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Bumble Bee Queen Plasticity and Social Regulation of Traits

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by

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DEDICATION

To all the bees who gave their lives for, or were otherwise a part of, this research.

ABSTRACT OF THE DISSERTATION

Bumble Bee Queen Plasticity and Social Regulation of Traits

by

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

This dissertation presents a deep dive into the behavior and physiology of spring queen bumble bees. Bumble bee queens emerge from diapause and initiate new nests independently in spring. As they lay eggs and subsequently rear offspring to adulthood, queens undergo a dramatic transition from a solitary, to subsocial, to eusocial lifestyle, which presents a unique opportunity to investigate phenotypic plasticity and life history transitions. This colony founding stage of their life cycle may also represent a particularly important demographic for bumble bee populations, many of which are experiencing dramatic declines. An improved understanding of this life stage may help inform conservation strategies and predict bumble bee responses to a changing world.

In this dissertation, I investigate behavioral and physiological plasticity in bumble bee queens across the spring colony founding stage. In Chapter One, I show that queen ovary development and nest searching and occupation occur simultaneously, which may enable rapid nest initiation. In Chapter Two, I show that the emergence of workers in incipient nests increases queen survival and reproduction, suggesting that the timing of early worker emergence in the nest likely impacts queen fitness, colony developmental trajectories, and ultimately nesting success. In Chapter Three, I show that queens respond to the emergence of workers in young nests by performing fewer brood care and food collection tasks, suggesting that queen maternal care behavior is tightly regulated by the number of helpers in the nest. And finally, in Chapter Four, I show that wild queens spend most of each day foraging via short, frequent trips, which highlights the heightened demands placed on early season queens.

Collectively, this work has yielded three major insights into spring queen bumble bee biology. First, it suggests that queens are highly plastic in the incipient stages of colony founding and have the capacity to change dynamically in response to social and environmental fluctuations. Second, it suggests that starting nests earlier in the season may be advantageous for bumble bees. Lastly, it underlines the importance of conservation interventions that support the early nesting period and facilitate the production and maintenance of workers in incipient nests.

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INTRODUCTION

The ability to synchronize behavior and physiology with environmental conditions is critical to individual survival and fitness. Phenotypic plasticity enables organisms to respond dynamically to their current environmental conditions. Plasticity encompasses both the capacity to modify developmental trajectories (e.g., environmental sex determinism in reptiles, (Leigh *et al.*, 1976); seasonal color polyphenism in butterflies, (Shapiro, 1976)) and respond to variable environments and experiences (e.g., learning, immune response, behavior). Phenotypic plasticity can also be organized as ontogenetic polyphenisms, whereby individuals progress through distinct life stages with unique phenotypes separated by transitional phases. For example, frogs (order Anura), true jellyfish (class Scyphozoa), and holometabolous insects (superorder Endopterygota) all transition through distinctive life phases such as larval and adult states characterized by prominent differences in behavior and morphology.

Eusocial animals exhibit complex suites of plastic traits, including social phenotypes, making them excellent systems in which to study phenotypic plasticity from a variety of perspectives. Eusocial animals are characterized by a reproductive division of labor, overlapping generations, and cooperative brood care (Batra, 1966). Individual castes express caste-based phenotypes such as specialized morphology or reproductive status. These differing phenotypes, however, are typically not based in genetic differences. Rather, social environmental factors such as nutrition (Slater *et al.*, 2020) and subsequent phenotypic plasticity control caste determination (Schwander *et al.*, 2010). Within a single caste, too, individuals exhibit ontogenetic polyphenisms as they progress through various life history stages, as well as temporally expressed phenotypes in response to the resource and social environments. For example, eusocial honey bee workers exhibit an age-based polyethism, in which they transition, as they age, from carrying out within-nest tasks such as nursing brood, to foraging tasks such as collecting pollen and nectar (Wilson, 1971).

The eusocial bumble bees (genus *Bombus*, family Apidae) are annually eusocial insects in which queens transition from solitary, to subsocial, to eusocial life stages (Fig 0-1). Queens emerge from diapause in the spring and subsequently initiate new nests solitarily. Once queens lay eggs in these nests, they transition to subsociality, in which they carry out all tasks related to larval rearing independently. After queens successfully rear their first offspring to adulthood, they together transition to eusociality as the newly emerged workers take over foraging and brood care tasks. This ontogeny of sociality coincides with a suite of (as of yet vastly understudied) behavioral and physiological changes in the queen (Free and Butler, 1959), and provides a unique opportunity to study phenotypic plasticity and the social regulation of traits.

The ontogeny of sociality in bumble bee queens also occurs during a foundational stage of the bumble bee life cycle: the colony founding life stage. This stage is thought to be particularly sensitive to environmental stressors, which may help explain national and global declines in bumble bee populations. However, the colony founding stage and the factors that limit nesting success remain severely understudied. Bumble bees are among the most economically important managed native pollinators in North America (National Research Council, 2007). Queen bumble bees play a vital role in early season pollination, because they emerge early in the season when temperatures are cool and few other pollinators are able to fly (Willmer *et al.*, 1994). Despite the economic importance of colony founding queens, current conservation strategies focus primarily on supporting bumble bee colonies during the eusocial phase of their life cycle (Goulson *et al.*, 2007). The needs of spring queens remain largely unknown and unaddressed, although this stage may represent a particularly important demographic for bumble bee populations. Subsocial queens must both forage and perform all the tasks required for colony success and reproduction, so this stage may respond particularly strongly to environmental stressors such as diminishing or degraded floral resources, urbanization, pesticide use, and higher temperatures, and may help explain national and global declines in bumble bee populations (Goulson *et al.*, 2007, 2015; Cameron *et al.*, 2011). An improved understanding of this life stage in variable environments may help predict bumble responses to a changing world.

In this dissertation, I use bumble bees as a model system to explore physiological and behavioral phenotypic plasticity and its connection to social regulation and the resource environment. In addition to investigating phenotypic plasticity in bumble bee queens from a purely biological perspective, I also aim to inform conservation strategies with my research, by elucidating life history details during this understudied, yet fundamental stage of the bumble bee life cycle. Here, I explore bumble bee queen life history, plasticity, and the social and environmental regulation of traits. Specifically, I explore the transition from solitary to subsocial to eusocial lifestyles in bumble bee queens as measured by behavioral plasticity in response to the social environment (Chapter One), physiological plasticity in response to the social environment (Chapter Two), and behavioral and physiological plasticity in response to the ecological environment (Chapters Three and Four).

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Figures

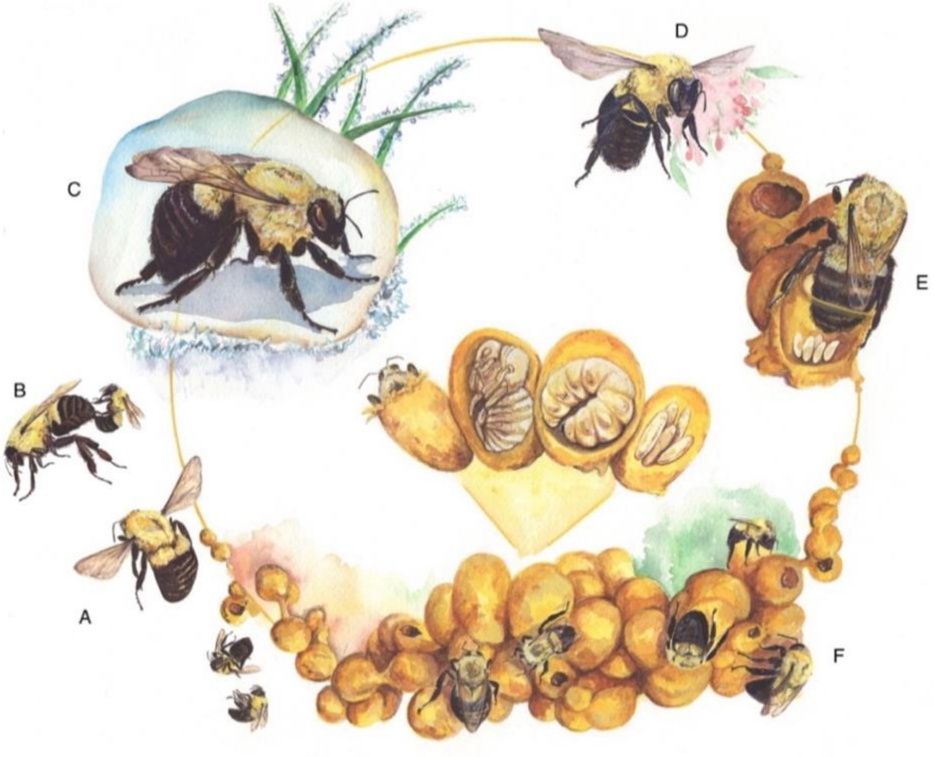

Figure 0-1. Diagram of bumble bee life cycle (Free and Butler 1959; artwork by T Jung, in collaboration with E Sarro). [A] Newly eclosed queens-to-be (gynes) emerge from their natal colony. Natal colony dies as winter approaches; [B] Gynes mate with a single male; [C] Mated gynes overwinter in a diapause state underground; [D] Overwintered gynes emerge from diapause in the spring, begin to develop their ovaries, and search for a suitable nesting site; [E] Gynes initiate a new nest independently and transition to subsociality; [F] Subsocial foundresses successfully rear their first set of adult offspring and transition to eusociality. They are now called queens. Workers take over the brood care and foraging tasks, queens focus on egg laying, and the colony grows over the course of the season.

CHAPTER 1: BUMBLE BEE (B. VOSNESENSKII) QUEEN NEST SEARCHING OCCURS INDEPENDENT OF OVARY DEVELOPMENTAL STATUS

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Abstract

Studies on the physiological states of wild-caught organisms are essential to uncovering the links between ecological and physiological processes. Bumble bee queens emerge from overwintering in the spring. At this time, queens develop their ovaries and search for a nest site in which to start a colony. Whether these two processes, ovary development and nest-searching, interact with or influence one another remains an unresolved question in behavioral physiology. We explored the hypothesis that ovary development and nest-searching might be mechanistically connected, by testing whether (1) ovary development precedes nest-searching behavior; (2) nest occupation precedes ovary development; or (3) ovary development and nest-searching occur independently, in bumble bee (*Bombus vosnesenskii*) queens.

We collected queens either nest-searching (and thus prior to occupying a nest) or pollen-collecting (and thus provisioning an occupied nest) and measured their degree of ovary activation. We further screened these queens for parasites or other symbionts, to identify additional factors that may impact their reproductive success at this time. We found that queens searched for and occupied nests at all stages of ovary development, indicating that these processes occur independently in this system. Nest-searching queens were more likely to have substantial mite loads than pollen-collecting queens, who had already located and occupied a nest. However, mite loads did not significantly predict ovary developmental status. Collectively, our work shows that nesting status and symbionts alone are insufficient to explain the variation in spring bumble bee queen ovary development. We propose that ovary development and nest-searching occur opportunistically, which may enable queens to begin laying eggs earlier in the season than if these processes occurred in discrete succession.

Introduction

Life history transitions are catalyzed by underlying physiological and behavioral processes, which often interact with and influence one another. The organizing influence of these underlying processes is considered adaptive in that it can help organisms respond to or synchronize with changes in environmental conditions (Snell-Rood, 2013). In female animals, the transition to reproductive maturation is dominated by the physiological process of ovary development. This process is mediated by age-related, intrinsic factors and also external environmental factors, such as parasite infestation or food resource availability (Labeyrie, 1978; Wheeler, 1996; Roy *et al.*, 2018). The onset of ovary development, in turn, can cause changes in behavior. For example, in vertebrate and invertebrate animals, mature ovaries produce the hormones estrogen and ecdysteroids, respectively, which impact behaviors as diverse as host competition (Mathiron *et al.*, 2020), aggression (Bolingbroke and Kass-Simon, 2001), and courtship (Ganter *et al.*, 2011). Identifying how the onset of ovary development is mediated, and how this organizes downstream changes in behavior and physiology, is a key goal in behavioral physiology research.

The bumble bees (family: Apidae, genus: *Bombus*) are an exemplary group to study life history transitions and changes in reproductive state. This is because their annual social colony cycle contains distinct, caste-specific phases that vary dramatically in reproductive output (Fig 1-1). Queens are the primary reproductive caste in bumble bees. During early adulthood, queens have entirely undeveloped ovaries (Heinrich, 2004), a status that is mediated in part by low levels of the gonadotropic hormone juvenile hormone (JH) (Larrere *et al.*, 1993). After leaving the nest and mating, queens overwinter, then emerge from diapause in spring. Across these stages, the ovaries continue to remain undeveloped (Heinrich, 2004). However, over the course of several weeks in the spring, JH levels rise (Larrere *et al.*, 1993), which precipitates the onset of ovary development. Around this time, queens also find a suitable place to lay their eggs and later begin to oviposit (Alford, 1971; Heinrich, 2004). Queen reproductive output accelerates during the early stages of nest development and is then sustained over much of the life of the colony (Frison, 1930). This process of reproductive acceleration is mediated in part by the social environment itself, as the presence of workers positively influences queen JH levels and ovary development (Woodard *et al.*, 2014; Sarro *et al.*, 2021). As the colony reaches its maximum size and the queen approaches the end of her life, queen egg laying can decline, and worker egg laying can increase (Alaux *et al.*, 2004, 2006). Studying systems such as bumble bees where a combination of developmental, social, and ecological conditions can impact ovarian development is especially important for understanding complexity in the regulation of reproduction. Furthermore, in important pollinators such as bumble bees, research into reproductive biology and plasticity can help inform life-stage-specific conservation regimes in this lineage (Woodard, 2017; Malfi *et al.*, 2019; Sarro *et al.*, 2021).

An unresolved question in bumble bee biology is whether the onset of queen ovary development might be a key driver, or a consequence, of locating and occupying a nest. Life history theory predicts that reproduction and nest-searching behaviors may be limiting to one another (e.g., the “flight-fecundity tradeoff” Zera and Denno, 1997; Tigreros and Davidowitz, 2019). Therefore, queens may invest in one process or the other, in discrete succession. Moreover, ovaries are thought to be involved in the master regulation of both social behavior and reproduction in social Hymenopteran systems (e.g., “ovarian ground plan hypothesis”; West-Eberhard, 1987). This is because changes in reproductive status coincide with differences in nesting behavior in social wasps (West-Eberhard, 1987, 1996). Pleiotropic links between reproductive and social traits have been detected in some social insect systems, such as honey bees (Amdam *et* *al.*, 2006; Oldroyd and Beekman, 2008), which supports this hypothesis, albeit not in bumble bees. Thus, ovary development may itself induce nest-searching behaviors in bumble bee queens, in which case queens should develop their ovaries prior to searching for a nest. Alternatively, queens may develop their ovaries subsequent to, or as a consequence of, locating and occupying a nest. This pattern has been observed in subsocial systems such as burying beetles, where the behaviors involved in preparing an oviposition site directly induce ovary development (Pellissier Scott and Traniello, 1987). It is also consistent with a pattern observed in bumble bees themselves, where social environmental changes that occur around the time of nest foundation (specifically, the emergence of workers) have direct, positive impacts on queen ovary development and egg production (Woodard *et al.*, 2013; Sarro *et al.*, 2021). This finding demonstrates that signals associated with early nest foundation can positively impact ovary development in queen bumble bees. A third possibility is that ovary development occurs independent of the behaviors involved in locating a nest. That these two phenomena occur around the same time and are both prerequisite to oviposition does not necessarily indicate that they are coregulated.

