to account for lighting or behavioral disturbances and for occasional inconsistencies in recording duration when cameras were reset, or new queens were placed under observation. From the remaining 22-hour interval, pre-manipulation behavioral observations were conducted over two hours total. These hours were chosen to examine queen behaviors during the time period that is closest to initial worker manipulation and close to the experiment end, the latter time point representing when queens have been given the most time to adjust to worker loss after worker removal. Post-treatment behavioral observations were conducted during one-hour sampling windows spaced every three hours (8 total observational hours). Analyses comparing pre- and post-treatment behavior were restricted to two matched timepoints, pre- and post-manipulation (hours 4–5 and 19–20 for both, Supplementary Figure 1).

In total, over 500 hours of video were watched across all nests. All behavioral observations were performed by a single observer to minimize potential observational bias. Across literature, there is a broad array of cooperative behaviors associated with

bumble bees in colonies, including, but not limited to: foraging, nest defense, nest construction and maintenance, incubation, and feeding (Heinrich, 1979; Oster and Wilson, 1978). In our study, queen brood care behaviors were limited to incubation and brood feeding and are defined as follows. For incubation: queen bee is stationary and perched on brood wax or cocoon in a flattened position with the thorax and abdomen contacting the brood; the abdomen is often pumping (Heinrich, B. 1972, Sladen, 1912; Michener, 1974). To ensure accurate scoring, incubation was recorded only when the queen's main body (excluding head and legs) was positioned directly on the brood. For feeding: stereotypical behavior in which the queen pierces the larval wax envelope, inserts her mouthparts through the opening, and regurgitates food, accompanied by an abdominal contraction (Woodard et al., 2013; Costa et al., 2021; Sarro Gustilo et al., 2023; Free and Butler, 1959; Fisher et al., 2022; Sladen, 1912).

Videos were analyzed with a standardized grid overlay consisting of labeled columns (A–D) and rows (1–6), resulting in 24 equally sized cells (~52 cm^2 per cell). All behaviors were recorded within specific grid cells to track spatial positioning. When the queen's body spanned multiple cells, all relevant cells were noted (see supplementary "definition and descriptions" for more information. Videos were viewed in the same video frame dimensions, with the same grid overlay for all nests to ensure spatial measurements were standardized (See supplementary Figure 2 for a visual example).

In addition to our primary experimental colonies described above, we also examined queen brood care behaviors within a set of nine (five treatments and four

controls) mature (pre-gyne emergence; average = 456.56 ± 32.62 workers) colonies, where queens are believed to be highly invested in reproductive behavior. Colonies were obtained from Koppert Biological Systems, maintained in the same room in UCR's insectary and quarantine, and provided pollen and artificial nectar ad libitum as described above. Nests were acclimated to laboratory conditions and developed for three weeks before observation. Due to the size and structural complexity of these fully developed colonies, complete worker removal from the W- colonies was not feasible, and approximately 20–40 workers remained in the nest following manipulation. Infrared cameras (described above) were also placed above these mature colonies; however, due to the substantially larger nest size and colony complexity, cameras were unable to capture the entire nest simultaneously. Consequently, these colonies were not included in statistical analyses, and behavioral data were instead used qualitatively. We also only observed incubation in these fully mature colonies, as feeding was more difficult to observe in these videos. For these observations, videos were monitored until the first occurrence of incubation was detected, after which the remaining footage was not analyzed.

Reproductive behavior and colony development:

After the 48-hour recording period, all nests were frozen and stored at -80 C until dissections were performed. During dissections, the number of eggs present in each nest was recorded as a metric of queen reproductive output. Eggs present in the nests at the end of the experiment were assumed to reflect queen egg laying (rather than eggs laid by

workers), given that queens lay the overwhelming majority of eggs in bumble bee nests (Fisher et al., 2022; Alaux et al., 2004, 2006; Amsalem et al., 2017; Bloch, 1999; Padilla et al., 2016).

In addition to egg counts, other, older brood were visually inspected and classified into developmental categories following Rozen et al. (2018) and Peto et al. (2025): (i) *eggs*; (ii) *younger larvae* corresponding to first and second instars housed in communal chambers; (iii) *older larvae* corresponding to third instar and most fourth instar larvae that begin spinning cocoons; and (iv) *late-stage brood*, which includes post-defecation fourth instar larvae, prepupae, and pupae. These same classifications were used when determining which brood stages were fed or incubated during behavioral observations and for subsequent analyses.

Statistical Analyses:

All statistical analyses were conducted in R version 4.3.1 and statistical significance was assessed at $\alpha = 0.05$. Data distributions were evaluated using Shapiro–Wilk tests and most response variables deviated from normality. Accordingly, we employed generalized linear mixed models (GLMMs) to examine how social conditions influenced queen brood care behavior. GLMMs were fitted using the *glmmTMB* package. Specifically, Gaussian models were used for approximately normally distributed responses (e.g., incubation frequency in some subsets). Gamma distributions were used for non-normal, skewed responses (e.g., incubation duration and total incubation effort), and negative binomial models (nbinom2) were used for overdispersed

count data (e.g., feeding frequency). In cases where response variables were bounded or proportional (e.g., standardized incubation), alternative distributions (e.g., ordered beta) were applied as appropriate. Across all models, natal colony was included as a random effect to account for non-independence among queens originating from the same natal colony. When relevant, queen-level repeated measures (e.g., pre- vs. post-manipulation) were incorporated within this structure. Fixed effects included treatment (worker presence versus removal), colony size (small, medium, and large), experimental phase (pre- versus post-manipulation), and their interactions, depending on the specific hypothesis being tested. See supplement for more detailed definitions.

For each response variable, we constructed a set of candidate models representing all biologically relevant additive and interactive combinations of fixed effects, while holding the random-effects structure constant. Model selection was performed using Akaike's Information Criterion corrected for small sample sizes (AIC) via the *model.sel* function in the MuMIn package. The model with the lowest AIC value was considered the best-supported model; however, when competing models differed by less than $\Delta AIC = 2$, biological interpretability was also considered in model selection (see supplementary information for more details). Following model selection, we evaluated overall model effects using Type II analysis of variance (ANOVA) from the *car* package. Post hoc pairwise comparisons were conducted using estimated marginal means (emmeans) with Tukey-adjusted contrasts. To examine relationships between correlated responses, such as total incubation effort and egg production, we accounted for collinearity by using residuals derived from regressions of incubation effort against treatment (following

Graham, 2003). This approach allowed us to isolate variation in incubation behavior independent of treatment effects.For comparisons involving single response variables between two groups (e.g., final egg counts between control and treatment colonies within a given colony developmental stage), we used Welch's two-sample t-tests.

For treatment queens, we calculated the change in cumulative incubation duration as Post − Pre and used a Gaussian GLMM to test whether the magnitude of change differed by group size, with colony ID included as a random effect.

To assess whether brood care behavior varied naturally across control colonies (to establish a baseline behavior), we analyzed post-manipulation control queens (post-W+) only. Incubation frequency, average incubation duration, total incubation duration, and total feeding events) were modeled with group size (S, M, or L) as a fixed effect and colony ID as a random effect. Feeding events are analyzed only as counts because each feeding event consists of a single, quick, regurgitation to a larva or set of larvae. Gaussian models were used for incubation frequency, Gamma models with log links were used for incubation duration variables, and a negative binomial model was used for feeding counts; candidate models were compared using AICc, followed by estimated marginal means for pairwise contrasts.

In addition to full factorial analyses, we conducted subset analyses to address specific biological questions. For example, to test whether colony size alone influenced behavior independent of experimental manipulation, we analyzed post-manipulation control colonies only using GLMMs with group size as the primary fixed effect.

Similarly, changes in behavior at the individual level were quantified using amplitude metrics (post–pre differences per queen), which were analyzed using Gaussian GLMMs.

All data processing and visualization were conducted in R using packages including *tidyverse* and *ggplot2*. Additional details regarding best-fitting models are provided in the Supplementary Materials (Tables 2.S1-2.S17).

For definitions of how analyses were conducted, see the "definitions and description of data used in each analysis" section in the supplement.

Results

Queen Brood Care Does Not Decline Across Colony Stages Tested (both Incubation and Feeding):

First, we asked if brood care behaviors (incubation and feeding) differed across colony developmental stages in our control queens only, to get a sense of the baseline amount of brood care that queens provide at different points in colony development. In general, queens spent between 22-24% (on average) of the hour incubating brood. For incubation, we examined three factors: baseline incubation duration (total minutes across post-manipulation phase), frequency per hour (total events divided by experimental phase hours), and average event duration (average duration of individual events). For more details, refer to the supplement. We did not detect any differences in queen brood care in our assessment of whether brood care differs across group sizes (in our control queens only). Cumulative incubation duration ($\chi^2 = 1.2895$, df = 2, p = 0.5248, Supplementary Figure 2-S3a-2-S3c), incubation frequency per hour ($\chi^2 = 1.0854$, df = 2, p = 0.5812), and the average incubation event duration ($\chi^2 = 0.0448$, df = 1, p = 0.8324) did not differ across the three control groups (S, M, L).

Additionally, we did not detect any differences in the total number of queen feeding events across the colony developmental stages in our unmanipulated queens (overall model: $\chi^2 = 0.102$, df = 2, p = 0.9503). Total feeding events between the matched timeframes for the control queens were generally rare (average number of feeding events observed per group: S: 1.86 ± 0.553; M: 1.62 ± 0.498; L/W+: 1.44 ± 0.475).

Queen Incubation, but not Feeding, Increases When Workers are Removed from the Nest

Cumulative duration of brood incubation provided by queens was greater in the post worker removal treatment (W-) versus the pre-manipulation treatment colonies, in all colony developmental stages (Overall model: S: $\chi^2 = 6.4357$, df =1, $p < 0.05$; M: $\chi^2 = 18.0507$, df = 1, $p < 0.001$; L: $\chi^2 = 13.0811$, df = 1, $p < 0.005$; post-hoc tests: small: Post/S/W− vs. Pre/S/W−: $T = -4.860$, $p < 0.0001$; medium: Post/M/W− vs. Pre/M/W−: $T = -5.058$, $p < 0.001$; large: Post/L/W− vs. Pre/L/W−: $T = -5.101$, $p < 0.001$; (Supplementary Figure 5). The unmanipulated control nests and worker-removal nests did not differ significantly before manipulation in all colony developmental stages (S: $T = -1.041$, $p = 0.7251$, M: $T = 0.805$, $p = 0.851$, L: $T = 0.796$, $p = 0.856$), and the control nests did not differ across experimental phases (S: $T = -0.917$, $p = 0.7956$, M: $T = 0.873$, $p = 0.8185$, L: $T = 0.014$, $p = 1.000$, pre vs. post controls).

Incubation frequency also increased following worker removal in all colony developmental stages (overall model: S: $\chi^2 = 38.007$, df = 1, $p < 0.001$; M: $\chi^2 = 17.5708$, df = 1, $p < 0.001$; L: $\chi^2 = 6.6729$, df = 1, $p < 0.05$; Supplementary Figure 2-S6). Post hoc comparisons for the medium and large colony developmental stages only are reported in Table 1, as incubation frequency did not differ between experimental phases in the small colony developmental stage (see Supplement for more information).

Table 2-1a-b. Post-hoc comparisons for incubation frequency for the medium and large group sizes.

Post-hoc comparisons for incubation frequency in the medium and large colony developmental stages. Values represent p-values comparing incubation frequency among control (W+) and worker-removal (W−) colonies across experimental phases. The small colony developmental stage is not included because post-hoc comparisons did not indicate phase-specific differences in incubation frequency; instead, treatment differences were observed across phases. Diagonal cells represent identical comparisons and are therefore not applicable.

Table 2-1a

INCUBATION FREQUENCY: MEDIUM COLONIES		Control (W+)		Treatment (W-)	
		Pre	Post	Pre	Post
Control (W+)	Pre	-	p = 0.6359	p = 0.2929	p = 0.033
	Post	p = 0.6359	-	p = 0.9271	p < 0.005
Treatment (W-)	Pre	p = 0.2929	p = 0.9271	-	p < 0.001
	Post	p = 0.033	p < 0.005	p < 0.001	-

Table 2-1b

INCUBATION FREQUENCY: LARGE COLONIES		Control (W+)		Treatment (W-)	
		Pre	Post	Pre	Post
Control (W+)	Pre	-	p = 1.000	p = 0.9554	p = 0.0208
	Post	p = 1.000	-	p = 0.0187	p < 0.05
Treatment (W-)	Pre	p = 0.9554	p = 0.0187	-	p = 0.0058
	Post	p = 0.0208	p < 0.05	p = 0.0058	-

Finally, the average duration of an individual incubation event (see supplement for more details) showed more complex differences across treatments and between experimental phases. In the smallest group, the average duration of an incubation event differed significantly between the worker-removal and control groups only during the pre-removal phase ($\chi^2 = 5.1664$, df = 1, $p < 0.05$, Supplementary Figure 2-S7). This effect was driven by a significant post hoc contrast between the pre-removal control and treatment groups ($Z = -2.635$, $p = 0.0418$), with no significant additional pairwise comparisons. In the medium and large groups, average incubation event duration was not affected by worker removal or experimental phase (overall models: M: $\chi^2 = 0.3397$, df = 1, $p = 0.5600$; L: $\chi^2 = 1.4923$, df = 1, $p = 0.2219$, Supplementary Figure 2-S7).