Additional factors, such as parasites, can further influence ovary development in bumble bee queens around the nest foundation life stage. For example, infection by the nematode *Sphaerularia bombi,* also known as the queen castrating parasite (Colgan *et al.*, 2020), can result in complete inhibition of ovary development in spring queens (Lundberg and Svensson, 1975). Additionally, fungal parasites such as *Vairimorpha* *bombi* and *Apicystis bombi* have negative effects on queen fat body reserves and survival (Schmid-Hempel, 2001; Mullins *et al.*, 2020), which might indirectly influence ovary development and nest initiation. In some bumble bee species, parasite infection is insufficient to explain failed bumble bee nest initiation (Mullins *et al.*, 2020), and parasites may impact nest founding queens differently than workers in established colonies, on which the majority of parasite research focuses (e.g. Macfarlane *et al.*, 1995; Malfi and Roulston, 2014). Life-stage-specific investigations of parasites on wild bumble bees, and particularly bumble bee queens, are rare.

Here, we examined queen bumble bee ovary development during the spring nest-founding period. Specifically, we quantified the degree of ovarian development in queens of the yellow-faced bumble bee, *B. vosnesenskii,* collected both before or after nest occupation. Our goal was to determine whether (and if so, how) reproductive physiology is synchronized with the process of locating and occupying a nest in wild bumble bee queens. If the onset of ovary development is a driver of nesting behavior in this system, we predicted that both queens before and after nest occupation would have highly developed ovaries. Alternatively, if the behaviors involved in locating and establishing a nest are a driver of ovary development, we predicted that queens who had not yet occupied a nest would have relatively undeveloped ovaries, and only those who did occupy a nest would have developed ovaries. If ovary development and nesting are uncoupled in bumble bee queens, however, we predicted that we would observe both queens with and without developed ovaries both before and after nest occupation.

It is important to note here that because our study is observational, data supporting any of these predictions would not directly indicate whether nest occupation and ovary development are co-regulated. Instead, we offer these predictions as a first step to investigating these hypotheses. In addition to measuring ovary development, we also screened all queens in the study for external and internal symbionts and determined the degree to which these spring queens were parasitized. This allowed us to explore how symbiotic relationships may act as an additional ecological factor that limits ovary development and/or nest founding in spring bumble bee queens.

Materials and Methods

Bee collections

B. vosnesenskii queens (n = 68) were collected from montane regions of Riverside and San Bernardino counties of California in the spring of 2020. All collection sites (n = 3 in Riverside County with <5 km between sites; n = 1 in San Bernardino County) were dominated by blooming manzanita (*Arctostaphylos* spp.) woodlands at 1700-2100 m elevation, with dry, rocky substrate. In addition to abundant manzanita, available floral resources included sparse California lilac (*Ceanothus* spp.) and lupine (*Lupinus* spp.). *B. vosnesenskii* is overwhelmingly the most common bumble bee species in California (Thorp *et al.*, 1983). In Southern California, queens of this species emerge from diapause in early spring (Williams *et al.*, 2014) and, as is true of most bumble bee species, select a suitable nesting site prior to the beginning of summer (Kearns and Thomson, 2001). *B. vosnesenskii*, like most bumble bees, typically nest in the ground (Kearns and Thomson, 2001). Queens were individually hand-netted between April 25 and May 30, 2020, from the hours of 09:00-18:00 on sunny days with ambient temperatures between 13-27 °C. Spring queens are readily differentiated from workers based on size (Chole *et al.*, 2019) and to a lesser extent phenology (Williams *et al.*, 2014), thus we are confident that all collected bees were queens and not workers. Queens were collected directly onto dry ice and subsequently stored at -80 °C until further processing. Queens from all three behavioral categories (see below) were observed on every collection day. Queens collected do not necessarily reflect the overall proportions of queens carrying out each behavior in the field on a given day. This is because the choice of individuals sought for collection on a given day were influenced by collections on previous days, in order to acquire comparable sample sizes across behavioral categories (see below).

We recorded the behavior of each queen at the time of collection and only included queens in our collections that fell into one of three clearly-defined categories: nest-searching, pollen-collecting, and nectaring (Table 1-1). The first two categories (nest-searching and pollen-collecting) are distinct, non-overlapping behavioral states that are reliable indicators of nest status (Figure 1-2). This is because pollen collection does not occur until a nest site has been located (Vogt *et al.*, 1994). Thus, these two categories were the main focus of our study.

Nest-searching queens (n = 26) were observed actively searching for a suitable nest site. Nest-searching is a readily identifiable, stereotypical behavior in which queens fly low to the ground in a zigzag pattern and occasionally stop to investigate potential nest site locations (Kearns and Thomson, 2001; Video 1-S1). This behavior has been well-described in previous studies, including ones where it has been examined as an indicator that queens are investigating potential nest sites prior to locating and occupying a nest (Vogt *et al.*, 1994; Svensson *et al.*, 2000; Lanterman *et al.*, 2019).

Pollen-collecting queens (n = 20) were observed with pollen loads within their corbiculae, the pollen-collecting structure located on the hind legs. These queens had already selected a nest site and were foraging for food resources to bring back to the nest, either to provision it before or after laying the first set of eggs, or to feed directly to developing larvae (Alford, 1971; Röseler, 1985). Although pollen collecting is a reliable indicator that a queen has located and occupied a nest site, it cannot be used to infer whether or not queens have initiated egg laying. This is because queens provision the nest with some food (pollen and nectar) before laying the first set of eggs, and then continue to forage as their first offspring develop.

Our third behavioral category (“nectaring”) consisted of queens observed manipulating flowers without collecting pollen, who were presumably feeding on or collecting nectar (n = 22). Queens in this category may or may not have yet located a nest. We collected this third set of queens to establish a more complete picture of spring queen ovary development, including queens outside of the nest-searching and pollen-collecting categories (e.g., prior to nest-searching). Exactly when queens begin to search for nests after emerging from diapause is not known, thus this category might encompass a broader timescale of queens that includes queens in a stage that is prior to our nest-searching category. As a result, we expected the greatest variation in ovary development within this group, relative to the nest-searching and pollen-collecting groups. Additionally, this set of queens enabled us to explore whether, given an individual's degree of ovary development and symbiont loads, we could predict an individual queen's nest status.

In addition to our collections of *B. vosnesenskii*, we also collected a small number of queens from seven additional species of the same subgenus (Pyrobombus: *B. bimaculatus, B. impatiens, B. melanopygus, B. perplexus, B. sandersoni, B. ternarius, B. vagans*) in the state of Maine, to explore cross-species patterns. Details on these collections can be found in the Supporting Information.

Bee dissections and symbiont screenings

First, we examined symbiotic organisms on or in the queens, noting the presence of both parasites and other symbionts such as mites that have an uncertain relationship with bumble bees. Queens were inspected for symbionts following the protocol in Mullins *et al.*, 2020. The exterior of each bee was inspected under a dissecting microscope prior to dissection, and any external organisms found were classified to the most refined taxonomic unit possible and individually stored in ethanol. Queens were pinned to a sterile dissecting dish, ventral side up, and an incision was made along the abdomen to expose the internal contents. The interior of the abdomen was then inspected for macroparasites, and again, any parasites found were individually identified and stored in ethanol. Tissue samples of the midgut, hindgut, fat body, and spermatheca were mounted in acid fuchsin stain on labeled glass microscope slides with coverslips for subsequent microparasite screenings (see below).

Next, we quantified ovary development in all queens. Ovaries were removed and the terminal oocyte in each ovariole (total of eight per bee) was subsequently measured in millimeters using an ocular micrometer and staged (I-IV) according to Duchateau and Velthuis, 1989. Staging quantifies the relative size of each oocyte and its associated trophocyte and thus measures oocyte maturity independent of body size. Binary oocyte resorption status (resorbed or unresorbed) was also recorded for each terminal oocyte. Oocyte resorption is common in *Bombus* queens, whereby females reabsorb the nutrients from mature egg cells they do not lay; resorption occurs in response to barriers to oviposition such as limited resources or social inhibition of egg laying (Cumber, 1949; Medler, 1962; Duchateau and Velthuis, 1989). Resorbed oocytes can be reliably identified by their yellow, misshapen appearance (Duchateau and Velthuis, 1989; Fig 1-S1).

Microparasite screenings

Lastly, we examined queen microparasite loads. Microparasite screenings were conducted following the visual protocol in Mullins *et al.*, 2020. Briefly, slides were inspected under 400x magnification for any spores of *Apicystis spp.* or *Vairimorpha spp.*, *Locustacarus buchnerii*, or other microparasites. Suspected positives were subsequently confirmed by two or more authors. We did not screen for *Crithidia,* a common bumble bee parasite, because it was not possible to distinguish using our methodology. *Crithidia* spp. can negatively impact nest initiation in lab-reared queens of *Bombus terrestris* (Brown *et al.*, 2003; but see Baron *et al.*, 2017), but has had no significant impact on nest initiation in *B. vosnesenskii, B. huntii*, (Mullins *et al.*, 2020), or *B. pratorum* (Rutrecht and Brown, 2008).

Statistical analyses

Analyses were carried out in R statistical software version 4.0.3. To assess the factors related to ovary development in *B. vosnesenskii* queens, we used generalized linear mixed models (GLMMs) using the glmer() function from the lme4 package (v. 1.1-23; Bates *et al*. 2015) with measures of ovarian activation (including all oocyte lengths per bee, maximum oocyte length per bee, and proportion of oocytes resorbed per bee) as response variables. We included behavioral category (nest-searching, pollen-collecting, or nectaring), collection date, and presence/absence of external mites as possible fixed effects. No other symbionts were found in more than one *B. vosnesenskii* queen, and thus no others were included in statistical analyses. Collection county was included as a random effect in all models. Bee identity was also included as a random effect in oocyte length models to account for multiple measurements per individual. We tested all possible models for each response variable based on all additive combinations of fixed effects, while holding random effects constant. The best fit model for each response variable was chosen based on the lowest Aikaike's Information Criterion (AICc) using the model.sel() function from the car package (v. 3.0-7; Fox and Weisberg, 2019). Resorbed oocytes were removed from oocyte length analyses, because resorption can result in misshapen oocytes with unreliable length measurements (Medler, 1962; Duchateau and Velthuis, 1989). We used a Chi-square test to determine whether symbiont presence was dependent on behavioral state across all species.

When all oocytes from all *B. vosnesenskii* bees were analyzed together with bee identity included as a random effect, oocyte length was highly correlated with oocyte stage (GLMM $p < 0.001$; Conditional $R^2 = 0.768$). Length measurements were more precise than stage measurements, thus we did not include oocyte stage in any statistical analyses.

All of the above analyses were also performed separately on queens of *B. ternarius*, which was the one species from the additional queen samples with a sufficient sample size ($N = 27$; $n = 6$-10 per behavioral category) for statistical analysis. Here, we excluded collection date and included presence/absence of *Sphaerularia bombi* as possible fixed effects, because all *B. ternarius* queens were collected on a single day and multiple individuals were infected with *S. bombi*.

Results

The best fit models predicting oocyte length and maximum oocyte length in *B. vosnesenskii* queens were the null models. The best fit model predicting oocyte resorption in *B. vosnesenskii* queens included collection date as the sole fixed effect. The random effect collection county explained very little variation ($\tau_{00} < 0.02$) in all three best fit models.

Degree of ovarian activation was independent of behavioral state in *B. vosnesenskii* queens. Oocyte length and resorption status did not differ among behavioral categories (GLMM behavioral category not included in best fit models for oocyte length, maximum oocyte length, or resorption; Fig 1-3). In all behavioral categories, queens had oocytes ranging from < 0.5 to > 3.5 mm length [representing a full distribution of undeveloped (stage 1-2) to fully developed (stage 4) oocytes], indicating that queens of all levels of ovarian development were observed in all behavioral states. Ovary development data from additional species corroborated the trends observed in *B. vosnesenskii* (Fig. 1-S2).

Among *B. vosnesenskii* queens, which were collected over the course of five weeks, collection date was not a significant predictor of oocyte length (not included in best fit models for oocyte length or maximum oocyte length; Fig 1-4A). The proportion of oocytes resorbed per queen, however, did significantly increase over the course of the season, independent of behavioral state (GLMM $p < 0.001$, estimate = 0.02, 95% CI = 0.01 - 0.02; Fig 1-4B).

Incidence of symbiont infection was low in queens of *B. vosnesenskii*, with only a single queen of this species showing signs of infection by *Vairimorpha* (Table 1-2). External mites were found on 19 of the 68 *B. vosnesenskii* queens (n = 12 nest-searching; 7 nectaring), and the presence of mites could be predicted by behavioral state, whereby pollen-collecting queens were less likely to have mites than nest-searching or nectaring queens (Chi-squared = 97.477, df = 2, p < 0.001, Fig 1-3). No mites were observed on any *B. vosnesenskii* queens in the pollen-collecting behavioral state. Mite infestation did not correspond to differences in *B. vosnesenskii* ovary activation (GLMM mite status not included in the best fit models for ovary length, maximum oocyte length, or resorption; Fig 1-3). *Sphaerularia bombi* was not observed in any *B. vosnesenskii* queens, but it was observed in nine queens of the species *B. ternarius* and *B. perplexus* (Table 1-S1).

Discussion

We examined patterns of ovary development in wild bumble bee queens in relation to nest foundation status, symbiont loads, and phenology, to identify the factors that influence reproductive state in solitary spring queens. Primarily, we explored the alternative scenarios that ovary activation either precedes nest-searching behavior, succeeds nest foundation, or is entirely uncoupled from nest occupation in early spring queens. Understanding whether and how these events are sequenced is an important first step in uncovering whether they are mechanistically linked. Collectively, our findings suggest that neither nesting status nor observed symbiont loads are major regulators of ovary development in spring queens of *B. vosnesenskii* in southern California. Instead, queens progressively develop their ovaries independent of nest status and symbiont loads, with some individual variation in this process that is driven by factors that are currently unknown.

We collected queens with oocytes ranging in their developmental status from fully developed, to fully undeveloped, in each of our three behavioral states. This indicates that bumble bee queens search for nest sites and also first occupy nests with ovaries at all levels of development. Although nest establishment and ovary development are physiologically linked in systems such as burying beetles (Pellissier Scott and Traniello, 1987), which have a social lifestyle similar to bumble bees at this life stage, it is perhaps not surprising that we did not find evidence for a similar linkage in bumble bee queens. There is no evidence that these processes are related in other hymenopteran systems, although this area remains relatively unexplored (but see Medler, 1962). Instead, social hymenopteran females are known to continuously develop their ovaries and resorb egg cells that they cannot or do not oviposit (Bell and Bohm, 1975). However, very little work has investigated oocyte resorption in early season bumble bee queens, prior to nest establishment. Although the ovaries of some bee species develop during winter diapause (Wasielewski *et al.*, 2011), bumble bee ovaries do not begin to mature until after queens have emerged from diapause in the spring (Palm, 1948; with the exception of some arctic species with very a short summer season, see Vogt *et al.*, 1994). Juvenile hormone, the primary gonadotropic hormone in insects, also increases following emergence from diapause (Larrere *et al.*, 1993; Sarro *et al.*, 2021) and catalyzes ovary development (Wigglesworth, 1934; Roy *et al.*, 2018; Shpigler *et al.*, 2020). Based on our data, we posit that queens might begin to develop their ovaries immediately upon emergence from diapause, if adequate nutritional resources are available (Vogt *et al.*, 1998; Heinrich, 2004), and subsequently resorb mature eggs if necessary until they find a suitable nest site. This is consistent with our observation that resorption status of *B. vosnesenskii* ovaries increased over the course of the five-week collection period in our study, irrespective of queen behavioral state.

While our proposed “develop-and-resorb” approach to ovary maturation might be inefficient with respect to resource allocation (Boggs, 2009), it may be adaptive in that it could enable rapid onset of egg production once nest sites are located. Egg laying opportunities can quickly change in annually and facultatively social species, and an individual’s ability to rapidly respond to such changes may provide a selective advantage. This might be particularly true in the context of enabling queens in annually eusocial species to establish nests earlier in the season. Bumble bee colonies have a limited season in which to grow and produce reproductives (males and new queens), and several lines of evidence suggest that establishing a nest earlier in the season is advantageous. For example, bumble bee species that emerge from overwintering and initiate nests earlier in the spring are less likely to be in decline, relative to species that emerge later in the season (Williams *et al.*, 2009). Colonies grow exponentially throughout the season, and the more time they have to grow, the more reproductives they are ultimately able to produce (Malfi *et al.*, 2019). Arctic-dwelling bumble bee species, which must establish their nests during an especially short season, appear to have evolved the strategy of diverting heat produced by the thorax to the abdomen to accelerate ovary development, to enable rapid nest establishment (Heinrich and Vogt, 1993; Vogt *et al.*, 1994, 1998). Moreover, the social environment itself can also increase queen survivorship (Sarro *et al.*, 2021), suggesting that the sooner a queen can establish a nest and produce offspring, the higher her chances of survival. Thus, spring queens may simultaneously develop their ovaries and search for a nest, dependent on more dynamic cues such as their nutritional state (Free and Butler, 1959; Heinrich, 2004) or environmental conditions. This approach may enable queens to colonize a nest and lay eggs earlier in the season than if these processes were dependent on one another or occurred in discrete succession. Although it is unclear to what extent nest sites are limited in natural systems, nest usurpation by congenerics appears to be commonplace, at least in some locations (Elliott, 2009; Koch *et al.*, 2021), suggesting there is competition for high quality nest sites. Queens who begin searching for nest sites earlier may have more options from which to choose, and they may be able to spend more time in the relative safety of the nest, buffered from exposure to extreme weather and predation. However, this early nest foundation may come at the cost of defending it. The ability to locate and occupy a nest opportunistically, without ovarian constraints, may provide queens the flexibility needed to select a high quality nest site at the most advantageous time in the season.

We observed substantial, unexplained variation in ovary development within our three behavioral states and across collection dates. These results are consistent with previous work on ovary development in early spring queens of several arctic and temperate bumble bee species (Richards, 1978; Vogt *et al.*, 1994), which found a wide range of ratios of ovary development to body size, both before and after nest foundation. The underlying drivers of the onset of ovary development have not been studied extensively in bumble bee queens (but see Palm, 1948; Vogt *et al.*, 1998; Bloch *et al.*, 2000; Heinrich, 2004; Geva *et al.*, 2005; Baron *et al.*, 2017b; Sarro *et al.*, 2021), as they have in workers (Larrere *et al.*, 1993; Bloch *et al.*, 1996, 2000a; Bloch and Hefetz, 1999; Cnaani *et al.*, 2007; Amsalem *et al.*, 2014; Shpigler *et al.*, 2014; Padilla *et al.*, 2016). Although we did not explore queen nutrition, it is possible that for queens in our study, ovary development was impacted by diet quality prior to overwintering or upon emergence in the early spring. Nutritional state has been shown to significantly influence ovary development in solitary bees (Cane, 2016) and also specifically in bumble bee queens (Vogt *et al.*, 1998). Successful ovary development requires proteins and lipids, which bees acquire primarily from pollen (Richards, 1994; Vogt *et al.*, 1998; Heinrich, 2004; Cane, 2016; Tanaka *et al.*, 2019). Bumble bee queens additionally require nectar to fuel abdominal heating (Vogt *et al.*, 1998). Queens thus require both nectar and pollen resources, both prior to and upon emergence from diapause, to become reproductive and to successfully establish a colony. Existing studies also suggest that myriad additional stressors, such as pesticide exposure (Baron *et al.*, 2017a; Leza *et al.*, 2018) and parasites (Lundberg and Svensson, 1975), can also limit or entirely inhibit ovary development and egg production in queens. In the wild, bumble bees can be exposed to all of these stressors, and laboratory studies have demonstrated that their effects on egg production can translate to nest failure and ultimately population decline (Baron *et al.*, 2017a). Future studies that explore the synergistic effects of multiple stressors on wild queen ovary development are needed to better determine the ecological mechanisms affecting reproductive physiology.

We caution that we were unable to differentiate between queens who were searching for or occupying their first nest from those who had previously occupied a failed nest. It is not uncommon for a first nest to fail due to usurpation or other means. As a result, some queens in our study (likely those with more developed ovaries) may have been searching for or occupying a second nest after a failed first attempt at nesting. These queens may artificially increase the prevalence of highly developed ovaries in all behavioral categories. The even distribution of ovary lengths across all behavioral categories, however, suggests that this did not substantially bias our results.

Parasites have been implicated in the inhibition or retardation of ovary development in bumble bees, as well as in bumble bee population declines (Cameron *et al.*, 2011; Goulson *et al.*, 2015). In our study, only a single *B. vosnesenskii* queen had a confirmed infection by *Vairimorpha bombi*, and none were infected by *Sphaerularia* *bombi* or *Apicystis bombi*. This is consistent with previous work showing low infection rates in this species (Graystock *et al.*, 2020; Mullins *et al.*, 2020) and may contribute to the relative success of this species throughout the western United States (Cameron *et al.*, 2011). Several queens of other species in our study, however, were infected with internal parasites. Interestingly, we found one queen of *B. perplexus* that was infected with *Sphaerularia bombi* and also had developed oocytes. This queen was even observed collecting pollen, indicating she had successfully located a nest. Although infection by *S. bombi* typically inhibits ovary development (Alford, 1971; Macfarlane and Griffin, 1990; Kubo *et al.*, 2016) and induces a suite of transcriptional changes in queens (Colgan *et al.*, 2020), there have been a few observations of infected queens with developed ovaries (Alford, 1969; Roseler, 2002; Mullins *et al.*, 2020). This suggests that *S. bombi* may invoke differential individual- or species-level responses in bumble bee queens. Alternatively, this differential response may be due to the timing of *S. bombi* invasion of queens relative to the onset of oviposition, whereby later invasions are less likely to result in castration (Roseler, 2002). More research is needed to clarify the individual and synergistic impacts of various parasites on the nest founding stage of different bumble bee species.

The presence of heterospecifics in or on bumble bees may not always indicate a parasitic relationship. In our study, external mites were prevalent in queens across all species, but mite loads were not associated with ovary developmental status. From our study, it is unclear whether these mites are parasitic. No mites were observed on pollen-collecting *B. vosnesenskii* queens, and we observed no substantial mite loads (>5 mites) on pollen-collecting queens of other species. This result indicates that only queens who had not yet located nests were subject to substantial mite infestation in our study. Although this could suggest that mites interfere with or prevent nest founding, we instead propose that mites dismounted from queens after nest establishment and did not interfere with nest founding. This idea is supported by our observation that all nest-searching queens with mites had substantial loads (between 9 to 100s of mites), whereas all pollen-collecting queens with mites had loads of five or fewer individuals. The majority of bumble bee-associated mite species do not parasitize bumble bees (Haas *et al.*, 2019), but instead are phoretic, using bumble bees as transportation between nest sites (Eickwort, 1994). Many mite species are closely associated with bumble bee nests and feed on pollen, microarthropods, and detritus within bumble bee colonies (Stebbing 1965; Richards and Richards 1976). To the best of our knowledge, no work has previously investigated the prevalence of mites on queens immediately before versus after nest foundation, but our results support previous studies that suggest mites use overwintering queens as transportation between colonies from year to year (Stebbing 1965; Huck *et al.*, 1998). If true, the presence of mites may simply be an indicator that a queen has not yet established a nest, rather than a signal of an inability to successfully establish a nest.

Studies on the physiological states of wild-caught organisms are essential to uncovering the links between ecological and physiological processes. Unfortunately, such studies are exceedingly rare in wild, non-managed bees (but see Alaux *et al.*, 2017). Abundant lab studies provide an important foundation for insights into the mechanisms driving animal behavior and physiology. However, lab studies are limited in their ability to subsequently link these mechanisms to complex, real-life ecological processes. This linkage can only be accomplished with studies of wild organisms. For example, studies involving nest-searching bumble bee queens cannot be conducted in the lab, because to the best of our knowledge, queens will not search for or independently colonize nests in a laboratory environment. Here, we investigated the physiological process of ovary development in wild, early spring bumble bee queens. Our study suggests that ovary development and nest initiation are uncoupled in bumble bee queens, at least in our focal species, and that myriad additional factors, such as nutritional state, instead drive variation in this fundamental physiological process.

Animal Welfare Statement: All bees collected for the purposes of this research were sacrificed as humanely as possible (either directly onto dry ice or directly into ethanol), to minimize suffering. Sample sizes were kept as low as possible, while still maintaining sufficient power to detect biologically relevant differences among groups, in order to minimize the number of bees collected and the impact of these collections on local populations.

Data Availability: Data and all associated code are available on Dryad https://doi.org/10.6086/D13H4P.

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Tables and Figures

Table 1-1. Behavioral states into which all collected queens were categorized. Based on these behaviors, we can infer that nest-searching queens have not yet established a nest, whereas pollen-collecting queens have. The nest status of nectaring queens is unknown.

Category	**Definition**
Nest-searching	Flying close to the ground (estimated at < 30 cm) in a stereotypical zigzag pattern for at least six seconds; typically observed landing and walking into shadows or holes in the ground between zigzag flights (Video 1-S1); no pollen observed in corbiculae
Pollen-collecting	Packed pollen loads easily observed in corbiculae; typically observed manipulating one or more flowers
Nectaring	Observed actively manipulating one or more flowers; no pollen observed in corbiculae

Table 1-2. Summary of sample sizes and symbionts detected in *B. vosnesenskii* queens, organized by behavioral category. Values in brackets in column "n" represent sample sizes of queens collected overall. Values in subsequent columns represent the number and percentage of those bees with confirmed symbiont infestations. The "total" column represents the number and percentage of bees infested with one or more symbionts, not the number of symbionts identified. The data in the "all behaviors summed" section are duplicates of the individual behavioral state data, summarized for convenience.

Behavioral state	**n**	**external mites (>5)**	***Sphaerularia bombi***	***Vairimorpha bombi***	***Apicystis bombi***	**TOTAL**
Nest searching	[26]	12 (46%)	0 (0%)	1 (4%)	0 (0%)	13 (50%)
Pollen collecting	[20]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Nectaring	[22]	7 (31%)	0 (0%)	0 (0%)	0 (0%)	7 (32%)
All behaviors summed	**[68]**	**20 (28%)**	**0 (0%)**	**1 (1%)**	**0 (0%)**	**21 (31%)**

Figure 1-1. Diagram of bumble bee life cycle. Young gynes (queens-to-be) emerge from their natal colonies in the fall (A), mate with unrelated males (B), and then overwinter underground in a diapause state (C). Queens emerge from diapause in the spring with undeveloped ovaries (D). During this time, they feed on pollen and nectar, develop their ovaries, and locate nests. After nest foundation, queens begin to oviposit in their newly formed nest (E) and increase their reproductive output over the course of the season as the social colony grows (F).

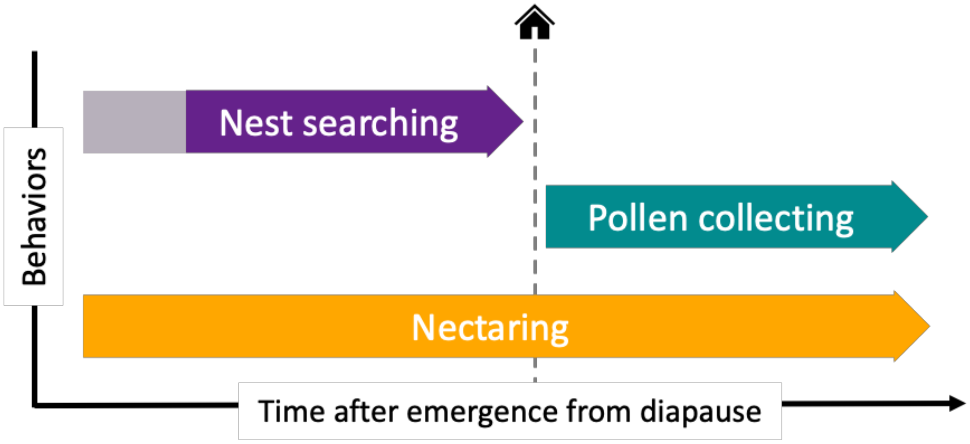

Figure 1-2. Visual timeline of early season queen behaviors outside the nest. Queens can be observed nectaring throughout the entirety of the early season (yellow arrow). Queens begin nest-searching shortly after emerging from diapause (purple arrow), but exactly how soon after diapause emergence nest-searching begins is unknown (indicated here by the light purple shaded area). Only after nest location occurs (vertical dashed line) do queens begin to collect pollen (green arrow). Pollen-collecting is an indication that a queen has located a nest but does not indicate whether the queen has begun oviposition within the nest. Nest-searching and pollen-collecting are distinct behavioral states that do not temporally overlap within an individual, whereas individuals can and do switch freely between nest-searching and nectaring, as well as between pollen-collecting and nectaring.

Figure 1-3. Oocyte length (A) and resorption status (B) of B. vosnesenskii queens. Large squares represent averages for a given behavior. Small points in plot A represent terminal oocytes and therefore include up to 8 data points per queen. Small points in plot B represent proportions and therefore include one data point per queen. All comparisons of ovary measurements among behavioral states were not significant. Small points are jittered to better visualize overlapping points (width +/- 0.4; height +/- 0.05).