Finally, in our mature colonies (n = 9), queens were observed incubating brood, regardless of whether workers were present or removed.

At the individual queen level, we also found that cumulative brood incubation provided by queens increased following the removal of workers from their nest, regardless of which colony developmental stage a queen belonged to (S, M, L / W- nests; all paired T-tests, $p < 0.05$; average percent increase in duration: small = 62.1%, medium = 115.6%, large = 92.6%; Figure 2-1). The magnitude of change in cumulative incubation, following worker removal, did not differ among queens in the different colony developmental stages (overall model: $\chi^2 = 1.57$, df = 2, $p = 0.46$; S: 0.231 ± 0.048 min.; M: 0.342 ± 0.067 min.; L: 0.269 ± 0.084 min.; Supplementary Figure 2- S4).

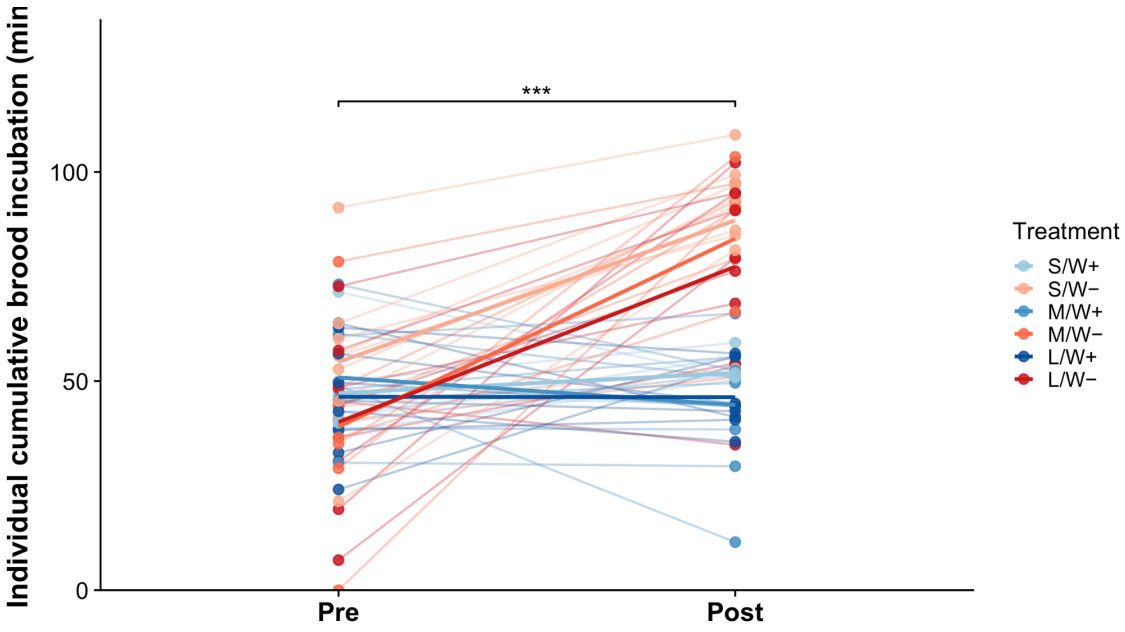

Figure 2-1. Paired line plot of cumulative incubation duration across the entire experimental phase (pre- and post-manipulation).

Data are shown for individual queens (individual points connected by thinner lines) with group means shown with thicker lines. Bracket indicates pairwise comparisons between pre- and post-manipulation periods; a single bracket is shown because all pairwise comparisons had an equivalent level of significance. Significance levels are denoted as ns (not significant),(*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Queen brood feeding behavior was not impacted by worker removal (overall model: S: $\chi^2 = 1.5209$, df = 1, p = 0.2; M: $\chi^2 = 2.6338$, df = 1, p = 0.05; L: $\chi^2 = 0.8157$, df = 1, p = 0.4). Queens were observed feeding brood across all colony developmental stages and experimental phases, but brood feeding was relatively infrequently observed (average number of feeding events observed per group and treatment: S/W+: 2.07 ± 0.57; S/W-: 1.44 ± 0.34; M/W+: 1.81 ± 0.31; M/W-: 1.00 ± 0.32; L/W+: 1.17 ± 0.28; L/W-: 0.83 ± 0.26).

Queens Incubate Late-stage Brood Most Often, But Feed Only the Youngest Brood:

Queens incubated late-stage brood the most (84% of events) of any brood type (n = 2994 incubation events were on late-stage brood, out of n = 3527 total events observed).

We then asked whether queens allocated a greater proportion of their cumulative post-manipulation incubation time to particular brood types. To account for variation among queens in total time spent incubating, we calculated the relative proportion of incubation time allocated to each brood type (hereby standardized incubation duration; see supplement for more details.)

Standardized cumulative incubation duration was significantly associated with brood type in the small ($\chi^2 = 26.99$, df = 3, $p < 0.001$) and large ($\chi^2 = 9.15$, df = 3, $p < 0.05$) colony developmental stages, but not the medium colony developmental stage ($\chi^2 = 7.08$, df = 3, p = 0.069, Figure 2-2). In contrast, treatment alone was not significantly associated with standardized cumulative incubation duration in any colony

developmental stage (S: $\chi^2 = 1.44$, df = 1, p = 0.229; M: $\chi^2 = 0.80$, df = 1, p = 0.372; L: $\chi^2 = 1.81$, df = 1, p = 0.179). Table 2-2a-c shows results of emmeans pairwise comparisons of colony developmental stages and treatment groups.

Table 2-2a. Results of emmeans pairwise comparisons of the small colony developmental stage and treatment groups.

Incubation by brood type: SMALL		Control (W+)				Treatment (W-)			
		Eggs	Younger larvae	Older larvae	Late-stage Brood	Eggs	Younger larvae	Older larvae	Late-stage Brood
Control (W+)	Eggs		0.8377	< 0.05	0.2006	0.9316	0.5911	< 0.05	0.8776
	Younger Larvae	0.8377		0.1686	< 0.01	1.0000	0.9316	0.0947	0.1763
	Older Lavae	< 0.05	0.1686		0.0001	0.1835	0.5783	0.9316	< 0.01
	Late-Stage Brood	0.2006	< 0.01	0.0001		0.1244	< 0.01	0.0001	0.9316
Treatment (W-)	Eggs	0.9316	1.0000	0.1835	0.1244		0.8377	< 0.05	0.2006
	Younger Larvae	0.5911	0.9316	0.5783	< 0.01	0.8377		0.1686	< 0.01
	Older Lavae	< 0.05	0.0947	0.9316	0.0001	< 0.05	0.1686		0.0001
	Late-Stage Brood	0.8776	0.1763	< 0.01	0.9316	0.2006	< 0.01	0.0001	

Table 2-2b. Results of emmeans pairwise comparisons of the medium colony developmental stage and treatment groups.

Incubation by brood type: MEDIUM		Control (W+)				Treatment (W-)			
		Eggs	Younger larvae	Older larvae	Late-stage Brood	Eggs	Younger larvae	Older larvae	Late-stage Brood
Control (W+)	Eggs		0.9971	1.000	< 0.001	0.5776	0.9852	0.9946	< 0.0001
	Younger Larvae	0.9971		1.000	< 0.001	0.8444	0.7205	1.0000	< 0.0001
	Older Lavae	1.000	1.000		< 0.01	0.8436	0.9489	0.9999	0.0001
	Late-Stage Brood	0.0002	< 0.001	< 0.01		0.0708	< 0.0001	0.0017	0.9061
Treatment (W-)	Eggs	0.5776	0.8444	0.8436	0.0708		0.1496	0.9341	< 0.01
	Younger Larvae	0.9852	0.7205	0.9489	< 0.0001	0.1496		0.7290	<0.0001
	Older Lavae	0.9946	1.0000	0.9999	0.0017	0.9341	0.7290		0.0001
	Late-Stage Brood	< 0.0001	< 0.0001	0.0001	0.9061	< 0.01	<0.0001	0.0001	

Table 2-2c. Results of emmeans pairwise comparisons of the large colony developmental stage and treatment groups.

Incubation by brood type: LARGE		Control (W+)				Treatment (W-)			
		Eggs	Younger larvae	Older larvae	Late-stage Brood	Eggs	Younger larvae	Older larvae	Late-stage Brood
Control (W+)	Eggs		0.8382	0.9999	0.0066	0.9060	1.000	1.000	< 0.001
	Younger Larvae	0.8382		< 0.001	< 0.01	0.1360	0.1723	0.5570	< 0.001
	Older Lavae	0.9999	< 0.001		< 0.001	0.6114	0.9824	1.000	< 0.0001
	Late-Stage Brood	0.0066	< 0.01	< 0.001		< 0.001	< 0.0001	< 0.0001	0.7169
Treatment (W-)	Eggs	0.9060	0.1360	0.6114	< 0.001		0.8958	0.7164	< 0.0001
	Younger Larvae	1.000	0.1723	0.9824	< 0.0001	0.8958		0.9980	< 0.0001
	Older Lavae	1.000	0.5570	1.000	< 0.0001	0.7164	0.9980		< 0.0001
	Late-Stage Brood	< 0.001	< 0.001	< 0.0001	0.7169	< 0.0001	< 0.0001	< 0.0001	

Tables 2-2a–c. Post-hoc comparisons for standardized incubation duration across brood types in the small, medium, and large colony developmental stages. Values represent p-values from emmeans pairwise comparisons of the proportion of cumulative post-manipulation incubation time allocated to each brood type, including eggs, younger larvae, older larvae, and late-stage brood. Comparisons are shown separately by colony developmental stage and treatment. Standardized incubation duration was calculated to account for variation among queens in total incubation time during the post-manipulation phase. Diagonal cells represent identical comparisons and are therefore not applicable.

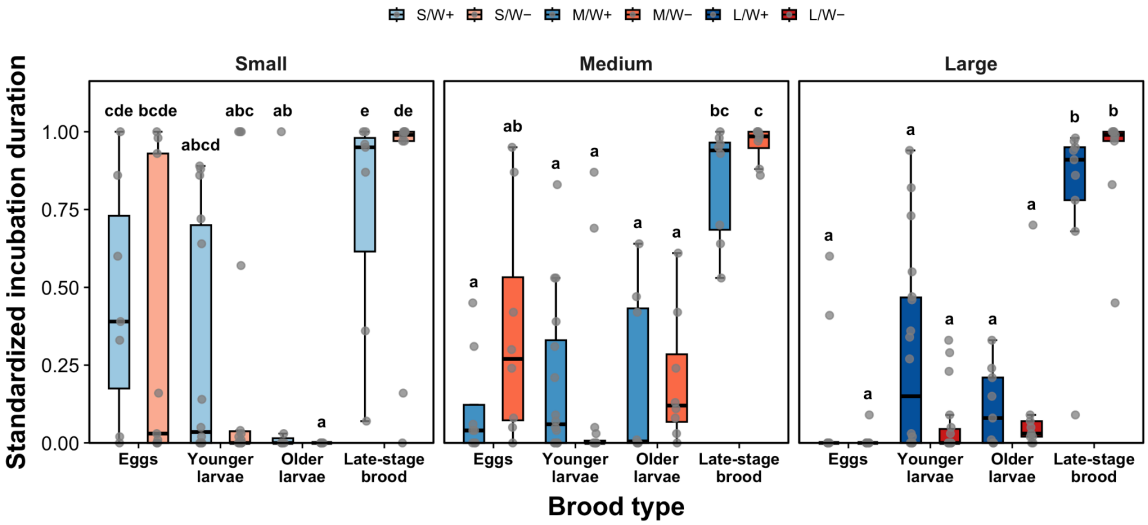

Figure 2-2. Box plot of standardized incubation duration across brood types for different group sizes, shown for the small, medium, and large colonies (see Tables 2a-c for more information).

Grey dots represent individual queens. Letters above groups indicate statistically significant differences; groups sharing the same letter do not differ significantly (Tukey-adjusted $p < 0.05$).

Unlike what was observed for incubation behavior, feeding behavior was directed primarily towards young larvae. Of the total feeding events observed across all nests (n= 310), only one event involved feeding older larvae, with the remaining (n=309) events for young larvae.

Tradeoff between Incubation and Egg Laying in Larger Nests:

Worker removal had a significant negative impact on the number of eggs in the nest at the end of the experiment in the large group size only (Welch's t-test: $t = 3.19$, $df = 14.58$, $p = 0.006$; L/W+ = 101.9 ± 14.3 eggs, L/W- = 72.5 ± 24.9 eggs; see Figure 2-3). In contrast, worker removal did not impact egg number in the small or intermediate group sizes (S/W- vs. S/W+: $Z = 0.33$, $df = 11.84$, $p = 0.748$; M/W+ vs. M/W-: $t = 0.18$, $df = 13.93$, $p = 0.862$).