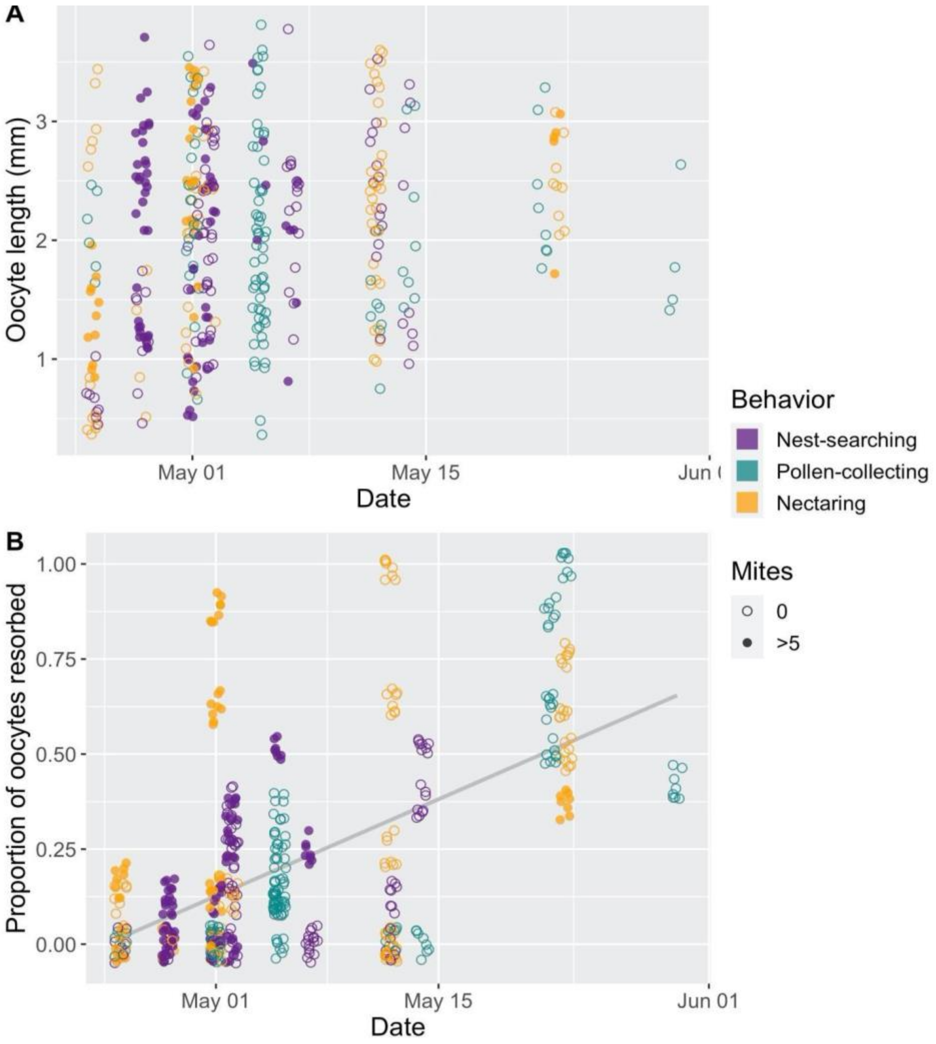

Figure 1-4. Length (A) and resorption (B) of *B. vosnesenskii* oocytes over time. Collection date did not significantly predict oocyte length (date not included in best fit models for oocyte length or maximum oocyte length). Resorption status, however, significantly increased over time (GLMM p < 0.001, estimate = 0.02, 95% CI = 0.01 - 0.02). Trendline in (B) is based on pooled data from all behavioral states; points are jittered to better visualize overlapping points (width +/- 0.4; height +/- 0.05).

Supporting Information

Methods for additional species collections

One *B. melanopygus* queen was collected in Southern California during the *B. vosnesenskii* collections and was processed alongside the *B. vosnesenskii* bees. Queens of the remaining species were collected in blueberry- and lupine- dominated fields in Franklin and Cumberland counties, Maine, respectively, over a single collection day: June 13, 2020. For these queens, we followed the methods in the main text with the exception that we collected these bees into 100% ethanol (rather than onto dry ice) and stored them at room temperature (rather than at -80 °C). The amount of information that can be obtained from this set of queens is more restricted, because there is limited replication across behavioral states for some species. Instead, we used data from these additional species to examine whether patterns were consistent with our findings from *B. vosnesenskii.* Sample sizes for these seven additional species are summarized in Table 1-S1.

Results for additional species collections

For *B. ternarius*, the additional species with sufficient sample size to perform statistical analyses, the best fit model predicting oocyte length included *S. bombi* as the sole fixed effect. The best fit model predicting oocyte resorption in *B. ternarius* included behavioral category and *S. bombi* as fixed effects.

B. ternarius ovary development could not be predicted by behavioral state (behavioral state not included in oocyte length models, GLMM p = 0.434, estimate = -

0.03, 95% CI = -0.10 - 0.04 in oocyte resorption model, Fig 1-S1). For all queens in this data set, we observed a range of ovary development in all behavioral categories, including nest-searching queens of *B. impatiens* and *B. ternarius* with fully developed (stage 4) oocytes, as well as pollen-collecting queens of *B. perplexus, B. ternarius,* and *B. vagans* with undeveloped (stage 1-2) oocytes.

Sphaerularia bombi was found in 9 queens of the species *B. ternarius* and *B. perplexus* (Table 1-S1). *S. bombi* was a significant predictor of oocyte length (GLMM $p < 0.001$, estimate = -1.82, 95% CI = -2.03 - -1.60, Fig 1-S1) and resorption (GLMM $p = 0.006$, estimate = -0.15, 95% CI = -0.24 - -0.05) in *B. ternarius* queens; all infected *B. ternarius* queens (n = 7) had fully undeveloped ovaries. *S. bombi* infection in queens of *B. perplexus* (n = 2), however, did not result in the inhibition of ovary development in all queens. Instead, one of these infected queens had developed ovaries comparable to those of uninfected queens (Fig 1-S1). Of the 9 bees infected with *S. bombi,* 8 had coinfestations with external mites. Substantial external mite loads (> 5 mites) were found on an additional 6 bees, for a total of 14 mite-infested queens of *B. impatiens, B. perplexus, B. ternarius,* and *B. vagans* (Table 1-S1). Similar to what we observed in *B. vosnesenskii,* no pollen-collecting queens were observed with substantial mite loads, whereas we observed several hundred mites on some individual nest-searching and nectaring queens. However, few mites (1-5) were found on 25 queens in the data set (n = 12 pollen-collecting; n = 13 nectaring), none of which were nest-searching. No *Vairimorpha bombi* or *Apicystis bombi* were detected in these queens. Additional symbionts identified in these queens included two globular springtails and one oribatid mite (Table 1-S1).

Tables & Figures

Table 1-S1. Summary of sample sizes and symbionts detected, organized by behavioral category and species. Data from all species collected (including *B. vosnesenskii*) are included here for completeness. Values in brackets in column “n” represent sample sizes of queens collected overall. Values in subsequent columns represent the number and percentage of those bees with confirmed symbiont infestations. The “total” column represents the number and percentage of those bees infested with one or more symbionts, therefore it does not always equate to the sum of symbiont infestations in that row (because some bees had coinfestations with multiple symbionts). In addition to the listed symbionts, we also found two globular springtails (on a nectaring *B. vagans* and nectaring *B. perplexus*), and one oribatid mite (on a nest-searching *B. impatiens*). NA values indicate an absence of data, where no queens were collected of that species in that behavioral state. The data in the “all behaviors summed” section are duplicates of the individual behavioral state data, summarized for convenience.

behavioral state	species	n	external mites (1-5)	external mites (>5)	*Sphaerularia bombi*	*Vairimorpha bombi*	TOTAL
nest searching	*B. bimaculatus*	[0]	NA	NA	NA	NA	NA
	B. melanopygus	[0]	NA	NA	NA	NA	NA
	B. impatiens	[1]	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1 (100%)
	B. perplexus	[0]	NA	NA	NA	NA	NA
	B. sandersoni	[0]	NA	NA	NA	NA	NA
	B. ternarius	[6]	0 (0%)	2 (33%)	2 (33%)	0 (0%)	2 (66%)
	B. vagans	[0]	NA	NA	NA	NA	NA
	B. vosnesenskii	[26]	0 (0%)	12 (46%)	0 (0%)	1 (4%)	13 (50%)
	TOTAL	**[33]**	**0 (0%)**	**15 (45%)**	**2 (6%)**	**1 (3%)**	**16 (48%)**
pollen collecting	*B. bimaculatus*	[2]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	B. melanopygus	[0]	NA	NA	NA	NA	NA
	B. impatiens	[2]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	B. perplexus	[8]	2 (25%)	0 (0%)	1 (13%)	0 (0%)	3 (38%)
	B. sandersoni	[1]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	B. ternarius	[10]	8 (80%)	0 (0%)	0 (0%)	0 (0%)	8 (80%)
	B. vagans	[9]	2 (22%)	0 (0%)	0 (0%)	0 (0%)	2 (22%)
	B. vosnesenskii	[20]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	TOTAL	**[52]**	**12 (23%)**	**0 (0%)**	**1 (2%)**	**0 (0%)**	**13 (25%)**
nectaring	*B. bimaculatus*	[4]	1 (25%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)
	B. melanopygus	[1]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	B. impatiens	[3]	2 (67%)	1 (33%)	0 (0%)	0 (0%)	3 (100%)
	B. perplexus	[14]	4 (29%)	4 (29%)	1 (14%)	0 (0%)	8 (57%)
	B. sandersoni	[0]	NA	NA	NA	NA	NA
	B. ternarius	[11]	4 (36%)	4 (36%)	5 (45%)	0 (0%)	9 (81%)
	B. vagans	[7]	2 (29%)	1 (14%)	0 (0%)	0 (0%)	3 (42%)
	B. vosnesenskii	[22]	0 (0%)	7 (31%)	0 (0%)	0 (0%)	7 (32%)
	TOTAL	**[61]**	**13 (20%)**	**18 (28%)**	**7 (11%)**	**0 (0%)**	**32 (49%)**
all behaviors summed		[6]					
	B. bimaculatus	[1]	1 (17%)	0 (0%)	0 (0%)	0 (0%)	1 (17%)
	B. melanopygus	[6]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	B. impatiens	[22]	2 (33%)	2 (33%)	0 (0%)	0 (0%)	4 (67%)
	B. perplexus	[1]	6 (27%)	4 (18%)	3 (14%)	0 (0%)	11 (50%)
	B. sandersoni	[27]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	B. ternarius	[16]	12 (44%)	6 (22%)	7 (26%)	0 (0%)	19 (70%)
	B. vagans	[68]	4 (25%)	2 (13%)	0 (0%)	0 (0%)	6 (38%)
	B. vosnesenskii	**[14**	0 (0%)	20 (28%)	0 (0%)	1 (1%)	21 (31%)
	TOTAL	**6]**	**25 (17%)**	**33 (22%)**	**10 (7%)**	**1 (1%)**	**62 (42%)**

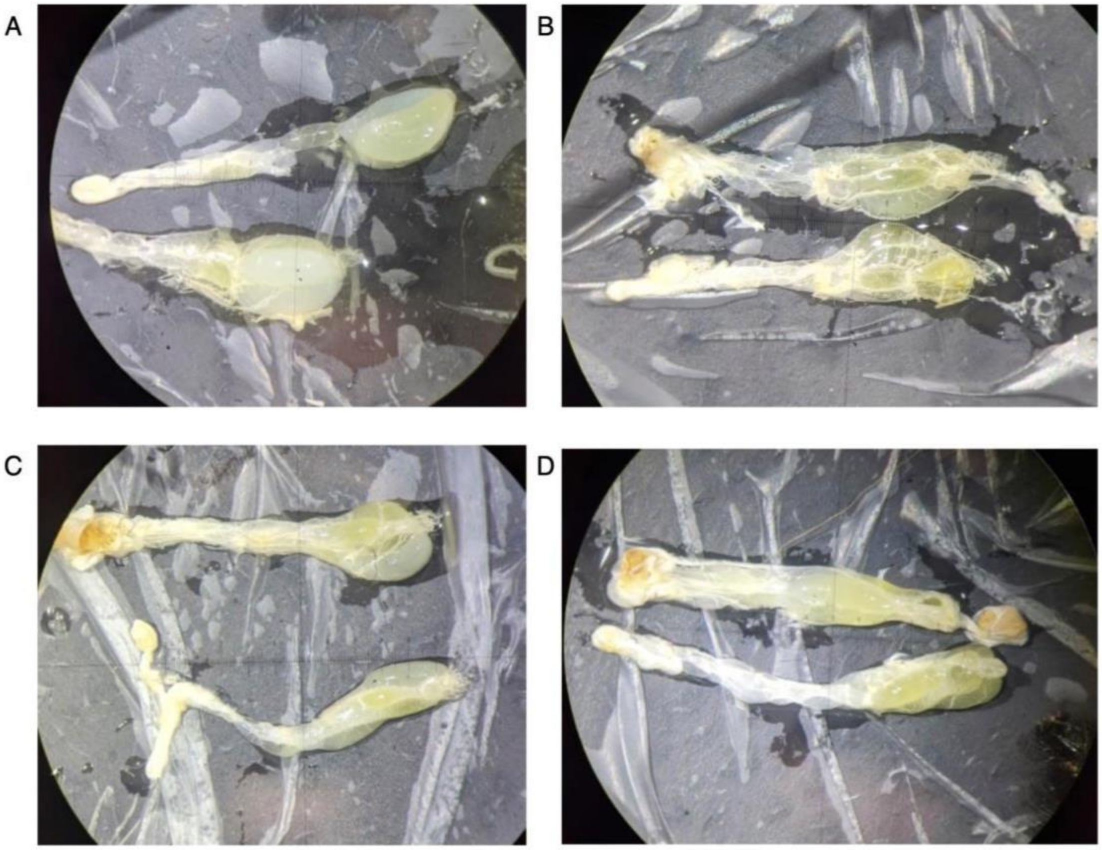

Figure 1-S1. Ovary resorption. Fully developed, unresorbed ovarioles (A) are white and evenly shaped. Ovarioles in various states of resorption (B-D) can be identified by their yellow, misshapen appearance.