Worker removal did not impact the amounts of other brood types in the nests (S: $Z= -2.037$, $p = 0.3212$; M: $Z = 2.195$, $p = 0.2399$; L: $Z = 1.072$, $p = 0.8925$). There was no relationship between the total amount of time a queen spent incubating and the number of eggs in her nest at the end of the experiment ($p = 0.6606$; see Supplementary Figure 2-S8).

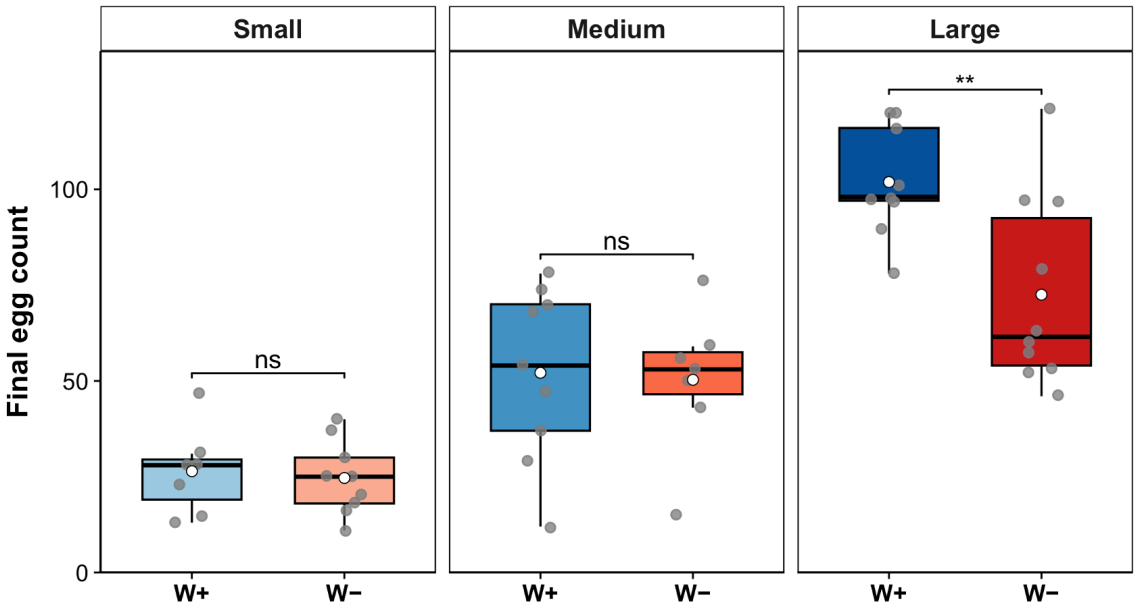

Figure 2-3. Box plot of the final egg counts per group size and treatment (control (W+) vs. worker removal (W-)).

Grey dots represent individual queens' final egg counts. Brackets indicate pairwise comparisons between treatments within each group size. Significance levels are denoted as ns (not significant),(paired T-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Overall, in the best-fit model total brood was significantly explained by the interaction between colony developmental stage and treatment ($\chi^2 = 9.14$, df = 2, p = 0.010). Treatment alone was not significant ($\chi^2 = 1.04$, df = 1, p = 0.308). The total amount of brood in the nest was greater in the large colonies and did not differ between the small and medium colony developmental stages (S vs. M: Z = -1.777, p = 0.18). On average, colonies within the small colony developmental stage contained 284 ± 81 total brood items (including eggs, larvae, and pupae), colonies within the medium colony developmental stage contained 429 ± 80 brood items, and the large colony developmental stage contained 923 ± 189 total brood items.

Discussion

In social insect systems, behavioral plasticity in brood care is often mediated by social cues, including brood-derived signals, that allow colony members to adjust caregiving in response to brood demand. Within these systems, social conditions can be experimentally manipulated to better understand the role of social regulation in mediating brood care behavior, including in the queen caste (Woodard et al. 2013; Sarro Gustilo et al., 2023; Johnson, 2003; Huang et al., 1994; Robinson, 1992). It has been previously described that queen bumble bees reduce their brood care behavior across early colony development, but the timescale upon which this happens, and the degree to which they cease providing care, are nuanced and vary across studies (Heinrich, 1979; Michener,1974; Sladen, 1912; Plath, 1934). There are also some previous reports that these changes in queen behavior are responsive to the social composition of the nest

(Heinrich, 1979; Sakagami, 1976; Benton, 2006; Michener,1974). Empirical studies of social regulation in bumble bees are, however, relatively limited and restricted to the influence of the earliest worker cohort on queens (Woodard et al., 2013; Gustilo Sarro et al., 2021). Here, we used a sociotomy experiment in *B. impatiens*, involving removal of all workers from the nest, to shed new light on the social regulation of queen bumble bee brood care behavior. We found that queen brood care behavior, and specifically incubation, is responsive to social conditions across nest sizes.

We also show that queens continue to provide brood care, particularly incubation, even in the presence of workers and even in highly developed nests, although at relatively low levels. To our knowledge, this study provides one of the first demonstrations that bumble bee queens continue to provide brood care even at the very mature colony stage. There are previous studies showing that during the earliest stages of nest development, when the first cohorts of workers emerge and nests first transition to eusociality, queen brood care behavior sharply diminishes but continues at relatively low levels (Shpigler et al., 2013; Woodard et al., 2013; Sarro Gustilo et al., 2023). We extended observations beyond this initial timeframe in *B. impatiens*. From the emergence of the first few workers up to nests of ~50 workers, we detected no further decline in queen brood care, and incubation persisted even in our most mature nests (~450 workers). Based on our data, we posit that queens never fully relinquish brood care across nest development. Instead, queens continue to provide care in the nest, at least incubation. This continuation of care may be advantageous as queens benefit from maintaining direct influence over developing brood, by shaping offspring developmental trajectories through their

care-related interactions with brood (Michener & Brothers, 1974; Shpigler et al., 2013; Costa et al., 2021; Franco et al., 2023; Chole et al., 2022).

We also show that queens can increase their expression of brood care behavior (specifically, incubation) if they are artificially induced to return to conditions where no other brood care-providing individuals are present in the nest. Even in our largest colony developmental stage, which was designed to reflect the mature nest stage where queens have had workers in the nest for many weeks, queens maintained the ability to increase their incubation behavior following worker removal. The magnitude of increase in queen incubation behavior was the same across all colony developmental stages. This demonstrates that queens remain equally plastic in their responses to social conditions, which is contrary to our original hypothesis that they would exhibit reduced plasticity at larger colony developmental stages, which reflect later stages in colony development.

The plasticity we observed in queens was, however, specific to incubation behavior. This suggests that incubation and feeding are regulated differently in bumble bee queens, with incubation being the more labile of the two forms of brood care. This is consistent with previous work, also in *B. impatiens*, showing that queens adjust incubation and brood-feeding behaviors differently following changes in worker number in the nest (Sarro Gustilo et al., 2023; Woodard et al.,2013). Incubation and feeding are both critical brood care behaviors required for brood development in the nest and thus overall nesting success (Hasselrot, 1960; Heinrich, 1972a; Grad and Gradišek, 2018; Vogt, 1986; Pendrel and Plowright, 1981; Pereboom et al., 2003). Incubation is likely the

more energetically demanding behavior (Heinrich, 1972, 1974; Goulson, 2003), requiring sustained heat production through thoracic muscle activity, with the queen pressing parts of her thorax and abdomen against the brood for extended periods of time (Vogt, 1986; Heinrich, 1979). In contrast, larval feeding is a brief (i.e., a few seconds) event in which caregivers regurgitate food to developing larvae (Free and Butler, 1959; Sarro Gustilo et al., 2023; Sladen, 1912). Incubation is broadly required across all brood stages, including all larval instars and pupae in early colonies (Heinrich, 1972; Heinrich, 1979). Even small decreases in brood temperature can substantially reduce brood developmental rate, survival, and colony growth (Stewart et al., 2021), and restoring heat to cooled brood is more energetically expensive than maintaining stable temperatures (Tulp & Schekkerman, 2006). In contrast, feeding, which occurs far more infrequently, appears to be released by the more acute needs of brood, as it is elicited by a larval hunger signal in bumble bees (Smeets & Duchateau, 2001; den Boer, SPA & Duchateau, MJHM., 2006). Overall, incubation and brood feeding may be uniquely regulated because they are fundamentally different behaviors, involving different physiological and behavioral processes, and because the needs for these different types of brood care differ based on the amount and type of brood in the nest.

We also saw that queen incubation and brood feeding behavior were directed differently with respect to brood type. In our large group, queens performed more incubation toward late-stage brood, which included late-stage larvae, prepupae, and pupae (Plath, 1934; Matsuyama, 2018). We did not detect this pattern in small or medium colony developmental stages, possibly because these colonies contained fewer brood

overall, allowing queens to incubate multiple brood stages simultaneously while covering much of the wax structure. Previous studies with bumble bee workers have similarly shown that workers show a preference towards interacting with pupae over larvae (Starkey et al., 2019), and here we find a consistent pattern in queens. In contrast, feeding was directed almost exclusively (99% of events) toward younger larvae, which are housed in a shared waxen cell. This is despite the fact that older larvae require more feeding (Ribeiro et al., 1999; Ribeiro, 1994; Pereboom et al., 2003). This distinction further highlights the modular nature of brood care, where different brood care behaviors can be targeted toward distinct developmental stages, rather than being uniformly applied across all brood. By directing incubation and feeding to specific brood stages, queens may influence brood developmental outcomes and, by extension, colony function. Queens have a stronger influence, mediated through feeding, on the development of younger larvae (Franco et al., 2023; Chole et al., 2022). Incubation of older brood may reduce the time until worker eclosion, at which point queens will have helpers in the nest. It has been posited that queens benefit from having workers emerge more quickly in the nest, as workers take on most brood care and other work-related tasks, which may allow queens to more fully specialize on egg laying (Peto et al. 2025).

Across animal systems, reproduction and parental care are governed by physiological and temporal trade-offs, in which increased investment in one function often comes at the expense of another (Monaghan et al., 1998; Visser and Lessells, 2001; O'Rourke, CF & Renn, SCP., 2015). In solitary-founding eusocial systems, these trade-offs are additionally mediated by the emergence of helpers, which allow the

primary reproductive individual (the queen) to shift investment away from care and toward reproduction. Within early bumble bee nests, worker removal reduces egg laying, whereas the premature addition of workers accelerates it (Peto et al., 2025; Sarro et al., 2021; Woodard et al., 2013). Consistent with this, we detected a significant decline in queen egg laying in our largest colony developmental stage, notably within just a ~24 hour window following worker removal, which is considerably shorter than the days or weeks examined in previous studies (Sarro et al., 2021; Sarro Gustilo et al., 2023; Shpigler et al., 2013; Fisher et al., 2022). These results suggest that queens do experience a reproductive constraint consistent with the existence of a trade-off between brood care and reproduction, including over short time periods.

Conclusion

In solitary-founding eusocial insect systems, during the onset of nest development, the queen's shift from a primarily caretaking to reproductive role can be interpreted as a developmental reallocation toward maximizing reproduction. Our findings show that this transition is not irreversible, and queens can increase their maternal care when social conditions demand it. This flexibility indicates that reproductive specialization does not necessarily entail a permanent loss of caregiving capacity. This reversibility in queen behavior may be particularly important in unpredictable environments, where offspring survival can be compromised by sudden changes, such as the loss of caregivers. The ability of bumble bee queens to re-engage in parental care under certain conditions highlights the potential advantages of behavioral flexibility in primitively eusocial systems

As a primitively eusocial model system, bumble bees offer a unique opportunity to study the regulation of queen brood care behavior and reproduction in ways that are not possible in many highly eusocial systems. For example, in swarm-founding systems, new queens never pass through a solitary nest-founding stage (Seeley and Buhrman, 1999; Winston, 1987), and therefore never face the caregiving demands that exist during early colony development in primitively eusocial species. The plasticity documented here may reflect behavioral flexibility retained from ancestral repertoires, persisting for up to 70-80 million years since this lineage is estimated to have first evolved sociality (Cardinal and Danforth, 2011) suggests it has been maintained by selection. Rather than representing a vestigial and non-functional trait, this plasticity may serve an adaptive role by enabling queens to adjust their behavior in response to changing social conditions, ultimately sustaining colony growth and reproductive success under variable environments.

Supporting Information:

Definitions and description of data used in each analysis:

Baseline incubation duration: total time (minutes) each queen spent incubating during the post-manipulation phase (8 hours) in control colonies. Only control queens with workers present were used to provide a baseline of incubation across group sizes.

Incubation frequency per hour: the number of incubation events per hour, calculated by dividing the total number of events by the total observation time across each experimental phase (either two (pre-manipulation) or eight (post-manipulation) For the baseline group-size comparison, we used only the post-manipulation control (W+) queens when comparing across group sizes.

Average incubation duration per event: the mean duration of individual incubation events (in minutes) for each queen throughout both experimental phases, calculated for each phase separately. This metric standardizes incubation behavior independent of the total number of events and possible observation hours. For the baseline group-size comparison, we only used the post-manipulation control (W+) queens across group sizes.

Cumulative feeding/incubation: Either the total number of feeding events or total time spent incubating (minutes) across all events observed during mirrored pre-post hours (4-5 and 19-20 pre- and post-manipulation, i.e., total experimental phase). These values were

calculated to enable direct comparisons for a given queen across specific brood care behaviors, as both hours 4-5 and 19-20 are present at both treatment points; see Supplementary Figure 1 for matched timeframes example.

Magnitude of change: the within-individual difference in cumulative incubation duration across experimental phases (calculated "Post" − "Pre"), for worker-removal treatment (W−) queens only, where positive values indicate increased incubation following worker removal, whereas negative values show decreased incubation.

Standardized incubation duration: The proportion of a queen's cumulative incubation time allocated to each brood type, including eggs, younger larvae, older larvae, and late-stage brood. For each queen in the post-manipulation phase, this metric was calculated by dividing the total time spent incubating a given brood type by the queen's total cumulative incubation duration across all brood types during that phase. This standardization allowed incubation allocation to be compared across brood types while accounting for differences in overall incubation effort among queens. Because bumble bee brood structures are inherently three-dimensional, with new brood cells often constructed on top of existing brood (e.g., eggs laid over late-stage brood; Heinrich, 2004; Alford, 1970; Rozen et al., 2018; Sladen, 1912; Michener, 1969), individual incubation events may involve multiple brood types located in close proximity or vertically stacked. Additionally, the top-down perspective of video recordings can make overlapping brood types appear two-dimensional, limiting the ability to distinguish among brood types during some incubation events. These factors were considered during

behavioral scoring, and each incubation event was assigned to the brood type most clearly associated with the queen's position and contact during that event.

Supplement figures:

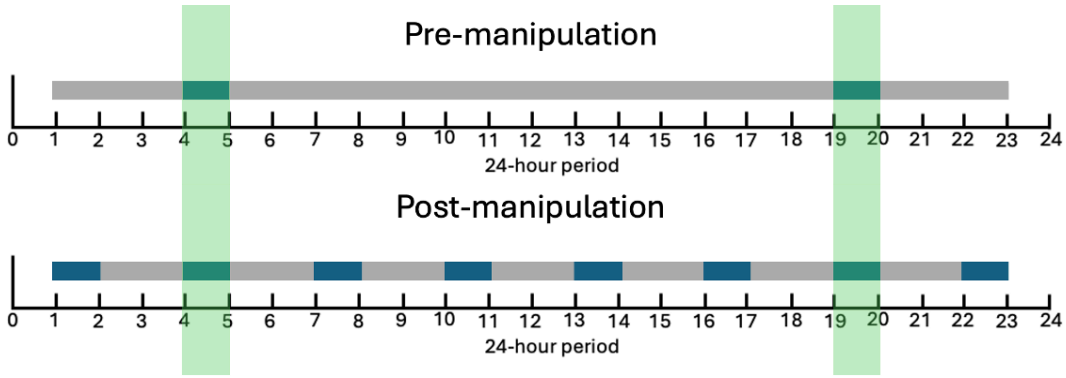

Figure 2-S1. Video sampling schedule used to quantify queen behavior across experimental phases.

One-hour video segments were used for behavioral observations during the 24-hour recording period. Grey horizontal bars indicate the usable recording window (hours 1–23), and dark blue bars represent the observation hours (every three hours). The light green shaded region highlights the two-hour periods used for matched timepoint comparisons between experimental phases. The same sampling scheme was applied for both control and treatment colonies.

Figure 2-S2. Representative video frame illustrating the grid system used for behavioral scoring.

The arena was divided into 24 equal cells (A–D × 1–6), and queen behaviors were assigned to grid cells based on body position, including multiple cells when spanning cells occurred. For visualization, the underlying image is shown at reduced transparency to highlight the grid.

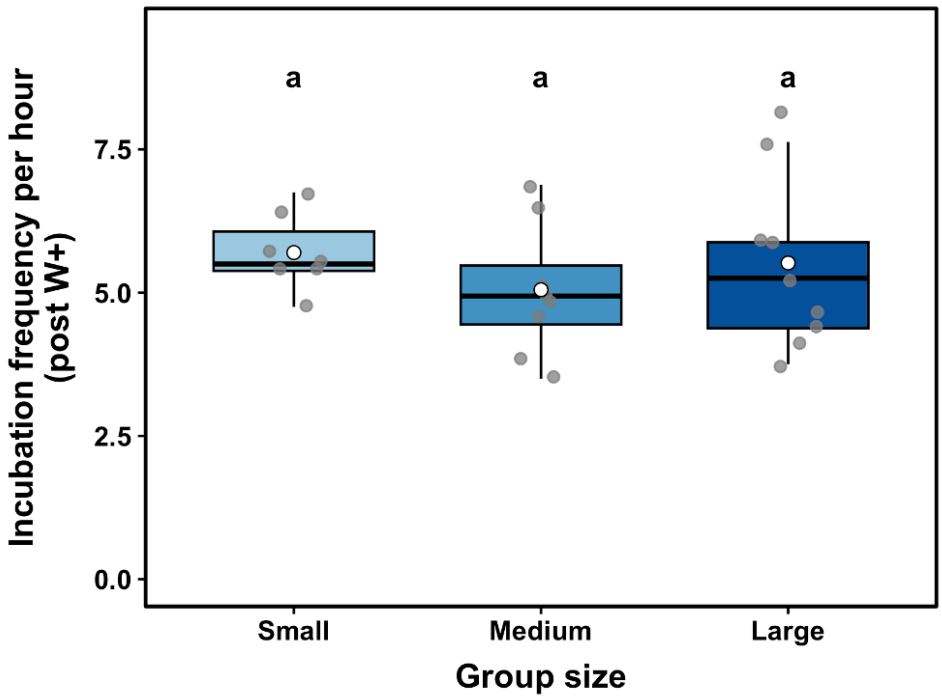

Figure 2-S3a. Incubation frequency in post-manipulation control colonies did not differ across group sizes.

Points represent individual queens, boxplots show the median and interquartile range, and white circles indicate group means. Identical letters above groups indicate no significant differences among group sizes based on *emmeans* pairwise comparisons.

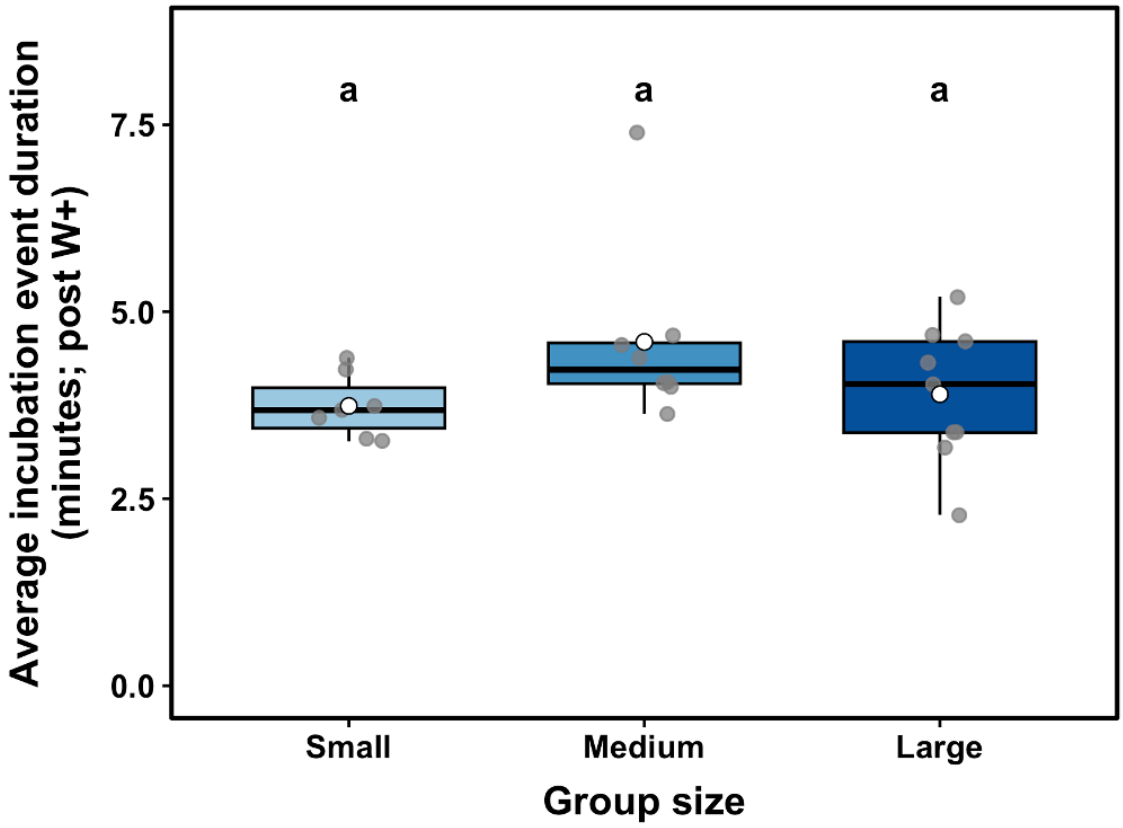

Figure 2-S3b. Average incubation event duration did not differ across group sizes in post-manipulation control colonies.

Points represent individual queens, boxplots show the median and interquartile range, and white circles indicate group means. Identical letters above groups indicate no significant differences among group sizes based on *emmeans* pairwise comparisons.

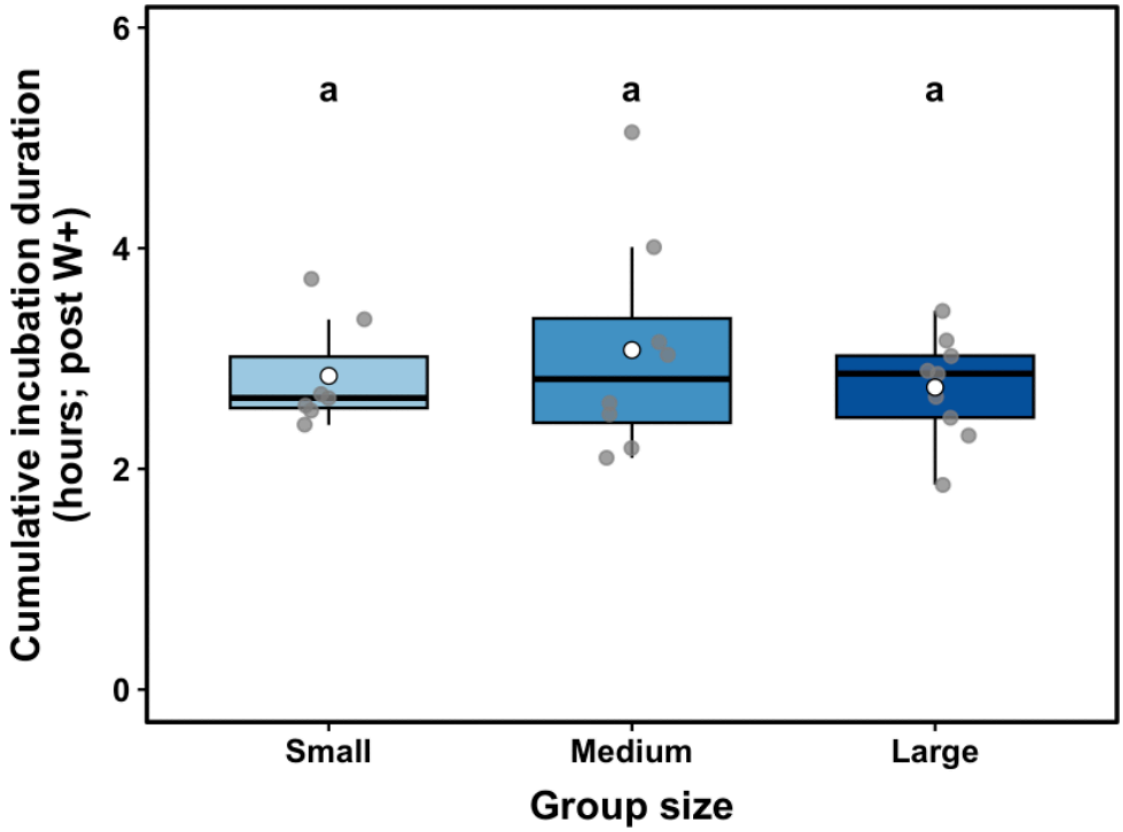

Figure 2-S3c. Cumulative incubation duration did not differ across group sizes in post-manipulation control colonies.

Points represent individual queens, boxplots show the median and interquartile range, and white circles indicate group means. Identical letters above groups indicate no significant differences among group sizes based on *emmeans* pairwise comparisons.