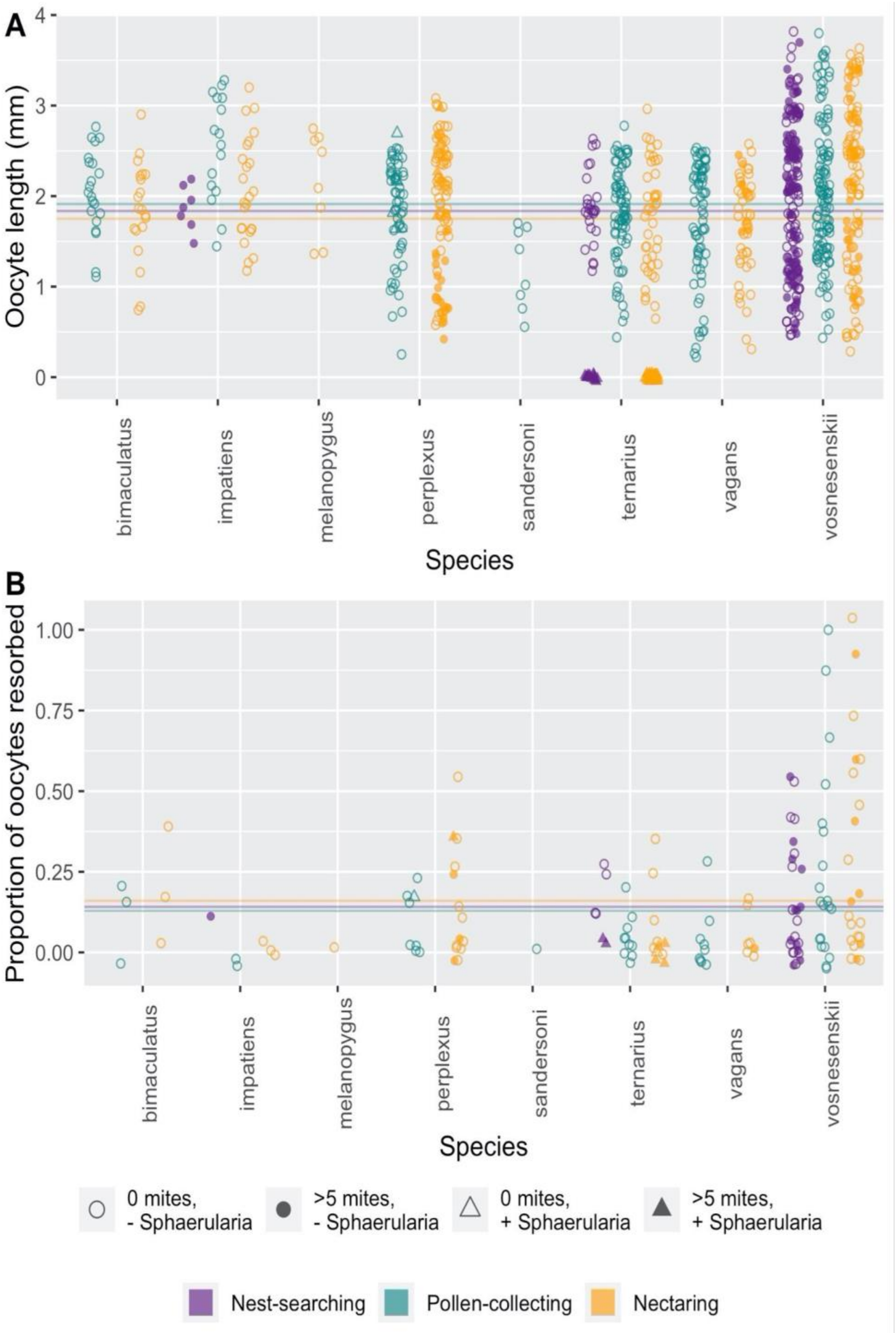
A
Oocyte length (mm)
0
1
2
3
4
bimaculatus
impatiens
melanopygus
perplexus
sandersoni
ternarius
vagans
vosnesenskii
Species
B
Proportion of oocytes resorbed
0.00
0.25
0.50
0.75
1.00
bimaculatus
impatiens
melanopygus
perplexus
sandersoni
ternarius
vagans
vosnesenskii
Species
0 mites, - Sphaerularia
>5 mites, - Sphaerularia
0 mites, + Sphaerularia
>5 mites, + Sphaerularia
Nest-searching
Pollen-collecting
Nectaring

Figure 1-S2. Oocyte length (A) and resorption (B) status of all collected queens. Horizontal lines represent averages for a given behavioral state across all species. Large points represent averages for a given species and behavior. Small points in plots A represent terminal oocytes and therefore include up to 8 data points per queen. Small points in plot B represent proportions and therefore include one data point per queen. All comparisons of ovary measurements among behavioral states were not significant (behavioral state not included in best fit GLMMs). In *B. ternarius* queens, *Sphaerularia bombi* significantly predicted oocyte length (GLMM $p < 0.001$, estimate = -1.82, 95%CI = -2.03 - -1.60, Fig 1-S1), but not resorption (*S. bombi* not included in best fit model for resorption). Small points are jittered to better visualize overlapping points (width +/- 0.4; height +/- 0.05 in A, +/- 0.3 in B).

Video 1-S1. Video recording of *B. vosnesenskii* queen nest-searching behavior.

CHAPTER 2: AN ORGANIZING FEATURE OF BUMBLE BEE LIFE HISTORY: QUEEN REPRODUCTION IS SYNCHRONIZED WITH WORKER EMERGENCE IN NESTS

(reprint of materials published in Conservation Physiology 2021 with co-authors Penglin Sun, Kerry Mauck, Damaris Rodriguez-Arellano, Naoki Yamanaka, and S Hollis Woodard; https://doi.org/10.1093/conphys/coab047)

Abstract

Bumble bee queens initiate nests solitarily and transition to living socially once they successfully rear their first cohort of offspring. Bumble bees are disproportionately important for early season pollination, and many populations are experiencing dramatic declines. In this system, the onset of the social stage is critical for nest survival, yet the mechanisms that facilitate this transition remain understudied. Further, the majority of conservation efforts target the social stage of the bumble bee life cycle and do not address the solitary founding stage. We experimentally manipulated the timing of worker emergence in young nests of bumble bee (Bombus impatiens) queens to determine whether and how queen fecundity and survival are impacted by the emergence of workers in the nest. We found that queens with workers added to the nest exhibit increased ovary activation, accelerated egg laying, elevated juvenile hormone (JH) titers and also lower mortality relative to solitary queens. We also show that JH is more strongly impacted by the social environment than associated with queen reproductive state, suggesting that this key regulator of insect reproduction has expanded its function in bumble bees to also influence social organization. We further demonstrate that these effects are independent of queen social history, suggesting that this underlying mechanism promoting queen fecundity is reversible and short lived. Synchronization between queen reproductive status and emergence of workers in the nest may ultimately increase the likelihood of early nesting success in social systems with solitary nest founding. Given that bumble bee workers regulate queen physiology as we have demonstrated, the timing of early worker emergence in the nest likely impacts queen fitness, colony developmental trajectories and ultimately nesting success. Collectively, our findings underline the importance of conservation interventions for bumble bees that support the early nesting period and facilitate the production and maintenance of workers in young nests.

Introduction

The ability to synchronize life history transitions with changes in the environment is essential to organismal survival and fitness. Disconnects between transient environmental characteristics and behavioral and physiological states can result in fitness declines (Stenseth and Mysterud, 2002). Conversely, closely coordinating key life history shifts with environmental changes allows organisms to better track resource availability and optimize fitness under current ecological conditions. For example, many animals emerge from winter diapause at the onset of spring, when food resources become available, thus aligning their heightened metabolic activity with access to adequate nutrition (Tauber and Tauber, 1976; Koštál, 2006). Behavioral and physiological changes associated with life history transitions are often responses to a suite of internal factors, such as nutritional or circadian state, as well as external cues, such as daylength, temperature, and chemical and visual stimuli (Flatt and Heyland, 2011). Often, these external cues are reliable indicators that directly reflect changes in ecological conditions. Identifying the proximate mechanisms that organize life history transitions, and understanding the adaptation of these physiological transitions to environmental variation, are major goals in conservation physiology research (Sinervo and Svensson, 1998).

In social animals, society members also regulate the behavior and physiology of one another in ways that promote cohesiveness between group members and support the survival of the group. The most extreme examples of this are seen in eusocial systems, which are defined by their reproductive division of labor, overlapping generations, and cooperative brood care (Batra, 1966; Michener, 1969). For example, reproductive division of labor is maintained by signaling among nestmates, such that reproductively dominant females use pheromones and/or aggression to inhibit worker reproduction and reinforce a reproductive skew between queens and workers (Van Oystaeyen *et al.*, 2014). This queen effect on worker reproduction promotes nesting success by reducing intra-nest conflict (Kocher and Grozinger, 2011). This form of social influence, whereby a queen regulates the physiology of her offspring to her benefit (Linksvayer and Wade, 2005), has been studied extensively in eusocial insects (Van Oystaeyen *et al.*, 2014).

Although much is known about how eusocial queens influence worker behavior and physiology (Keller and Nonacs, 1993; Grüter and Keller, 2016), little work has investigated the ways in which workers influence queens. Bumble bees (genus *Bombus,* family Apidae) are one of several social insect lineages in which queens live and reproduce under both solitary and social conditions. In these systems, nests are first initiated solitarily by queens, then transition to sociality when the first offspring eclose. This form of sociality is also seen in lineages such as some ponerine ants, halictine and xylocopine bees, and vespid wasps (Wilson, 1971). In these systems, emergence of the first workers in the nest exposes queens to an array of new social signals not present during the solitary nest-founding stage. In bumble bees, like most other solitary nest-founding social lineages, worker emergence coincides with a transition in queens from performing a broad task repertoire (e.g., brood feeding, foraging, nest maintenance, defense) to almost exclusively producing and laying eggs (Shpigler *et al.*, 2013; Woodard *et al.*, 2013). This transition is likely directly facilitated by the onset of the social environment, as bumble bee queens that are experimentally manipulated to become social through the addition of workers (Sladen 1912; Röseler, 1968; Kwon *et al.*, 2006; Woodard *et al.*, 2013), brood (Kwon and Saeed, 2003; Kwon *et al.*, 2006), conspecific queens (Strange, 2010), or even honey bee workers (Strange, 2010), lay eggs earlier and in greater numbers than solitary queens. Thus, it appears that queen bumble bees synchronize their transition to a more reproductive state with the emergence of helpers in the nest who will rear those offspring. However, the physiological underpinnings of this reproductive acceleration remain unknown. Further, it is unclear what factors influence the onset and persistence of queen reproduction in this pollinator lineage, which might translate to similarly solitary-founding social species.

Bumble bees are one insect lineage for which there is strong evidence of decline (Williams, 1982; Cameron and Sadd *et al.*, 2020; Colla *et al.*, 2012). Insights into the factors that ensure nesting success are particularly important to derive for this annually nesting lineage. An estimated 25% of species in this group are considered threatened (IUCN Red List). The solitary nest founding stage represents a unique challenge for the social insects in which this occurs, such as bumble bees, because queens are not yet buffered by the social environment and must do all work for the nest, including risking exposure when foraging for resources (Oster and Wilson, 1978). Existing studies suggest that nests at this stage are particularly sensitive to pesticides (Baron *et al.*, 2017; Leza *et al.*, 2018), parasites (Rutrecht and Brown, 2008; Elliott, 2009), and other stressors (Watrous *et al.*, 2019; Mola *et al.*, 2021). Studies on the physiological basis of bumble bee decline are important for understanding the mechanistic drivers of population health and population declines, which can be leveraged to target effective conservation strategies (Woodard, 2017). Examining the physiological impact of the social environment on early nesting queens, specifically, will elucidate the proximate mechanisms shaping population dynamics at this fundamental life stage.

Here, we explored the hypothesis that the emergence of workers in the nest promotes queen physiological changes related to early nesting success, by examining worker regulation of queen survival and reproductive behavior and physiology in recently-founded nests of the bumble bee *Bombus impatiens*. In the bumble bees, workers do not feed or groom the queen, as is the case in some other social lineages (Naumann, 1991). This provides a unique opportunity to investigate both direct and indirect impacts of workers in the nest, independent of nutritional or hygienic factors. We experimentally manipulated the timing of worker emergence in the nest and measured queen physiological responses to this social manipulation over multiple time scales during the early nesting stage. To assess queen reproductive physiology, we quantified egg laying, degree of ovarian activation, and juvenile hormone (JH) titers in hemolymph. JH is a key regulator of ovary development and reproduction in female insects (Wigglesworth, 1934; Roy *et al.*, 2018). We predicted that worker presence would elevate JH titers and expedite ovarian development in social relative to solitary queens, thus facilitating the previously observed acceleration of functional reproduction in bumble bee queens (Röseler, 1968; Kwon *et al.*, 2006; Woodard *et al.*, 2013). We further predicted that JH titers and ovarian activation would be positively associated with one another, irrespective of queen social status, which would indicate that JH has maintained its gonadotropic qualities in bumble bees.

We also experimentally removed brood and/or workers from a separate subset of queens to explore the impact of social history on queen reproduction. Here, we examined the rate of nest re-initiation after a simulated loss of brood and/or workers. In this experiment, we asked whether queens who were historically social would maintain elevated reproductive output relative to previously solitary queens. This was predicated on the idea that social environments might have enduring positive effects on reproduction. If our results support this, it would suggest that queens who successfully rear one set of offspring have a reproductive advantage over those who have not, even if they subsequently lose those offspring and must reinitiate a new nest. Alternatively, the effects of the social environment may be more transitory, in which case a queen who loses her first brood would have no measurable reproductive advantage. To our knowledge, this is the first study to investigate the proximate mechanisms underlying worker-induced queen reproduction in bumble bees and to explore the persistence of social effects on fecundity in an imperiled insect group.

Materials and Methods

Experiment 1: Queen reproductive physiology in response to the presence of workers

a) Bee rearing and experimental design

We first explored the impact of worker presence on queen reproductive maturation and physiology across time. Thirteen mature *Bombus impatiens* Cresson colonies (containing a queen and ≥ 50 workers) were acquired from Koppert Biological Systems (Howell, MI, USA) and kept in the University of California Riverside's Insectary and Quarantine Facility under dim red light at 27°C and 60% RH. A subset of these colonies were at the developmental stage where new reproductives (queens and males) are produced**.** These colonies were used to source queens for this experiment. The remaining colonies were younger and were used to source female workers for this experiment. Bees were fed *ad libitum* artificial nectar (recipe in Boyle et al. 2018) and pollen balls consisting of honey bee-collected, mixed-source pollen (Brushy Mountain Bee Farm, Moravian Falls, NC, USA) blended with artificial nectar.

Callow queens (< 24 hours old, identified by their silvery appearance) were removed from their natal colonies and arranged in groups containing either a single solitary queen, or a queen and five workers, in the following four configurations: *early-social* (workers added prematurely, before the queen has become reproductive); *early-solitary* (no workers added at this early stage, before the queens has become reproductive); *late-social* (workers added after the queen has established a nest and the first adult offspring have eclosed); and *late-solitary* (no workers added at this later stage, after the queen has established a nest and the first adult offspring have eclosed) (Fig 2-1). Throughout the experiment, the cages were kept under dim red lights (which are not visible to bees) at 27°C and 70% RH. All cages were fed the diet described above, with the exception that the first pollen ball provided was coated in honey bee wax.

As in previous studies (Röseler and Röseler, 1988; Amsalem and Grozinger, 2017; Leza *et al.*, 2018), queens were not mated to minimize variation introduced by this process (Baer and Schmid-Hempel, 2005) and to control the number and source of workers in the nests of late-stage queens. All queens were treated with CO_2 gas for 30 minutes per day at adult ages 12 and 13 days to cause them to bypass diapause and initiate egg laying (Röseler, 1985). CO_2 treatment is a widely-used technique that causes queens to bypass diapause in a way that is largely indistinguishable from true diapause (Amsalem *et al.*, 2015; Amsalem and Grozinger, 2017). CO_2-treated queens become reproductive irrespective of mating status (Amsalem *et al.*, 2017; Leza *et al.*, 2018), but produce only haploid male offspring when unmated.