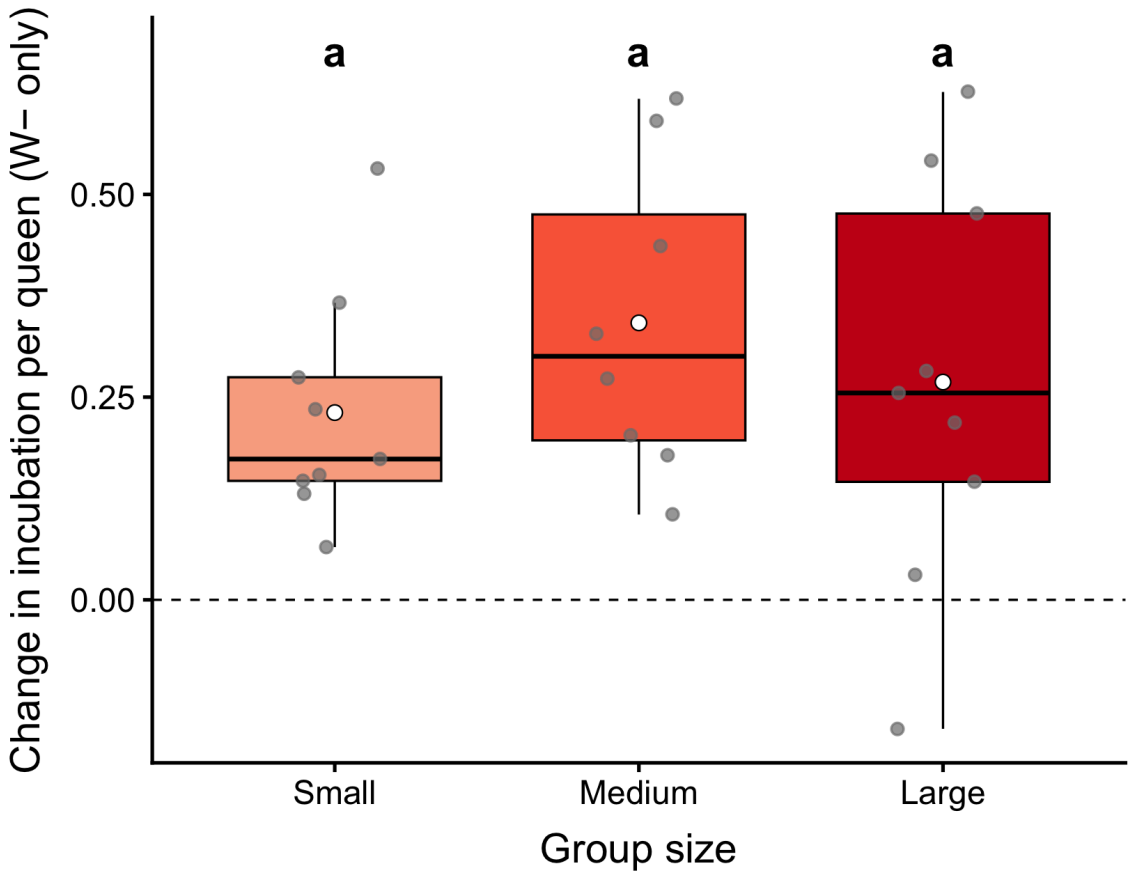

Figure 2-S4. Magnitude of change in cumulative incubation duration per queen across colony sizes following worker removal.

The change in incubation (Post − Pre) did not differ among the small, medium, and large group sizes (all $p > 0.05$). Points represent individual queens, boxplots show median and interquartile range, and white circles indicate group means. The dashed line indicates no change (zero) between experimental phases, and identical letters denote no significant differences among groups per *emmeans* comparison.

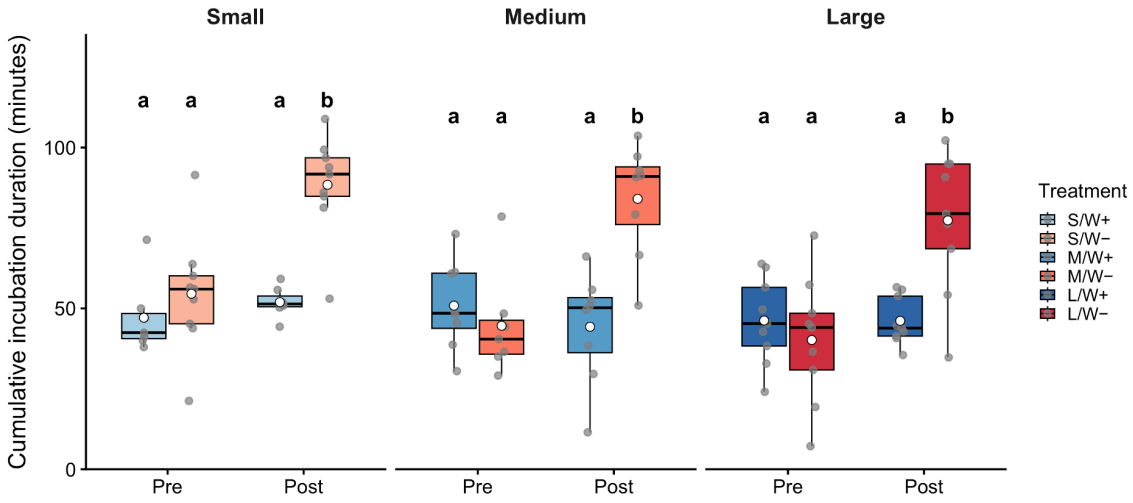

Figure 2-S5. Box plot of cumulative incubation duration (in minutes) across group size and treatment before and after manipulation.

Group sizes are shown in small, medium, and large colonies, and treatments are shown as control colonies with workers present (W+) and worker-removal colonies (W-). Points represent individual queens, and white circles represent group means. Letters indicate post hoc groupings, via emmeans, where groups that do not share a letter are significantly different from one another.

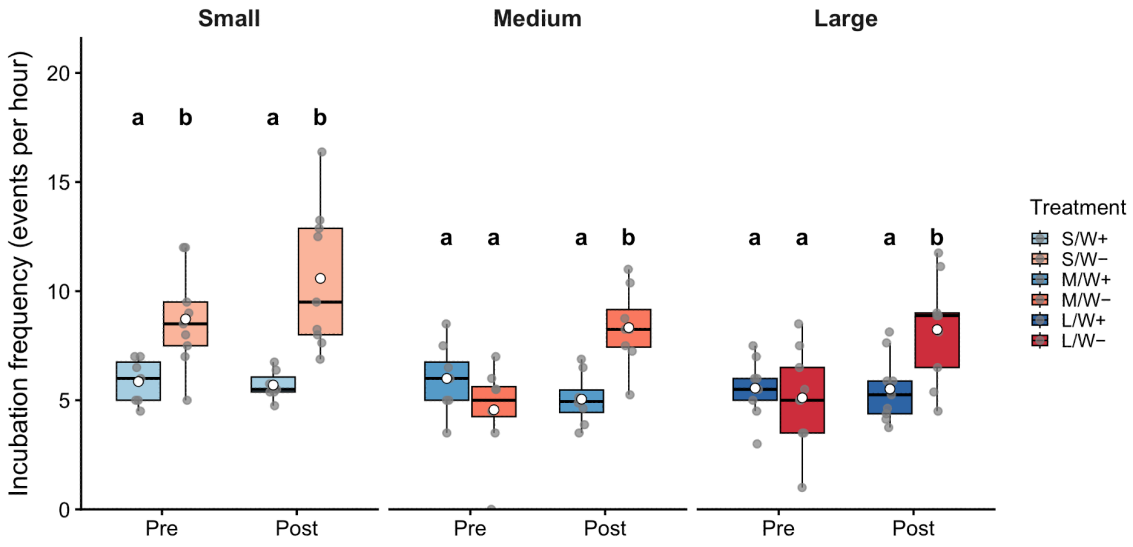

Figure 2-S6. Box plot of incubation frequency (events per hour) across group size and treatment, before and after manipulation.

Group sizes are shown as small, medium, and large colonies, and treatments are shown as control colonies with workers present (W+) and worker-removal colonies (W-). Points represent individual queens, and white circles represent group means. Letters indicate post hoc groupings, where groups that do not share a letter are significantly different from one another.

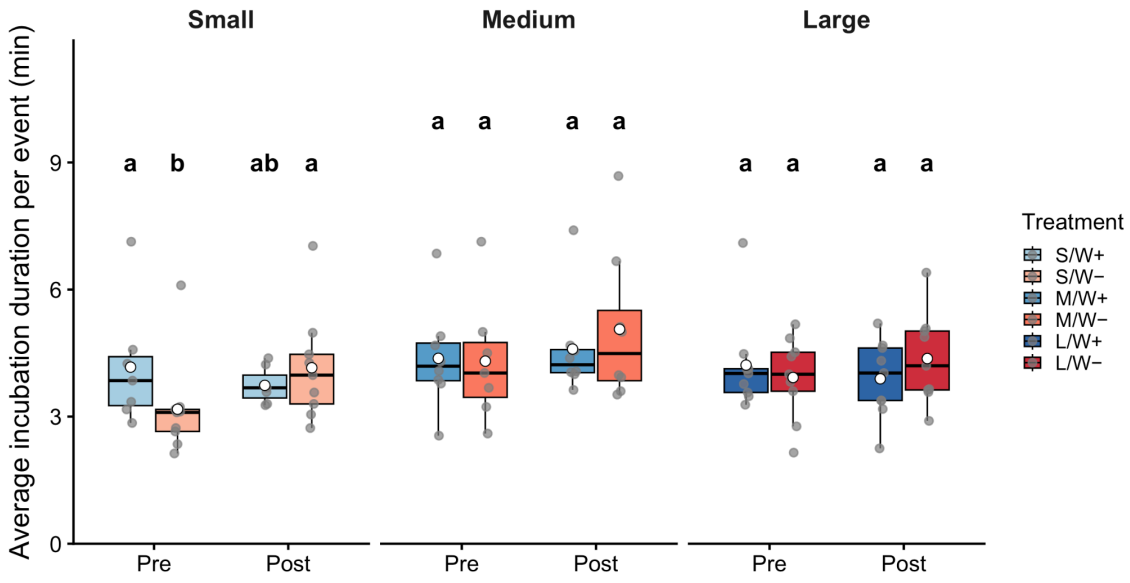

Figure 2-S7. Box plot of average incubation duration per event (minutes) across group size and treatment (control vs. worker removal) before and after manipulation.

Group sizes are shown as small, medium, and large colonies, and treatments are shown as control colonies with workers present (W+) and worker-removal colonies (W-). Points represent individual queens, and white circles represent group means. Letters indicate post hoc groupings, where groups that do not share a letter are significantly different from one another.

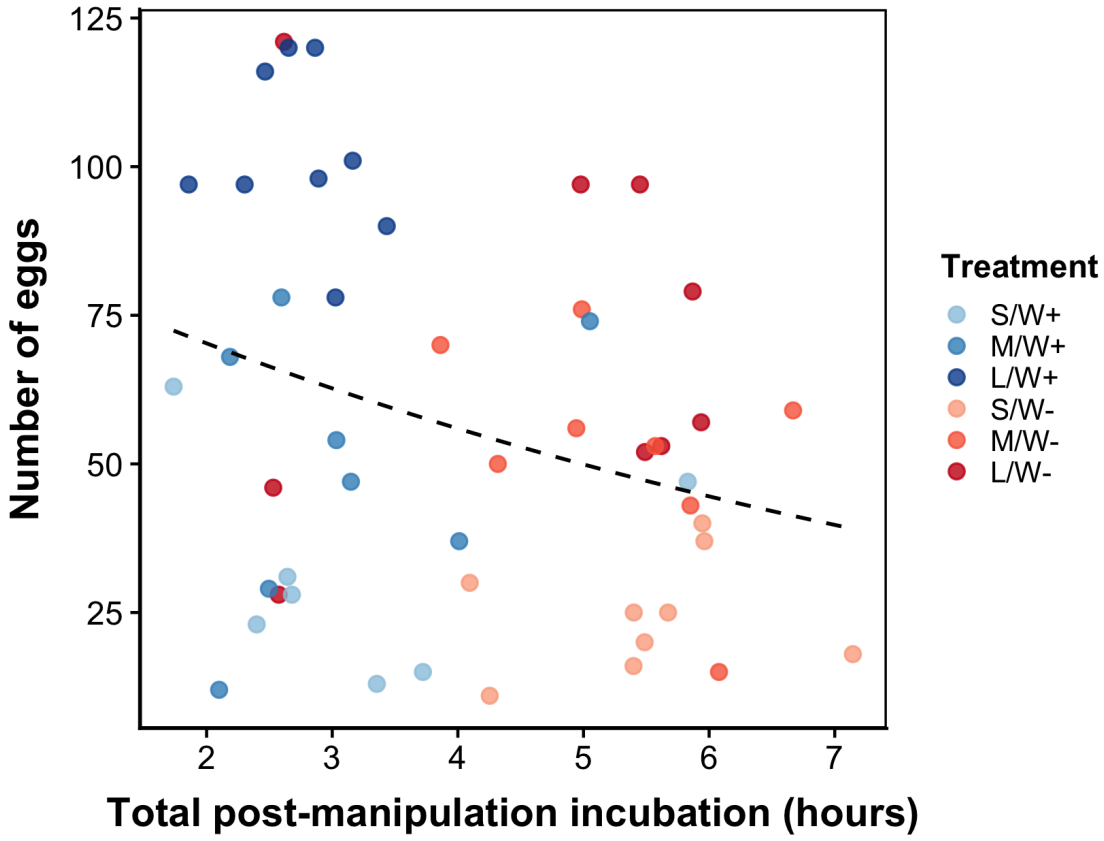

Figure 2-S8. Relationship between total post-manipulation incubation time (hours) and final egg production across colonies.

Each point represents an individual queen, with colors indicating treatment group across colony size and manipulation category (S/W+, M/W+, L/W+, S/W−, M/W−, L/W−). The dashed line represents the overall fitted trend across all queens; significance was assessed using a Wald χ^2 test.

Best-fitting models:

Table 2-S1. Incubation Frequency (50 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	NA	NA	NA	3	166.3
IncFreq1	NA	+	NA	4	164.2
IncFreq2	+	NA	NA	4	166.4
IncFreq3	NA	NA	+	5	164.1
IncFreq4	**NA**	**NA**	*****	**6**	**160.9**

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S2. Incubation Duration (50 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	**NA**	**NA**	**NA**	**3**	**106.5**
IncDur1	NA	+	NA	4	109.0
IncDur2	+	NA	NA	4	109.0
IncDur3	NA	NA	+	5	111.6
IncDur4	NA	NA	*	6	113.1

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S3. Incubation Effort (50 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	NA	NA	NA	3	329.4
IncDur1	NA	+	NA	4	324.5
IncDur2	+	NA	NA	4	328.7
IncDur3	NA	NA	+	5	323.1
IncDur4	**NA**	**NA**	*****	**6**	**315.1**

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S4. Incubation Frequency (15 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	NA	NA	NA	3	146.7
IncFreq1	NA	+	NA	4	145.7
IncFreq2	+	NA	NA	4	147.8
IncFreq3	NA	NA	+	5	146.9
IncFreq4	**NA**	**NA**	*****	**6**	**135.9**

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S5. Incubation Duration (15 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	**NA**	**NA**	**NA**	**3**	**106.3**
IncDur1	NA	+	NA	4	107.3
IncDur2	+	NA	NA	4	108.8
IncDur3	NA	NA	+	5	110.0
IncDur4	NA	NA	*	6	112.8

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S6. Incubation Effort 15 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	NA	NA	NA	3	287.8
IncDur1	NA	+	NA	4	286.1
IncDur2	+	NA	NA	4	285.0
IncDur3	NA	NA	+	5	283.0
IncDur4	**NA**	**NA**	*****	**6**	**272.0**

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S7. Incubation Frequency (5 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	NA	NA	NA	3	158.8
IncFreq1	NA	+	NA	4	160.4
IncFreq2	**+**	**NA**	**NA**	**4**	**136.6**
IncFreq3	NA	NA	+	5	138.0
IncFreq4	NA	NA	*	6	139.2

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S8. Incubation Duration (5 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	**NA**	**NA**	**NA**	**3**	**96.9**
IncDur1	NA	+	NA	4	98.1
IncDur2	+	NA	NA	4	98.0
IncDur3	NA	NA	+	5	99.0
IncDur4	NA	NA	*	6	97.3

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S9. Incubation Effort (5 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	NA	NA	NA	3	290.0
IncDur1	NA	+	NA	4	281.7
IncDur2	+	NA	NA	4	283.2
IncDur3	NA	NA	+	5	274.6
IncDur4	**NA**	**NA**	*****	**6**	**271.8**

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S10. Incubation duration (standardized, 50 workers)					
Model	**T**	**B**	**T:B**	**df**	**AIC**
Null	NA	NA	NA	4	148.2
Brood1B	NA	+	NA	7	96.8
Brood2CT	+	NA	NA	5	148.7
Brood3+	NA	NA	+	8	97.6
Brood4*	**NA**	**NA**	*****	**11**	**95.4**

Included T i.e., Treatment type (W+ or W-), B i.e., Brood type added as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S11. Incubation duration (standardized, 15 workers)					
Model	**T**	**B**	**T:B**	**df**	**AIC**
Null	NA	NA	NA	4	130.9
Brood2CT	+	NA	NA	5	133.0
Brood3+	NA	NA	+	8	90.1
Brood4*	**NA**	**NA**	*****	**11**	**90.4**

Included T i.e., Treatment type (W+ or W-), B i.e., Brood type added as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S12. Incubation duration (standardized, 5 workers)					
Model	**T**	**B**	**T:B**	**df**	**AIC**
Null	NA	NA	NA	4	152.8
Brood1B	NA	+	NA	7	128.5
Brood2CT	+	NA	NA	5	154.2
Brood3+	**NA**	**NA**	**+**	**8**	**129.5**
Brood4*	NA	NA	*	11	131.1

Included T i.e., Treatment type (W+ or W-), B i.e., Brood type added as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold. Best AIC score within one point and biologically relevant

Table 2-S13. Feeding events (50 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	**NA**	**NA**	**NA**	**2**	**104.6**
Feed1	+	NA	NA	3	105.9
Feed2	NA	+	NA	3	106.5
Feed3	NA	NA	+	5	109.9
Feed4	NA	NA	*	4	108.0

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S14. Feeding events (15 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	NA	NA	NA	2	102.0
Feed1	+	NA	NA	3	100.3
Feed2	NA	+	NA	3	101.8
Feed3	NA	NA	+	5	101.3
Feed4	**NA**	**NA**	*	**4**	**100.2**

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S15. Feeding events (5 workers)					
Model	**T**	**EP**	**T:EP**	**df**	**AIC**
Null	**NA**	**NA**	**NA**	**2**	**126.0**
Feed1	+	NA	NA	3	126.6
Feed2	NA	+	NA	3	128.2
Feed3	NA	NA	+	5	130.4
Feed4	**NA**	**NA**	*****	**4**	**129.1**

Included T i.e. Treatment type (W+ or W-), EP i.e. Experimental phase (pre- or post-manipulation time points) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S16. Total Brood					
Model	**G**	**T**	**G:T**	**df**	**AIC**
Null	NA	NA	NA	3	394.3
Brood1	NA	NA	+	6	376.7
Brood2	**NA**	**NA**	*****	**8**	**376.3**

Included T i.e. Treatment type (W+ or W-), G i.e. Group size (5,15 or ~50) as fixed factors and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 2-S17. Incubation vs. Eggs			
Model	**R**	**df**	**AIC**
Null	**NA**	**3**	**488.5**
EggsInc	+	4	490.9

Included T i.e. R (residuls of incubation and treatment) as a fixed factor and natal colony as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

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CHAPTER 3: BODY SIZE VARIATION ACROSS CALIFORNIA ECOREGIONS IN THE YELLOW-FACED BUMBLE BEE (*Bombus vosnesenskii*)

Abstract

Body size variation is a key trait in bumble bee systems that links physiology and environment. Geographic features, such as ecoregions may influence this trait. Studying body-size variation across these geographic axes can provide an understanding of how large-scale landscape features structure phenotypic diversity. *Bombus vosnesenskii* has a broad distribution along the west coast of the United States and is an ideal model for understanding how environmental variation is associated with phenotypic traits as well as population genetics. Using specimens collected from four projects between 2017-2025, we analyzed individuals spanning five distinct ecoregions to evaluate how environmental gradients influence bumble bee body size and population distribution across diverse landscapes. This project, to the best of our knowledge, provides the first large-scale synthesis of population-level variation of *B. vosnesenskii* across the whole state of California.

Introduction

Bumble bees are found worldwide, predominantly in temperate, subarctic, and montane regions of the northern hemisphere, with a small number of species in the lowland tropics (Williams, 1998). Bumble bees are often considered keystone species, providing important pollination services, and visiting a wide variety of flowering species to collect nectar and pollen, which provides essential nutrients for developing larvae

within the colony (Watrous et al., 2019; Woodard et al., 2017). Thus, the spatial and seasonal distribution of floral resources across landscapes can greatly influence not only foraging success, but ultimately colony growth and viability (Dukas and Edelstein-Keshet 1998; Carvell et al., 2007; Cresswell et al., 2000; Goulson et al., 2010). The resource landscapes in which bumble bees naturally inhabit vary among regions, especially across areas differing in climate, vegetation, elevation, and seasonality. Other natural conditions, such as temperature, humidity, and elevation, additionally vary among these landscapes. What is currently unknown is how differences between these landscape features impact bumble bee populations.

California provides a useful system for examining patterns of bumble bee health across different climates. In particular, California encompasses much of the California Floristic Province, one of the world's top 25 biodiverse hotspots, recognized for its high levels of plant and pollinator endemism (Myers et al., 2000). The state also contains a mosaic of 13 EPA Level III Ecoregions, which represent large areas with broadly similar ecosystem types and environmental conditions, including quality and availability of resources (Griffith et al., 2016; Strange and Tripodi, 2019; Omernik, 1987). From coastal chaparral and desert to coniferous forest, these regions reflect spatially structured combinations of interacting environmental elements, including climate, geology, soil, and vegetation and other interacting environmental features. In California, these variable ecoregions provide habitat for a multitude of endemic bumble bee species (Fisher et al., 2021). The highest diversity of bumble bees is found in the coastal, northern, and

mountain regions. Of the ~260 bumble bee species found globally, California is home to twenty-five species found across vastly distinct habitats with highly variable densities (Thorp et al., 1983; Fisher et al., 2022). Among them, *Bombus vosnesenskii* is one of the most widespread and abundant species in western North America, found across diverse habitats, but the highest abundance is found in coastal, northern, and montane regions, and thus a suitable species to investigate how environmental gradients influence size variation.

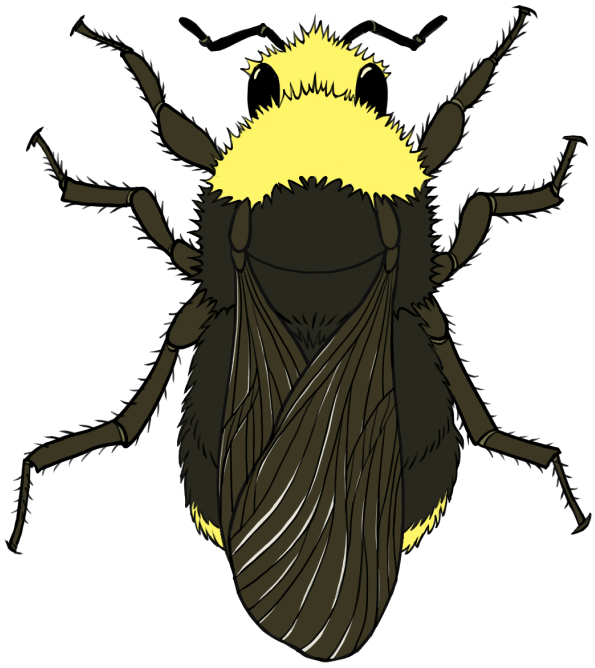

Figure 3-1. Illustration of *Bombus vosnesenskii* worker.

This species was used as the focal organism for examining worker body size variation across California ecoregion. Artwork by Luis F. Garcia.

Broad landscape differences may influence bumble bees in multiple ways, including their distribution, abundance, phenology, and morphology. In particular, body size may be especially responsive to environmental conditions as it is a fundamental biological trait that shapes resource acquisition, survival, and interactions with other organisms (Chown and Gaston, 2010). Across insects, larger body size is often associated with improved performance, competitive ability, and capacity to acquire resources or mates (Chole et al., 2019; Chown and Gaston, 2010). Additionally, larger individuals may live longer or achieve higher reproductive success than smaller individuals, although the strength and direction vary across taxa and ecological contexts (Beukeboom et al., 2018). In bumble bees, worker body size variation is biologically significant as it is associated with differences in behavior, physiology, and colony-level task performance, including social interactions within the nest and foraging activity (Chole et al., 2019). Despite the importance of body size for bumble bee ecology, relatively little is known about how worker size varies across broad landscape features. Many previous studies have focused on body size variation at smaller spatial scales, or across narrower ecological gradients (Retzlaff, 2018; Theodorou et al., 2020; Austin et al., 2022; Persson, AS and Smith, HG, 2011, but see Fitzgerald et al's review (2022), Greenleaf et al's review (2007) and Gérard et al.'s study of body size across time (2020). These gaps limit our understanding of whether broad regional differences in climate, vegetation, elevation, and seasonality are associated with morphological variation in bumble bee populations.

Here, we examine how body size varies across ecoregions in *Bombus vosnesenskii* across California. In bumble bees, variation in worker body size can have important consequences for colony function, including social interactions, brood care, and foraging (Chole et al., 2019). Important gaps remain in our understanding of how dynamic worker size variation is across natural landscapes and how this variation is shaped by ecological factors. Examining body size across ecoregions may provide a useful framework for examining these patterns, as they represent broad landscape areas with similar environmental conditions, including climate, vegetation, elevation, and seasonality. These conditions can influence the timing, abundance, and diversity of floral resources available to bumble bees throughout the season, which may in turn affect worker development and body size. By comparing body size across ecoregions, we can test whether broad habitat differences are associated with variation in worker body size. Specifically, we examine whether variation in worker body size is associated with ecoregion identity, and whether measured geographic variables such as elevation and latitude help explain additional variation within or across ecoregions. To do this, we combined mixed-effects modeling with multivariate analyses to (1) quantify differences in body size among ecoregions and (2) evaluate whether relationships between body size and environmental variables are consistent across regions or vary by ecological context. We hypothesized that body size would be structured by environmental conditions associated with ecoregions, which encompass gradients such as elevation and geographic variation. If environmental factors are the primary drivers, we expect their effects to be consistent across regions. Alternatively, if regional differences persist after accounting for environmental variables,

this would suggest that additional ecological or biogeographic factors contribute to variation in body size.