Within 24 hours of the second CO_2 treatment, five callow workers were added to the nests of queens in the *early-social* group. The remaining queens (*early-solitary*, *late-solitary*, *late-social*) were reared solitarily at this stage. Early-stage nests (i.e., *early-social* and *early-solitary*) were collected either one, four, or seven days after CO_2 treatment (n = 6-9). On each nest's collection day, the queen was sacrificed, hemolymph was collected from the queen (see methods below), and the queen and nest were stored at -80°C. These early-stage time points capture data prior to queen reproductive maturation (early day 1), at the approximate day social queens begin laying eggs based on preliminary observations (early day 7), and at an intermediate stage between these two time points to capture the onset of reproductive development (early day 4). The remaining queens (in the two late-stage groups) were allowed to continue developing their nests and rear their first brood cohort to adulthood (Fig 2-1). For the *late-social* queens, five callow workers were added to nests within 24 hours of the first male eclosing in the nest (simulating natural emergence of workers in the nest) following the above methods for worker additions. The remaining queens (*late-solitary*) were left solitary. Late-stage nests were collected one, four, or seven days after the first male eclosed (n = 5-9) to match the length of time queens were exposed to workers in the early-stage treatment groups. In this way, we could directly compare worker effects on queen reproduction in early- versus late-stage queens. Nests were inspected every 1-2 days and all eclosed males were removed from late-stage nests as soon as they were detected to control the number of adult offspring in nests. Additional sampling details are provided in the Supporting Information.

b) JH-III quantification

Live queens were briefly restrained in plastic marking tubes (Betterbee, Greenwich, CT, USA). Using forceps, heads were swiftly removed to expose the open neck cavity. Using a graduated glass capillary tube, a measured quantity of hemolymph (5-20 ul per bee) was collected from the cavity and placed into a mix of 50 ul acetonitrile (Fisher Scientific A998-4, Waltham, MA, USA) and 50 ul 0.9% sodium chloride solution (Fisher Scientific S271-500) contained within a 9 mm autosampler insert (Fisher Scientific C4010-630) inside an autosampler vial (Fisher Scientific C5000-1W) with a vial cap (Fisher Scientific C5000-54B). This method prevents hemolymph melanization and preserves JH in suspension, following Kai et al., 2018. Samples were vortexed and JH was twice extracted into 100 µL volumes of hexanes (Fisher Scientific H306-1) containing 10 ng citronellol (Sigma-Aldrich W230915, St. Louis, MO, USA) as an internal standard. After each extraction, the JH-hexane-citronellol phase (upper layer) was transferred into a new autosampler vial (with insert and cap). JH extracts were stored at -80°C until they were run on a gas chromatography-mass spectrometry machine according to methods in Kai et al., 2018 (see Supporting Information for details). Bees were stored at -80°C until they were processed for dissection.

c) Ovary dissections and measurements

Queen abdomens were soaked in RNAlater®-ICE (Ambion Life Technologies, Austin, TX, USA) at -20°C for 24 hours prior to dissection to enable wet dissection while maintaining fat body RNA integrity for potential future use. RNAlater®-ICE does not cause histological or morphological changes to tissues (Florell *et al.*, 2001). Ovaries were removed and lengths of all eight terminal oocytes were measured with an ocular micrometer. Any oocyte resorption (characterized by yellow coloration and misshapen oocytes lacking a trophocyte, Fig 2-S1) in terminal oocytes was recorded. Oocyte resorption, in which females reabsorb the nutrients from egg cells that they do not or cannot oviposit, is commonplace in insects, including bumble bees (Bell and Bohm, 1975; Duchateau and Velthuis, 1989). Individuals may resorb eggs due to the lack of suitable oviposition sites, unfavorable environmental conditions, or pheromone- or aggression-induced functional sterility (Medler, 1962; Duchateau and Velthuis, 1989). To prevent measurement bias, dissectors were blind to queen treatment group. We also confirmed that workers were non-reproductive when collected by categorically staging all worker ovarioles according to Duchateau and Velthuis (1989). No workers had ovarioles developed beyond stage two, indicating that their ovaries did not contain mature eggs.

d) Body size measurements and nest dissections

The length of bumble bee marginal wing cells is highly correlated to overall body size (Medler, 1962) and was used here as a proxy for body size to be included in statistical analyses. Queen forewings were removed and the marginal cell length of each wing was measured with an ocular micrometer. Cell lengths were averaged together to establish a single measurement per individual. Nests were dissected on dry ice and the number of eggs was recorded.

e) Statistical analyses

All statistical analyses were carried out in R version 4.0.0. Results were visualized using the ggplot2 package (v. 3.3.0 Wickham, 2011). Generalized linear mixed models (GLMMs) were used to determine predictors for JH titer, oocyte length, and egg laying rate. GLMMs were carried out using the lme4 package (v. 1.1-23 Bates et al., 2015). Details on model formation are provided in the Supporting Information. For each analysis, Akaike's Information Criterion for small sample sizes (AICc) was used to select the best fit model based on the model.sel() function from the car package (v. 3.0-7 Fox and Weisberg, 2019), and the model with the lowest AICc score that was not rank deficient was used for subsequent analyses. P-values were acquired using the tab_model() function from the sjPlot package (v. 2.8.3 Lüdecke *et al.*, 2009) and pairwise comparisons were carried out using the lsmeans() function from the lsmeans package (v. 2.30-0 Lenth, 2018) with a Tukey p-value adjustment, Welch's two-sample t-tests using the t.test() function with a Bonferroni p-value adjustment, or Wilcoxon rank sum exact test using the wilcox.test() function with a Bonferroni p-value adjustment. Levene's test for homogeneity of variance was used to measure variance of samples using the leveneTest() function from the car package.

Experiment 2: Uncoupling current social environment from social history

a) Bee rearing and experimental design

We performed a second experiment to examine whether there are persistent effects of the social environment on queen reproductive physiology. Here, bees were sourced from 14 mature *B. impatiens* colonies (also from Koppert Biological Systems) reared as described above, with the exception that nests were kept at ambient room temperature and humidity (22 +/- 2°C; 35 +/- 10% RH). All queens (n = 39) were allowed to initiate two consecutive nests to enable repeated measurements. First, queens were randomly assigned to one of four groups: *solitary-solitary* (queens remained solitary for the duration of the experiment), *solitary-social* (five callow workers added to the second nest), *social-solitary* (five callow workers added to the first nest, but not transferred to the second nest) and *social-social* (five callow workers added to the first nest and subsequently transferred to the second nest) (Fig 2-2, n = 9-10).

Five callow workers were added to the nests of *social-solitary* and *social-social* queens on the same day the queen was added to the first nesting box (adult age 13 days, immediately following second CO_2 treatment). Nests were monitored every 2-3 days to record the presence or absence of brood. Twenty-two days after the first eggs were observed in the first nest (approximately ¾ of pre-adult worker development time; Cnaani et al. 2002), queens, but not brood, were transferred to new nesting boxes. This simulated the loss of brood and removed any related chemical cues from nests. Hereafter, we refer to these pre- and post-brood removal nests as first and second nests, respectively. When queens were transferred to second nests, workers in *social-social* nests were also transferred to second nests, five callow workers were added to *solitary-social* second nests, and workers from *social-solitary* nests were removed and sacrificed. Second nests were monitored until queens re-initiated egg laying, and entire nests were subsequently collected 22 days after eggs were first observed (the same time frame as in the first nest). This allowed brood to develop for as long as possible, while ensuring no offspring eclosed, allowing us to control the number of workers in nests. All collected brood and bees were stored at -80 °C until further processing.

Any queens that survived the duration of the experiment but did not lay eggs in the first (n = 2) or second (n = 1) nests were collected after 60 or 30 days, respectively, and were not included in statistical analyses. Queen mortality and the number of days until the first eggs were observed were recorded. Nests were dissected over dry ice and the number of eggs, larvae, and pupae were recorded.

b) Statistical analyses

GLMMs were used to determine predictors for two response variables: number of days until first eggs were observed in the nest, and total number of brood items (eggs, larvae, and/or pupae) in the nest. Details on model formation are provided in the Supporting Information. Best fit models and p-values were identified according to the methods in Experiment 1. Mortality was analyzed with a mixed-effects Cox regression model using the coxme package (v. 2.2-16; Therneau, 2014) and survival package (v. 3.1-12; Therneau, 2010), for which significance was calculated by performing an Analysis of Variance (anova()) on the best fit model, and data were visualized using survminer (v. 0.4.0) and ggplot2 (v. 2.2.1).

Results

Experiment 1

a) Effect of workers on queen ovary development

The presence of workers in the nest positively impacted queen ovary development, as evidenced by an increase in mean oocyte lengths (GLMM $p = 0.027$, estimate = 0.65, 95% CI [0.08, 1.22], Fig 2-3a) and a decrease in variability among oocyte lengths in social relative to solitary queens (pairwise Levene's tests: early day 4 $p < 0.001$; early day 7 $p = 0.77$; late day 4 $p < 0.001$; late day 7 $p < 0.001$). Maximum oocyte lengths did not differ between social and solitary queens at any single time point, but presence of workers was associated with a higher minimum (and therefore greater average) queen oocyte length on day 4 in both early- and late-stage nests (pairwise Welch's two sample t-tests: early day 4 $p < 0.001$; late day 4 $p < 0.001$, Fig 2-3a). By day 7 in both early- and late-stage nests, solitary and social queen oocyte lengths no longer differed (pairwise Welch's two sample t-tests: early day 7 $p = 0.90$; late day 7 $p = 0.79$, Fig 2-3a). The best fit model predicting oocyte lengths included social treatment, nest stage, collection day, and the interaction between nest stage and collection day as fixed effects.

In the late-stage groups, the presence of workers was associated with decreased oocyte resorption, whereby *late-social* queens had fewer resorbed oocytes than *late-solitary* queens (GLMM pairwise, Tukey-adjusted lsmeans: $p < 0.01$, estimate = 4.65, 95% CI [3.00, 6.30], Fig 2-3b). Because most early-stage queens did not yet have mature oocytes, oocyte resorption was infrequent in these groups and did not differ between *early-social* and *-solitary* queens (GLMM pairwise, Tukey-adjusted lsmeans: $p = 0.096$, Fig 2-3b). The best fit model predicting oocyte resorption included social treatment, nest stage, and their interaction as fixed effects.

b) Effect of workers on queen juvenile hormone levels

Social status strongly impacted queen JH titers, as the presence of workers in the nest resulted in elevated titers, irrespective of queen reproductive state (GLMM $p < 0.001$, estimate = 0.36, 95% CI [0.27, 0.45], Fig 2-4). There was a significant interaction between social status and nest stage, where JH levels in early-stage queens were more strongly impacted by social status than late-stage queens (GLMM $p = 0.002$, estimate = -

0.21, 95% CI [-0.34, -0.08], Fig 2-4). Solitary queens maintained relatively low JH titers at all time points (all Bonferroni-adjusted pairwise Wilcoxon rank sum tests among solitary queens $p > 0.1$), irrespective of reproductive state, although reproductive state, represented by oocyte length, was a weak predictor of JH titer independent of social status (GLMM $p = 0.002$, estimate = 0.05, 95% CI [0.02, 0.08]). The best fit model predicting JH titer included social treatment, oocyte length, nest stage, and the interaction between social treatment and nest stage as fixed effects.

c) Effect of workers on queen functional reproduction

Social nests contained significantly more eggs than solitary nests (GLMM $p < 0.001$, incidence rate ratio = 15388.64, 95% CI [52.85, 4480383.51], Fig 2-5). No eggs were detected in any of the 26 nests of *early-solitary* queens. Of the seven *early-social* nests collected on day 4, one contained eggs, and of the seven *early-social* nests collected on day 7, three nests contained eggs. Social and solitary early-stage nests did not differ from one another with respect to the number of eggs (GLMM pairwise, Tukey-adjusted lsmeans: $p = 0.7$, Fig 2-5) and were excluded from additional statistical analyses because so few nests contained eggs. All *late-social* nests contained eggs, whereas eggs were detected in only 13 out of 21 (62%) of *late-solitary* nests (late day 1, $n = 5$; late day 4, $n = 5$; late day 7, $n = 3$). *Late-social* queens had, on average, approximately twice as many eggs at day 4 (mean +/- s.e.m. 21.86 +/- 4.21 eggs) and four times as many eggs at day 7 (30.00 +/- 4.36) relative to *late-solitary* queens at matching time points (day 4 8.7 +/- 4.65, day 7 6.57 +/- 4.00). The best fit model predicting egg number included social treatment, oocyte length, queen body size, collection day, and the interaction between social treatment and oocyte length as fixed effects.

Experiment 2

a) Effect of workers on queen functional reproduction

Social queens laid eggs sooner than solitary queens in their first and second nests, irrespective of social history (GLMM $p < 0.001$, estimate = 1.15, 95% CI [1.05, 1.25], Fig 2-6a). Queens also laid eggs sooner in second nests relative to first nests, irrespective of social history (GLMM $p < 0.001$, estimate = -0.44, 95% CI [-0.80, -0.36], Fig 2-6a). Nest, social treatment, and their interaction were included as fixed effects in the best fit model to predict the number of days to lay eggs. The number of days until eggs were first observed was more variable in solitary nests relative to social nests (Levene's test for equal variances $p < 0.001$).

Brood were observed in all social nests, but in only 85% of solitary first-nest and second-nest queens. Of the nests that did contain brood, social nests contained on average more brood items (eggs, larvae, and/or pupae) than solitary nests (GLMM social treatment $p < 0.001$, estimate = 36.93, 95% CI [2.80, 33.48], Fig 2-6b). Number of brood items in the second nest was not impacted by social history (GLMM pairwise comparisons via Tukey-adjusted lsmeans *social-solitary* vs. *solitary-solitary* second nests $p = 0.94$; *social-social* vs. *solitary-social* second nests $p = 0.99$, Fig 2-6b). The best fit model predicting brood number included social treatment, social history, and their interaction as fixed effects.

b) Effect of workers on queen mortality

Solitary queens had higher mortality than social queens (mixed effects cox regression $p < 0.001$, chisq = 49.9, Fig 2-7), with the overwhelming majority of mortality occurring in the first nest (seven out of eight total deaths, mixed effects cox regression $p < 0.001$, chisq = 1149.25). No queens in the experiment died while in the presence of workers. Nest and social treatment were included as fixed effects in the best fit model to predict mortality.