Materials and Methods

Site Selection and Shared Sampling:

Leveraging specimens collected from four projects between 2017 and 2025, we integrated data from approximately 66 sites spanning California's coastal, agricultural, urban, and mountain regions (Figure 3-2). For the Southern California, Sierra Nevada, and APHIS projects, sampling followed standardized Woodard Lab field protocols. Sites were selected based on accessibility, permitted access, evidence of local bumble bee activity, and the presence of flowering plants that are visited by *Bombus vosnesenskii*. At each site, workers were collected while actively foraging on flowers using a sweep net under suitable conditions, avoiding periods of rain or high winds. GPS coordinates and elevation were recorded at the time of collection. Collected individuals were transferred to tubes and immediately placed on dry ice (López-Uribe et al., 2025) to ensure rapid euthanasia and preservation for later analyses. By integrating specimens from multiple existing projects, this study captured broad spatial and ecological variation across California while reducing the need for additional collection effort. All sites were assigned to EPA Level III Ecoregions following established classification frameworks (Fisher et al., 2022; Strange and Tripodi., 2019).

Southern California collections

Bees from Southern California were collected over two consecutive years, from 2024 to 2025. During May–July 2024, *Bombus vosnesenskii* were sampled across 12 sites spanning both natural and urban environments. At each site, a target of approximately 15 workers was collected by two or three collectors. Building on this initial effort, additional sites were sampled in May-July 2025, seven additional sites were sampled using the same field collection methods described above. These additional sites were selected based on prior collections and observational records to increase sampling coverage and capture additional spatial variation in *B. vosnesenskii* populations across Southern California.

Sierra Nevada collection:

During April–July 2017, specimens were collected in the Sierra Nevada mountains. Twenty workers were collected at each of the 15 sites.

APHIS Project collections:

During April and July 2021, specimens were collected by collaborators, with sampling efforts focused primarily along the coastal regions of California. Twenty workers per site (n = 14) were captured.

Cascade mountain range collection:

Sampling was conducted by collaborators from Oregon State University during May–August of 2023–2024 in the Cascades ecoregion of northeastern California. Using

their own lab-specific sampling protocol, collaborators collected *Bombus vosnesenskii* workers (n = 15 per site) from a total of 18 sites that were selected on privately managed lands, including both treated fuel breaks and untreated reference sites, to capture environmental variation across the landscape. Individuals were collected throughout the summer flight season using a combination of standardized hand-netting surveys and passive trapping methods, including blue vane traps and colored pan traps. Specimens were collected, dried, and pinned prior to this study, body size measurements were taken from preserved specimens rather than freshly collected individuals.

Body size measurements

We measured the marginal cell size of all adult workers (n=969) collected using the Minitool Measuring Scale (in mm). The length of the second marginal cell of both wings was measured and averaged for each bee as a proxy for body size. In bumble bees, the second marginal cell is highly correlated with overall body size (Medler, 1962; Owen, 1988; Costa et al., 2021; Costa et al., 2025; Shpigler et al., 2013).

Statistical Analysis

All statistical analyses were conducted in R (2025.05.1+513), and statistical significance was assessed at $\alpha = 0.05$. Average wing size via marginal cell size was used as a continuous proxy for body size. Prior to analysis, individuals with missing wing size, collection, site, latitude, altitude, or ecoregion information were removed.

To assign individuals to ecoregions, georeferenced collection records were converted to spatial point data using latitude and longitude coordinates. These points were spatially joined to California Level III ecoregion shapefiles using the sf package. Ecoregion names were simplified for interpretation, and analyses focused on five focal ecoregions: Coast Range, Coastal Sage, SoCal Mountains, Sierra Nevada, and Cascades. Spatial data processing and map visualization were conducted using the sf and *tmap* packages.

Data distributions were evaluated using visual diagnostics, density plots, histograms, and Shapiro–Wilk tests. Because average wing size was modeled as a continuous response variable and model residuals were evaluated directly, we used linear mixed-effects models to test predictors of marginal cell size. Models were fitted using the lme4 package, with average wing size as the response variable and collection site included as a random intercept.

To test whether marginal cell size varied across ecoregions, we constructed a set of candidate models representing biologically relevant predictors of average wing size. Candidate models included a null model with only site as a random effect, an ecoregion model with ecoregion as a fixed effect, a temporal model including collection month and collection year, and a full model including ecoregion, collection month, and collection year. Model selection was performed using Akaike's Information Criterion corrected for small sample size (AICc) with the model.sel function in the MuMIn package. The model

with the lowest AICc value was considered the best-supported model. Fixed effects in selected models were evaluated using Type II analysis of variance from the car package.

To determine whether temporal variation influenced patterns of marginal cell size, we first assessed the effects of collection month and year. The additive temporal model provided the best fit, indicating that seasonal and interannual variation contributed to observed body size patterns (see Supplement). With that in mind, we used the two factors (month and year) for the remaining analyses as fixed effects.

Following model selection, post hoc pairwise comparisons among ecoregions were conducted using estimated marginal means with the emmeans package. Pairwise contrasts were adjusted for multiple comparisons using Tukey-adjusted tests. Compact letter displays were generated to visualize statistically distinct ecoregion groups in figures.

To evaluate whether broad environmental gradients explained variation in marginal cell size, we fit additional linear mixed-effects models testing latitude, altitude, and their combined effects while retaining the collection site as a random effect. These models included a null model, a latitude model, an altitude model, and a combined latitude and altitude model, with collection month and collection year included as covariates where appropriate. Models were compared using AICc to determine whether latitude and/or altitude improved model fit relative to the null model.

Because preliminary results indicated strong differences among mountainous regions, we also conducted a subset analysis including only individuals from the Cascades, Sierra Nevada, and SoCal Mountains. For this analysis, we compared additive and interaction models including ecoregion, latitude, altitude, and their interactions. The best-supported mountain-region model was selected using AICc, and fixed effects were evaluated using Type II analysis of variance. Pairwise comparisons among mountain ecoregions were conducted using estimated marginal means, and compact letter displays were used to visualize significant differences among regions.

Model assumptions were assessed using residual diagnostic plots, quantile-quantile plots, fitted-versus-residual plots, and simulated residuals using the DHARMa package. Data wrangling, summaries, and visualization were conducted using packages including tidyverse, dplyr, ggplot2, ggpubr, and RColorBrewer. Full candidate model structures, model selection results, diagnostic outputs, and pairwise comparisons are provided in the Supplementary Materials.

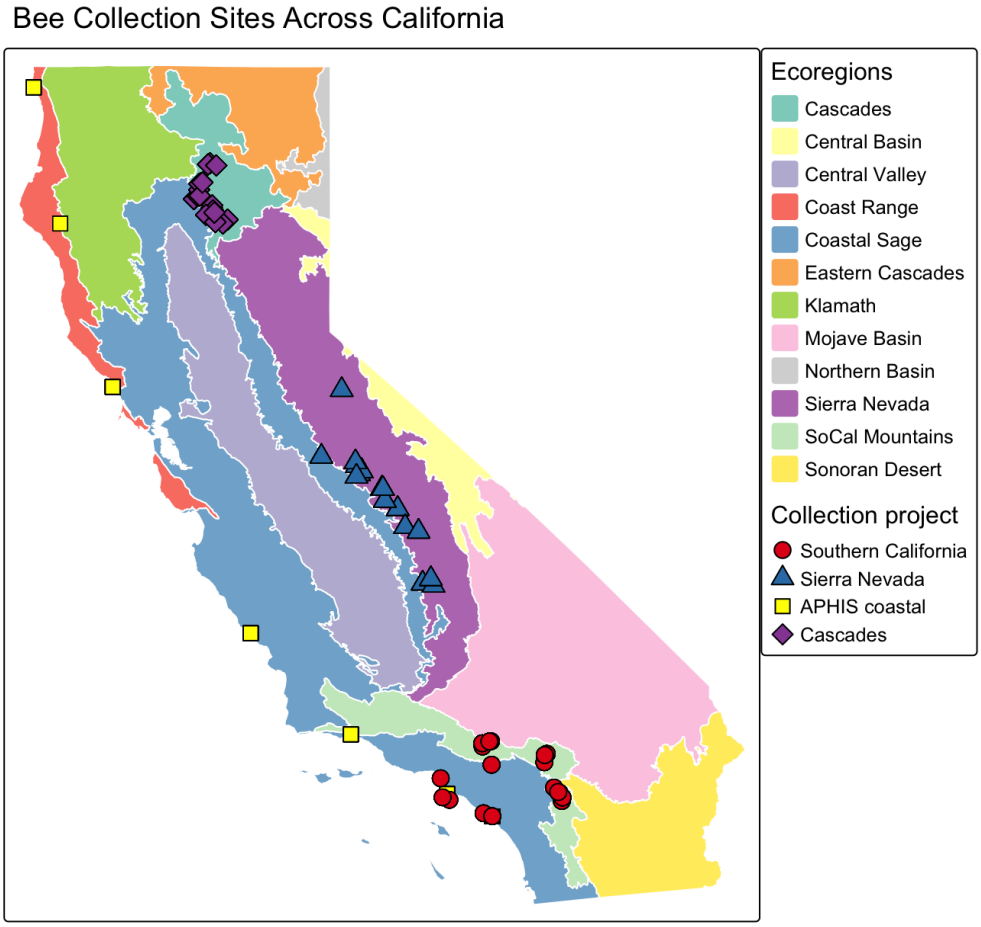

Figure 3-2. Map of *Bombus vosnesenskii* collection sites across California.

Points represent sampling locations (n = 65) included in this study and are differentiated by collection project: Southern California collections are shown as red circles, Sierra Nevada collections as blue triangles, APHIS coastal collections as yellow squares, and Cascades collections as purple diamonds. Sites span 5 of the 13 EPA Level III Ecoregions in California, with ecoregions indicated by background color (see legend).

Results

Covariates of eco-region:

Latitude and altitude were included as fixed-effect predictors to assess whether wing size varied along geographic gradients. Model selection strongly supported the null model, while models containing altitude, latitude, their additive or interaction received no support. This suggests that simple geographic gradients alone did not explain variation in marginal cell size in our dataset. Therefore, neither latitude nor altitude was included in the primary ecoregion models (see below).

Eco-region and body size:

Marginal cell size differed significantly among eco-regions ($\chi^2_4 = 57.91$, $p < 0.0001$). Mean wing size was largest in the Cascades (3.07 ± 0.27 mm) and smallest in the Southern California Mountains (2.72 ± 0.29 mm), with intermediate values observed in the Coast Range (2.92 ± 0.18 mm), Coastal Sage (2.87 ± 0.22 mm), and Sierra Nevada (2.80 ± 0.28 mm; Figure 1). Pairwise comparisons using emmeans revealed significant differences among several ecoregions (Tukey-adjusted comparisons; see Figure 3-3), with bees in the Cascades exhibiting significantly larger wing sizes than those in the Southern California Mountains ($p < 0.0001$) and Sierra Nevada ($p < 0.0001$), and bees in Coastal Sage exhibiting larger wing sizes than those in the Southern California Mountains ($p = 0.047$). However, there were no significant differences between Coast Range and any

other ecoregion ($p = 0.31$), nor between Coastal Sage and the Sierra Nevada ($p = 0.78$), or between the Southern California Mountains and the Sierra Nevada ($p = 0.36$).

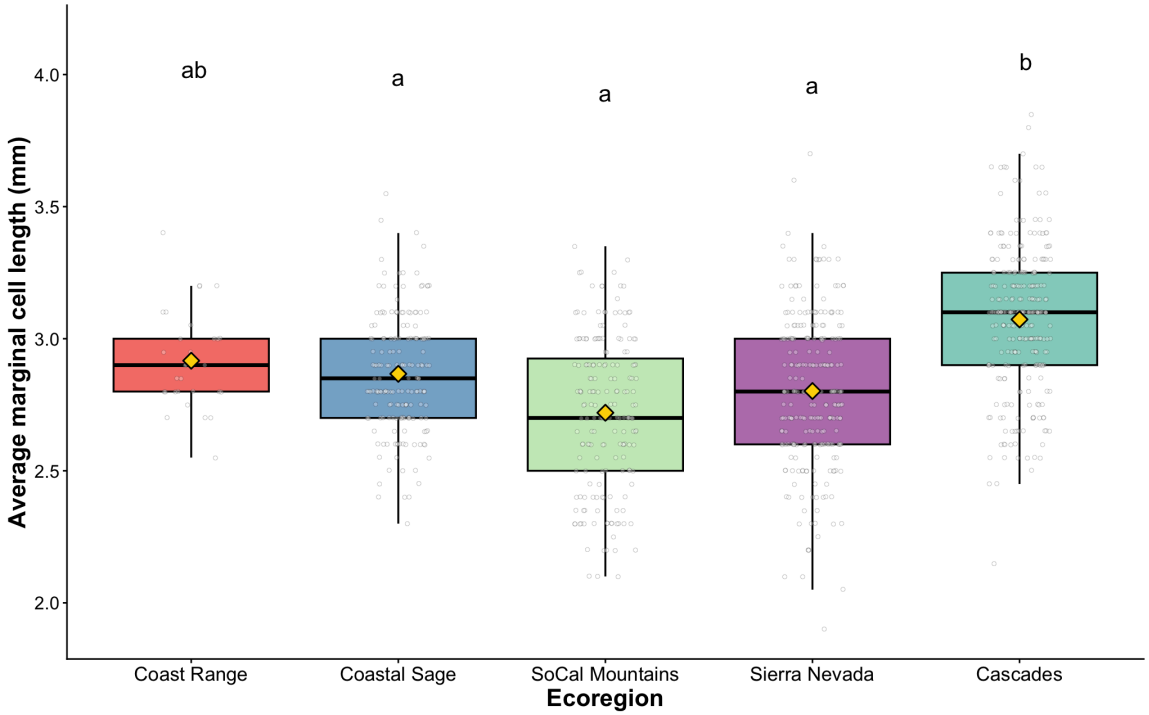

Figure 3-3. Marginal cell size variation across California ecoregions.