Discussion

In social insects with a solitary nest-founding stage, the onset of the social stage is critical for nest survival, yet the mechanisms that facilitate this transition remain understudied. Here, we manipulated the social environment of early nesting queen bumble bees (*B. impatiens*) to explore how the life history transition from living solitarily to socially influences queen reproduction and survival. Our study is predicated on the hypothesis that the presence of workers accelerates queen functional reproduction in bumble bees (Röseler, 1968; Kwon *et al.*, 2006; Woodard *et al.*, 2013), thus aligning queen egg production with the emergence of helpers in the nest, who around this time assume the task of rearing offspring. This alignment is proposed to be adaptive, because it helps ensure nesting success by rapidly increasing the number of workers, and therefore the productivity (Malfi *et al.*, 2019), of the nest. Collectively, across both of our experiments, we found that queens in a social environment exhibit increased ovary activation, elevated juvenile hormone titers, accelerated egg laying, and higher survival, relative to solitary queens. This supports our hypothesis that workers positively impact queen fecundity and survival during the early nesting stage. However, we found that these positive effects are transitory, in that they reflect only the current social environment and not social history. Our experimental design also allowed us to uncouple the social environment from reproductive status, and our data demonstrate that queen juvenile hormone levels are strongly positively impacted by the social environment irrespective of queen reproductive state.

Bumble bee workers promote reproduction and survivorship in queens

In our study, queen ovarian development and egg laying were both increased by the artificial addition of workers to the nest. For example, eggs were observed, on average, three-fold sooner in the first nests of social relative to solitary queens. Similarly, social queens laid on average 2-3 times as many eggs as their solitary counterparts, a finding also observed in the bumble bee *B. terrestris* (Woodard *et al.*, 2013). Conversely, we also observed reduced fecundity in queens who lacked workers in their nests at around the time point in the colony cycle that they would typically emerge. Specifically, we detected fewer eggs and more resorbed oocytes in relevant solitary nests (i.e., nests of *late-solitary* and *social-solitary* queens). These data are consistent with a previous study in *B. terrestris* that found that queens whose workers are removed upon emergence (much like the *late-solitary* group here) exhibit delayed reproduction and increased mortality relative to queens that retain their workers (Engels, 1990). Social insect queens have evolved to produce large numbers of eggs throughout their lives, with queens of some species laying hundreds of thousands, or even millions, of eggs over their lifetime (Winston, 1987). We propose that worker regulation of queen reproductive status is a related, under-studied aspect of eusocial evolution, particularly in lineages with solitary nest founding.

From an evolutionary perspective, queens may perceive workers in the nest as an honest indicator of helpers, and selection might have favored the ability to adjust reproductive output accordingly. However, a queen's first cohort of workers could be lost to events such as extreme weather, predation, or habitat destruction, and the ability to reverse worker-induced reproductive acceleration, as we observed in our experiments, may also be advantageous for queens. Thus, intensive selective pressures might have shaped the evolution of mechanisms that promote synchronization between the acceleration of queen reproduction and a social environment capable of rearing those offspring.

Given enough time (specifically, seven days), solitary queens in our study ultimately did reach similar levels of ovary development as seen in social queens. However, that some social queens, and no solitary queens, were laying eggs at this time point suggests that in addition to their impacts on queen ovarian development, workers may also accelerate or release queen egg laying behavior. Thus, reproductive maturation and egg laying behavior may be controlled independently in the bumble bees, consistent with previous observations that workers sometimes develop their ovaries but do not lay eggs (Duchateau and Velthuis, 1989). Although we did not explicitly test this, the positive association between accelerated reproduction and increased survival in social queens in our study differs from what has been observed in many animals, in which trade offs exist between longevity and fecundity (Stearns, 1992). This relationship is reversed in many eusocial lineages; reproductive queens often live orders of magnitude longer than nonreproductive nestmates (Carey, 2001). Indeed, one study in eusocial ants found that the activation of queen reproduction itself promotes longevity in queens, as reproductive queens outlived their nonreproductive counterparts, irrespective of their social environment (Rueppell *et al.*, 2015). From the results of our study, we are unable to determine whether queen reproductive activation (prompted by worker presence) promotes longevity, as it does in other eusocial insects (Rueppell *et al.*, 2015), or whether there is some alternative mechanism operating that enhances survival. However, the fact that we detected a difference in mortality in this buffered laboratory environment containing unlimited food and no exposure to predators or weather events suggests a physiological, rather than environmental, mechanism. Overall, our data are consistent with the hypothesis that in social insects, sociality promotes queen survival and resilience and decreases variation in the number of offspring queens produce, an idea thus far supported primarily by theoretical rather than empirical studies (Stevens *et al.*, 2007;

Kennedy *et al.*, 2018). Our finding that social queens had the same maximum, but a higher minimum, oocyte length relative to solitary queens suggests that workers may advance queens toward a personal physiological maximum rate of reproduction. Additionally, our data suggest that, while queens can reinitiate new nests after losing or abandoning their offspring, they have no observed reproductive advantage over queens who are starting their first nests. Instead, the reproductive benefits of sociality are conditional on a continuous social input in this species. This finding highlights the importance of producing and maintaining early season workers, and therefore a reproductive and survival advantage, for queens in young nests.

Juvenile hormone is involved in bumble bee social organization

JH likely mediates the accelerated reproduction observed in social queens in our study, which is consistent with its role as a gonadotropin in other insects (Roy *et al.*, 2018). However, JH titers were most strongly impacted by the social environment in our study, irrespective of queen reproductive state. In addition to its conserved role as an adult gonadotropin (Adams, 2009) and regulator of early-life development (Jindra *et al.*, 2013; Truman, 2019), JH has evolved to take on new functions in some insects. For example, it has been co-opted to play a role in reproductive dominance in many social insects (reviewed in Kapheim and Johnson 2017), although this has not yet been demonstrated in bumble bees. Previous studies on JH in bumble bees have focused almost exclusively on workers in the first week of their lives (Röseler, 1977; Röseler and Röseler, 1978; Duchateau and Velthuis, 1989; Bloch *et al.*, 2000a, 2000b; Bloch and Grozinger, 2011;

Hartfelder *et al.*, 2013; Amsalem *et al.*, 2014; Shpigler *et al.*, 2014, 2016), or newly emerged gynes (young queens) prior to diapause (Röseler and Röseler, 1988; Amsalem *et al.*, 2014), rather than nesting queens (but see Amsalem *et al.* 2014). Further, in these previous studies, workers are always maintained in social groups (e.g., Bloch, Hefetz, *et al.* 2000; Shpigler *et al.* 2014), and reproductive individuals in these groups are nearly always considered dominant. Here, by investigating JH in early nesting queens (rather than workers or gynes) across solitary and social conditions, we were able to uncouple the social environment from reproductive state to disentangle dominance, reproduction, and social status.

JH may indirectly promote reproduction in social individuals through its involvement in dominance establishment (consistent with the "challenge hypothesis"; Tibbetts and Huang 2010). This is supported by our finding that *early-social* queens, which were not yet reproducing when workers were added to the nest and were likely in the process of establishing reproductive dominance (Amsalem and Hefetz, 2010), had higher JH titers than *late-social* queens, which were reproductively mature at the time of worker introduction and therefore may have been able to establish dominance more readily. Our data further suggest that relatively high JH levels are not necessary for oogenesis to proceed in solitary individuals. High JH levels are also not necessary for worker ovary development in social colonies (Röseler, 1977). Alternatively, the high JH levels observed in *early-social* relative to *late-social* queens in our study may indicate the involvement of JH in the initiation of egg laying. JH acts broadly on the insect nervous system (Fahrbach and Robinson, 1996; Anton *et al.*, 1999) and plays a major role in the control of oviposition behavior and pheromone production in many insects (Nijhout and Wheeler, 1982). A more thorough investigation of the interaction between JH, oogenesis, and oviposition across a broader spectrum of social configurations is needed to clarify this interaction.

A remaining question is how workers cause the observed changes in queen reproductive physiology, upstream of their effects on JH. In other eusocial insects, nestmates frequently communicate with, and socially regulate, one another through an array of chemical, visual, tactile, and other signals (Billen, 2006). In bumble bees, brood attenuate queen circadian rhythmicity (Eban-Rothschild *et al.*, 2011), but beyond this, the social signals that impact queens are largely unknown. With respect to regulation of reproduction, tactile cues have been shown to stimulate reproduction in cockroach females (Uzsák *et al.*, 2014), whereas pheromones and aggressive interactions have been broadly shown to limit reproduction in eusocial workers (Van Oystaeyen *et al.*, 2014). Further, JH levels are regulated by factors such as temperature, nutrition, and insulin signaling in other systems (Flatt *et al.*, 2005), any of which may be impacted by the social environment in bumble bees. Alternative to, or in addition to, these direct mechanisms, workers may elicit the observed physiological changes in queens indirectly, by altering the queen's energy balance. As the workers take over brood care and nest maintenance tasks, this may free the queen to invest more energy into reproduction. In our study, queen reproductive status reflected the current social environment only, suggesting the social environment has immediate, but not persistent, effects on queen fecundity. Indeed, *social-social* queens reinitiated egg laying in their second nest almost immediately (range 1-5 days) whereas *social-solitary* queens took an average of 13 days to do so (range 3-46 days). Thus, the underlying mechanism promoting queen fecundity is seemingly reversible, and likely requires some continuous input.

Our results are based on one domesticated species of bumble bee, *Bombus impatiens*. Working with domesticated species is essential for the types of experiments we conducted, which are difficult to carry out in the field or with at-risk species. Although we cannot rule out the possibility that artificial selection in the domestication process has impacted our results, we think it unlikely that our results are an artifact of bumble bee domestication. Natural history notes observing accelerated queen reproduction in the presence of workers were first recorded in *B. terrestris* prior to domestication (Sladen 1912), suggesting that this phenomenon is present in some form in wild, undomesticated bees. Further, domestication might be predicted to dampen the observed effects, rather than enhance them. This is because, in the wild, queens experience additional stressors such as overwintering, foraging, nest defense, and a shorter summer season in which to grow their colonies. Any of these added stressors may result in stronger selective pressures for workers to induce accelerated reproduction and increase survival of queens in the ways we have demonstrated. The relatively short (~100 years) process of domestication has minimized, or altogether eliminated, many of these natural stressors for captive, commercial bumble bee populations (Velthuis and van Doorn, 2006). For example, domesticated queens have less selective pressure to expedite their reproduction in the early season, because they are not bound by the short summer season. This may in turn result in a dampening of worker-induced queen reproduction in domesticated relative to wild lineages.

Conclusions

Bumble bee queens, like other annually social insects, initiate colonies in spring that will perish by fall, and there is a limited window of time for colonies to grow and ultimately produce reproductives (males and new queens). Multiple lines of evidence suggest that the earliest stages of colony development are especially important for ultimate colony growth and success. For example, early season resources have disproportionate impacts on colony growth and reproductive success (Williams *et al.*, 2012; Malfi *et al.*, 2019; Mola *et al.*, 2021). Colonies grow exponentially throughout the nesting season, and the number of workers produced directly corresponds to the number of reproductives produced (Crone and Williams, 2016). Thus, queens likely benefit from being able to rapidly establish nests in spring. This also is consistent with the pattern that bumble bee species that emerge from diapause and begin nesting earlier in the spring are less likely to be declining, relative to those that emerge later in the season (Williams *et al.*, 2009). This evidence, along with our finding that queen survival and reproduction increase upon emergence of the social environment, collectively suggest that intervention strategies that target this early nesting stage and promote the production and maintenance of early season workers are needed for effective conservation of this solitary nest-founding, social lineage.

Bumble bees are the most economically important native pollinators in North America (National Research Council 2007) and play essential roles in pollination networks in wild plant communities (Ollerton *et al.*, 2012; Brosi *et al.*, 2017). Early nesting queen bumble bees play a vital role in early season pollination of wild plants and crops such as blueberry, because they emerge early in the season when temperatures are relatively cool and few other pollinators are able to fly (Willmer *et al.*, 1994; Tuell and Isaacs, 2010). Despite the economic and ecological importance of early nesting queens, current conservation strategies focus primarily on supporting bumble bee colonies during the social phase of their life cycle (Goulson *et al.*, 2007). Thus, the needs and unique biology of early nesting queens remain largely unknown and unaddressed (but see Baron *et al*. 2017; Bogo *et al*. 2017; Kells and Goulson 2003; Leza *et al*. 2018; Tripodi and Strange 2019; Watrous *et al.* 2019; Costa *et al.* 2021), although this stage may represent a particularly important demographic stage for bumble bee populations. Solitary queens must both forage and perform all the tasks required for colony success and reproduction, so this stage may respond strongly to environmental stressors such as diminishing or degraded floral and habitat resources, urbanization, pesticide use, and higher temperatures. Ultimately, the sensitivity of this life stage may help explain global declines in bumble bee populations (Goulson *et al.*, 2007, 2015; Cameron *et al.*, 2011).

Given that workers regulate queen physiology in the ways we have demonstrated, the timing of worker emergence in the nest, as well as the maintenance of those workers, likely impacts queen fitness, colony developmental trajectories, and ultimately nesting success in bumble bees. Thus, we propose that bumble bee conservation regimes should focus more heavily on the early nesting period to support the emergence and maintenance of early-season workers in young colonies. For example, ensuring ample, pesticide-free forage and nesting resources in the early spring, particularly in agricultural, urban, and other degraded and disturbed habitats, is one concrete action that would be predicted to have substantial positive impacts on nesting success. Current conservation regimes often focus on mitigating stressors in mid-summer (Goulson *et al.*, 2007), but focusing on the early spring may be just as important, if not more important, for supporting bumble bee population success. Additionally, more research investigating the unique needs and stressors affecting early season queens is essential to developing targeted conservation regimes specific to this life stage. For example, the effects of increased environmental stochasticity (Lande 1993), potential phenological mismatches between queen emergence and floral blooms (Kudo and Cooper, 2019), and warming temperatures (Soroye *et al.* 2020)), on early season queens remain open areas for future climate-change related research. A more in depth understanding of the impacts of parasites and pathogens on early season queens, specifically (as opposed to social colonies), is also needed (but see Mullins *et al.* 2020). Our findings highlight unique aspects of the solitary nest-founding stage in social insects and underscore the importance of conservation interventions that support this early nesting period.

Data availability: Data and code are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.jdfn2z383.