Points represent individual bees, and boxplots summarize the distribution of marginal cell length as a proxy for wing size within each region. Boxes represent the interquartile range (25th–75th percentiles), the horizontal line indicates the median, and whiskers extend to the 5th and 95th percentiles. Golden diamonds indicate mean wing size. Letters denote significant differences among ecoregions based on post hoc comparisons from a mixed-effects model that included collection month and year as covariates.

Regional differences persist within mountain systems:

To determine whether regional differences in body size persisted within broadly similar habitat types, we restricted analyses to montane ecoregions (Southern California Mountains, Sierra Nevada, and Cascades). Within montane ecoregions, average wing size differed significantly among regions ($\chi^2_2 = 14.65$, $p < 0.001$; Figure 3-3). Marginal cell size ranged from 2.72 ± 0.29 mm in the Southern California Mountains to 3.07 ± 0.27 mm in the Cascades, with an average of 2.80 ± 0.28 mm observed in the Sierra Nevada. Altitude was associated with wing size ($\chi^2_1 = 4.75$, $p = 0.029$), whereas latitude showed no significant effect ($\chi^2_1 = 3.54$, $p = 0.060$). Models including interactions between geographic variables and ecoregion were not better supported than simpler models.

Discussion

Body size is an important trait that can influence how animals interact with and survive in their environments. Here, we demonstrate that body size is variable across California's different eco-regions in *Bombus vosnesenskii*, with larger bees generally found in the Cascades and Coast range. Both the Cascades and Coast range receive relatively high annual precipitation compared with many other California regions, suggesting that broad climatic differences, such as precipitation, may contribute to regional variation in body size by influencing floral resource availability. Differences were especially pronounced among montane ecoregions, where bees from the Cascades were larger than individuals from the Sierra Nevada and Southern California mountains.

These patterns suggest that body size variation in *B. vosnesenskii* may be shaped by spatially structured ecological processes across California landscapes, including broad climatic differences among regions.

One possible contributor to body size variation across ecoregions is regional variation in floral resources. As these ecoregions differ in climate, vegetation, and seasonality, they may strongly influence key aspects of bumble bee colony development. Floral diversity, bloom timing, and plant community composition vary regionally and can influence both food availability and nutritional quality (Sutcliffe and Plowright, 1998; Schmid-Hempel and Schmid-Hempel, 1998; Wray et al., 2014). For example, montane regions such as the Cascades may support extensive meadow systems that provide relatively consistent, high-quality floral resources throughout the season. At the community level, greater floral diversity and higher abundances of native plant species have also been linked to larger-bodied bees, suggesting that resource-rich environments may facilitate the production of larger individuals (Fitzgerald et al., 2022). In bumble bees, adult body size is generally determined during larval development and fixed after eclosion. While caregivers (either workers or the mother queen) can impact brood size (Shpigler et al., 2013; Costa et al., 2021; Knee and Medler, 1965) as well as the location of the larvae with the nest (Couvillon and Dornhaus, 2009), external factors, such as floral nutrition and developmental temperature, can also influence larval growth, survival, and body size (Heinrich 1972; Plowright and Jay, 1977; Plowright and Pendrel, 1977; Couvillon and Dornhaus, 2009). In particular, differences in quantity and pollen quality, particularly protein content, can have cascading effects on body size distributions

(Pendrel and Plowright, 1981; Tasei and Aupinel, 2008; Vaudo et al., 2020). Consistent with this, larvae fed higher-protein pollen tend to develop into larger adults, a pattern observed both in bumble bees and other bee taxa (Rotheray et al., 2017; Li et al., 2014; Zerbo et al., 2001). As such, regional differences in floral resources, driven by climatic factors, may contribute to body size variation across California ecoregions.

In addition to floral resource availability, abiotic conditions may also contribute to differences in body size across Californian landscapes. In bumble bees, exposure to higher developmental temperatures can be associated with smaller adult size, particularly under warmer or more extreme thermal conditions (Guiraud et al. 2021). As bumble bees are generally adapted for the cooler temperatures of the temperate and montane habitats, a bigger body size is generally considered beneficial for these regions for heat retention, flight performance, and foraging efficiency (Bishop and Armbruster, 1999; Peat et al. 2005). Consistent with Bergmann's rule and a broader pattern across organisms, larger individuals are often associated with colder environments, including high-elevation habitats (Peters et al., 2016; Osorio-Canadas et al., 2016). In our study, bees from the Cascades were larger on average than individuals from the Sierra Nevada and Southern California mountains, suggesting that body size differences specifically among mountainous ecoregions may reflect a broader environmental difference across these regions. We did not find strong support for altitude as an independent predictor of marginal wing cell size across the full dataset, indicating that elevation alone does not explain the observed patterns. Instead, differences among mountainous ecoregions may reflect regional conditions, including temperature regimes, season length, floral

phenology, level of urbanization, etc. While resource availability may contribute to these patterns, temperature and persistent abiotic conditions may also contribute to development and final adult morphology. Larger workers may benefit from greater flight capacity and the ability to collect more resources during a single foraging trip, although a larger size can have higher energetic costs (Pyke, 1978). As such, body size differences are likely tied to persistent environmental conditions associated with where bees occur (Fitzgerald et al., 2022).

One aspect to consider is that bumble bees, in particular, exhibit tremendous variation in body size distribution both within and among species (Michener, 2000). Within a single colony, the body size distribution can vary by as much as a factor of ten (Couvillon et al. 2010), whereas other non-*Bombus* eusocial bee species demonstrate a narrower size variation (Roulston, 2000). This size variation also greatly varies across the nest development timescale. Workers within early nests are generally smaller than in larger nests. Thus, collections in the early season (early-late spring) are expected to be generally smaller when compared to later in the season (late summer/early fall) (Goulson, D. 2010). Although there was some variation in body size across the season, we did not find that body size significantly differed.

Together, there are many factors that can influence body size variation in bumble bees. In this study, we focus on broad landscape-level patterns in natural settings, demonstrating that body size in *Bombus vosnesenskii* varies across eco-regions. However, body size can be influenced by additional factors not directly measured here, including

colony age, colony size, brood composition, level of queen and worker care, and genetic background. As bumble bees face declines due to shifts in climate, land use, habitat loss, and reduced floral resource availability, assessing body size variation can be especially important. Future work should examine whether observed phenotypic differences among regions may also be associated with underlying genetic structure or differences in population connectivity. Understanding these patterns can provide critical insight into how bumble bee populations are shaped by their environments and how they may respond to ongoing changing landscapes.

Conclusion

Bumble bees exhibit natural variation in body size within and among colonies due to factors such as genetic background, nutrition, development, and colony conditions. Here, we examined body size variation in the yellow-faced bumble bee (*Bombus vosnesesnkii*) across California to determine whether broad geographic patterns in body size are associated with ecoregional identity. Across the five ecoregions assessed, bees from the Cascades and Coast Range were generally larger than those from other regions. When focusing specifically on montane ecoregions, bees from the northern part of the state were significantly larger than those from other mountainous areas. A more in-depth understanding of the underlying genetic structure may help elucidate why these distributional patterns occur. Together, our findings provide an initial step toward understanding how landscape-level variation may shape bumble bee body size and broader population dynamics across a large geographic area.

Supporting Information

Collection Date and Body Size:

We tested whether the body size of the bumble bees collected was influenced by the month or year they were collected. Average wing size as measured by marginal cell length varied across collection periods, with both collection month ($\chi^2 = 23.594$, df = 4, $p<0.001$) and year ($\chi^2 = 13.825$, df = 4, $p<0.001$) contributing to variation in wing size. Due to this, we included both "month" and "year" in the remaining analyses.

Best-fitting models:

Table 3-S1. Check if the month/year influences body size

Model	**M**	**Y**	**M:Y**	**df**	**AIC**
Null	NA	NA	NA	3	111.4
m1	+	NA	NA	7	89.9
m2	NA	+	NA	7	98.3
m3	**NA**	**NA**	**+**	**11**	**84.8**
m4	NA	NA	*	15	87.5
m5	NA	NA	NA	5	95.1

Included M, i.e., Month collected, Y i.e., Year collected as fixed factors and collection site as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.
*Note: In model 5, site, collection month, and collection year were all included as random effects to evaluate how much variation in average wing size was attributable to sampling location and temporal grouping factors, rather than to fixed environmental predictors.

Table 3-S2. Testing predictors of body size across California ecoregions					
Model	**E**	**Y**	**E:Y**	**df**	**AIC**
Null	NA	NA	NA	3	116.2
eco	**+**	**NA**	**NA**	**7**	**97.2**
time	NA	+	NA	11	119.6
full	NA	NA	+	15	116.5

Included E, i.e., ecoregion, Y i.e., Time collected (collected month + collected year) as fixed factors and collection site as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.

Table 3-S3. Environmental covariate model set					
Model	**L**	**A**	**L:A**	**df**	**AIC**
Null	**NA**	**NA**	**NA**	**3**	**116.2**
lat	+	NA	NA	12	120.7
altitude	NA	+	NA	12	139.2
both	NA	NA	+	13	140.8
mix	NA	NA	*	14	158.1

Included L, i.e., latitude, and A i.e., altitude, as fixed factors and collection site as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.
*Note: Collection month and collection year were included as fixed-effect covariates in all non-null models to account for temporal variation in sampling.

Table 3-S4. Montane ecoregion effects on wing size					
Model	**E**	**A/L**	**E:A/L**	**df**	**AIC**
Null	NA	NA	NA	3	148.1
add	+	+	**NA**	**7**	**142.2**
int	NA	NA	+	11	178.3

Included E, i.e., ecoregion, A/L i.e., latitude and altitude together, as fixed factors and collection site as a random factor. The best model fitting our data was selected based on the Akaike's Information Criterion (AIC). (+) parameters included in the model, and NA, not included parameters. The selected best model is given in bold.
*Note: for the "int" model included ecoregion-by-altitude and ecoregion-by-latitude terms to test whether environmental gradients were associated with wing size differently across montane ecoregions.

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Conclusions

In this dissertation, I explore several understudied aspects of bumble bee biology, including the dynamic interactions among workers, brood, and queens within developing colonies. I also begin to examine how colonies interact with their broader environments, by examining the relationship between worker body size and environmental variation across landscapes. Together, my work suggests that bumble bee queens retain plasticity in both their reproductive and brood-care providing behaviors, and that this flexibility may be important for supporting colony development under changing social conditions. Additionally, variation in worker body size across ecoregions suggests that colony members may also differ in response to broader environmental conditions.

In my first chapter, I found that *B. impatiens* queen egg-laying is socially regulated by both workers and developing brood and follows a dynamic yet stereotyped pattern during the early stages of colony development. During the initial stages of nest initiation, accelerated egg-laying in queens was associated with the presence of workers or older larvae and pupae. Additionally, the presence of workers was needed for queens to maintain increased levels of egg laying across this early developmental stage. In the second chapter, I found the shift from brood care to reproductive specialization is not fixed after worker emergence; queens perform these behaviors in the presence of workers and increased incubation after workers were experimentally removed. Finally, in the third chapter, I explored body size variation in a native bumble bee species, *Bombus vosnesenskii*, across five different ecoregions of California. I found that workers were

generally larger in the Cascade region, particularly when comparing mountainous ecoregions.

Together these findings contribute to a broader understanding of plasticity in social organisms across different contexts. Previous work on bumble bee social behavior has largely focused on worker dynamics within the nest, with less attention to how queens respond across different stages of sociality. My work shows that the transition between subsocial and eusocial colony organization is not necessarily fixed or irreversible. Instead, queens can remain responsive to social cues from both workers and brood, suggesting that flexibility may be an important feature of early colony development.

This work also provides insight into how a common, native bumble bee species varies across environmental contexts. By using specimens collected through previous projects, this dissertation also gives existing collections new scientific value while helping rescue the need for additional collection. Overall, this work contributes to the field of bumble bee biology and ecology by improving our understanding of how bumble bees interact with one another within colonies and how colony members vary across landscapes.

Future work should build on these findings by examining whether observed body size differences across ecoregions are associated with underlying genetic structure, environmental variation or both. More broadly, linking behavior, physiology, morphology and multivariable environmental change and how plasticity may contribute to colony success and population persistence.