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Figures

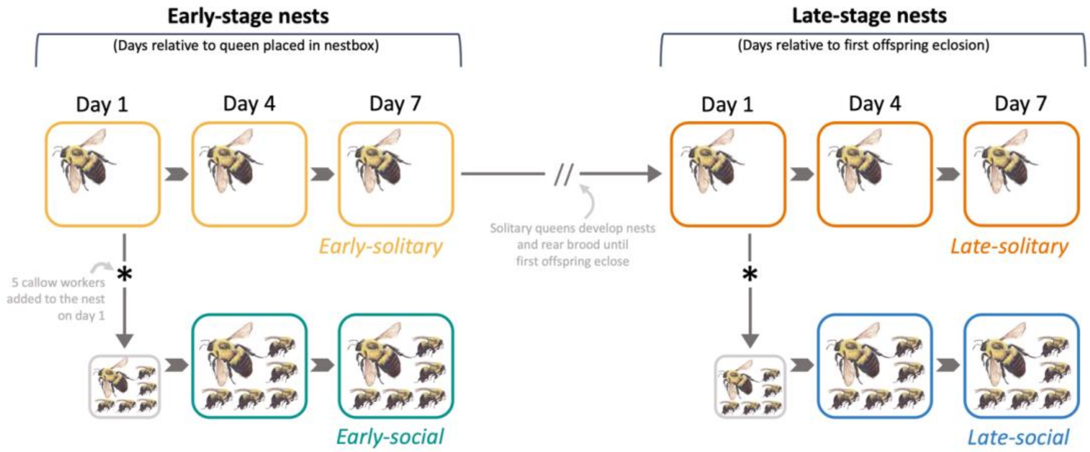

Figure 2-1. Experimental design implemented in Experiment 1. Early-stage nests were collected (i.e., sacrificed and processed) 1, 4, or 7 days after they were placed in a new next box following their second CO_2 treatment. Late-stage nests were collected (i.e., sacrificed and processed) 1, 4, or 7 days after their first offspring eclosed in the nest. All males were removed from nests as soon as they were observed. Solitary queens did not receive workers and were solitary for the duration of the experiment. For social queens, five callow workers were added to nests either one day after the second CO_2 treatment (*early-social*) or one day after the first male offspring eclosed in the nest (*late-social*). *Early-solitary* and *late-social* groups represent the natural development of sociality in young bumble bee nests, whereas *early-social* and *late-solitary* queens represent social manipulations. Colors indicate treatment groups; small boxes indicate days in which workers were added to nests, but no bees or data were collected.

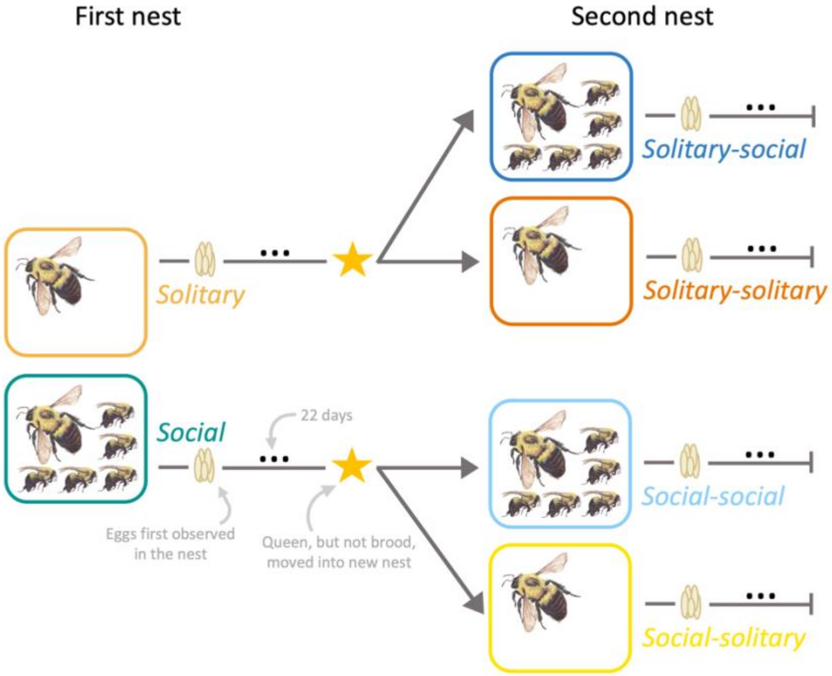

Figure 2-2. Experimental design implemented in Experiment 2. All queens in Experiment 2 were given the opportunity to initiate two independent nests. First nest refers to the nest immediately after the second CO_2 treatment, pre-brood removal. Second nest refers to the nest immediately following brood removal. For social queens, five callow workers were added to nests either immediately following the queen's second CO_2 treatment (first nest) or at the time of transfer to the second nest (second nest). The *solitary-social* group represents the natural development of sociality in young bumble bee nests, whereas the *solitary-solitary*, *social-solitary,* and *social-social* groups represent social manipulations. Colors indicate treatment groups.

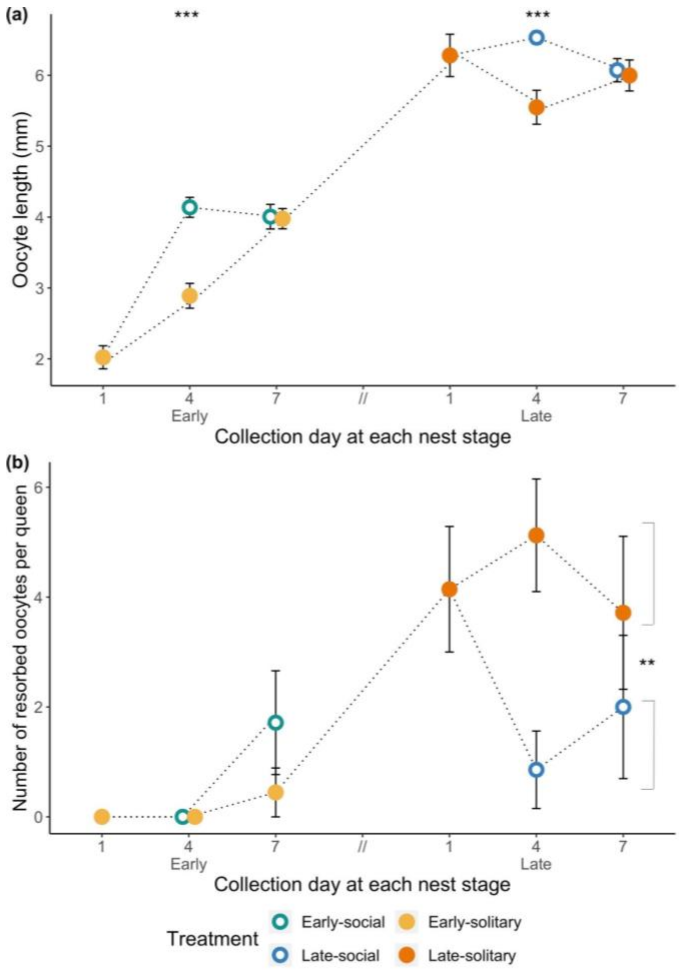

Figure 2-3. Ovary development in queens from Experiment 1. Dotted lines represent the social history of each treatment group. Overlapping points are horizontally jittered for easier visualization. (a) Mean oocyte lengths (+/- s.e.m.) for terminal oocytes. Asterisks represent p-values (*** $p < 0.001$, n = 5-9 queens, 40-71 oocytes) for pairwise Welch's two-sample t-tests between solitary and social queens at each timepoint. (b) Mean number of resorbed oocytes (+/- s.e.m) per queen. Late-social queens had significantly fewer resorbed oocytes than late-solitary queens (post hoc Tukey ** $p < 0.01$, n = 12-24).

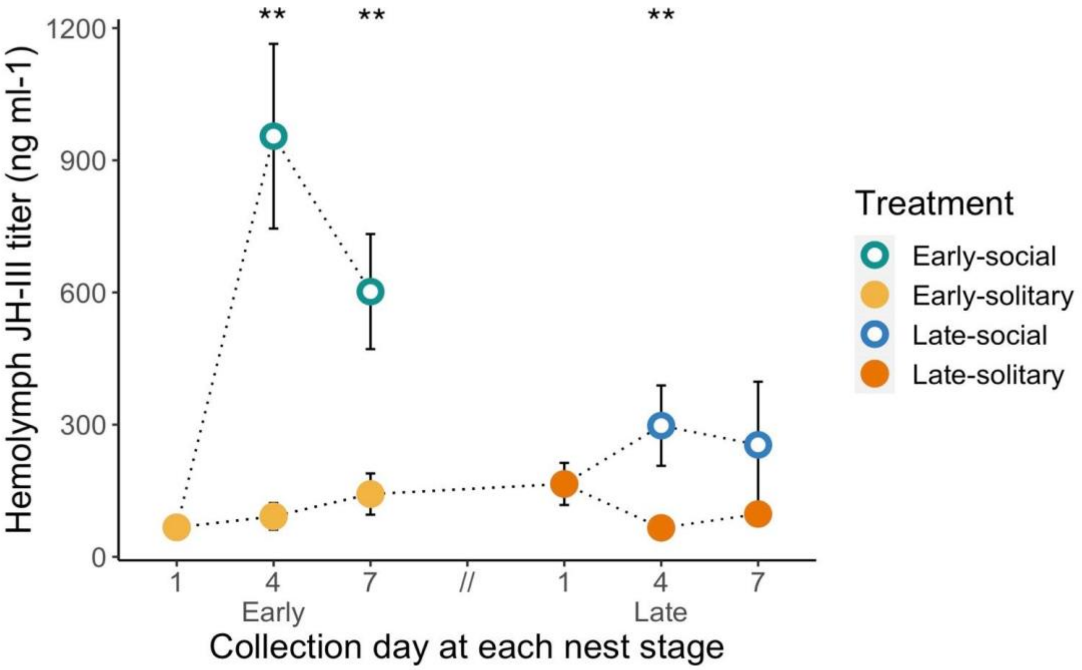

Figure 2-4. Mean juvenile hormone titer (+/- s.e.m.) in queen hemolymph from Experiment 1. Dotted lines represent the social history of each treatment group. Asterisks represent Bonferroni-corrected p-values (** $p < 0.01$, n = 5-9) for pairwise Wilcoxon rank sum exact tests between solitary and social queens at each time point.

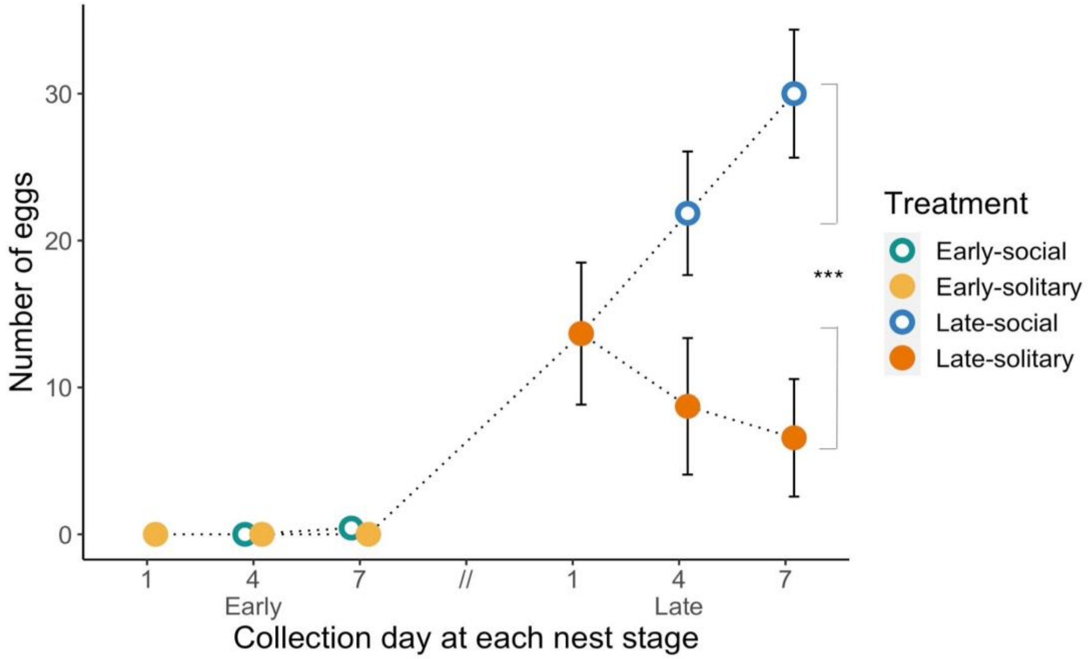

Figure 2-5. Mean number of eggs (+/- s.e.m.) in nests from Experiment 1. Dotted lines represent the social history of each treatment group. Overlapping points are horizontally jittered for easier visualization. No *early-solitary* nests contained eggs at any time point. Nests of *late-social* queens had significantly more eggs than those of *late-solitary* queens (post hoc Tukey *** $p < 0.001$, n = 12-24).

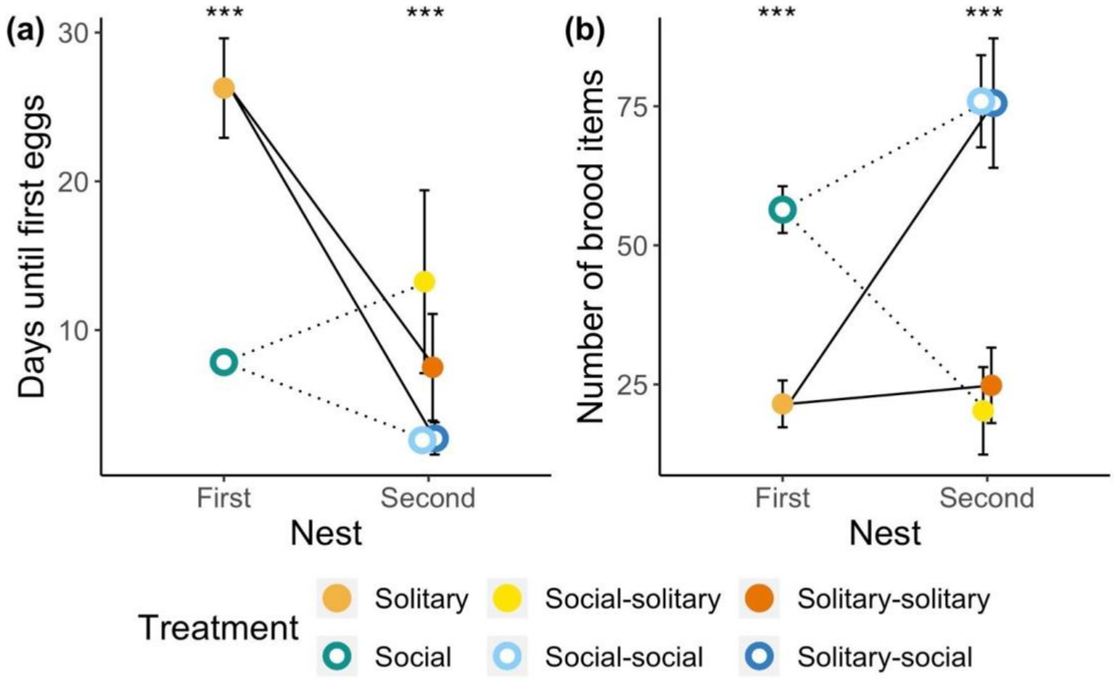

Figure 2-6. Functional reproduction in Experiment 2. Lines represent the repeated measures trajectory and social history of each treatment group: solid lines = first nest was solitary (solitary-solitary and solitary-social groups); dotted lines = first nest was social (social-solitary and social-social groups). Asterisks represent p-values (*** $p < 0.001$, n = 14-19) for post hoc Tukey tests of all solitary versus all social nests at each time point. Overlapping points are horizontally jittered for easier visualization. (a) Mean number of days (+/- s.e.m.) until eggs were first observed. (b) Mean number of brood items (+/- s.e.m.) on the day of collection.

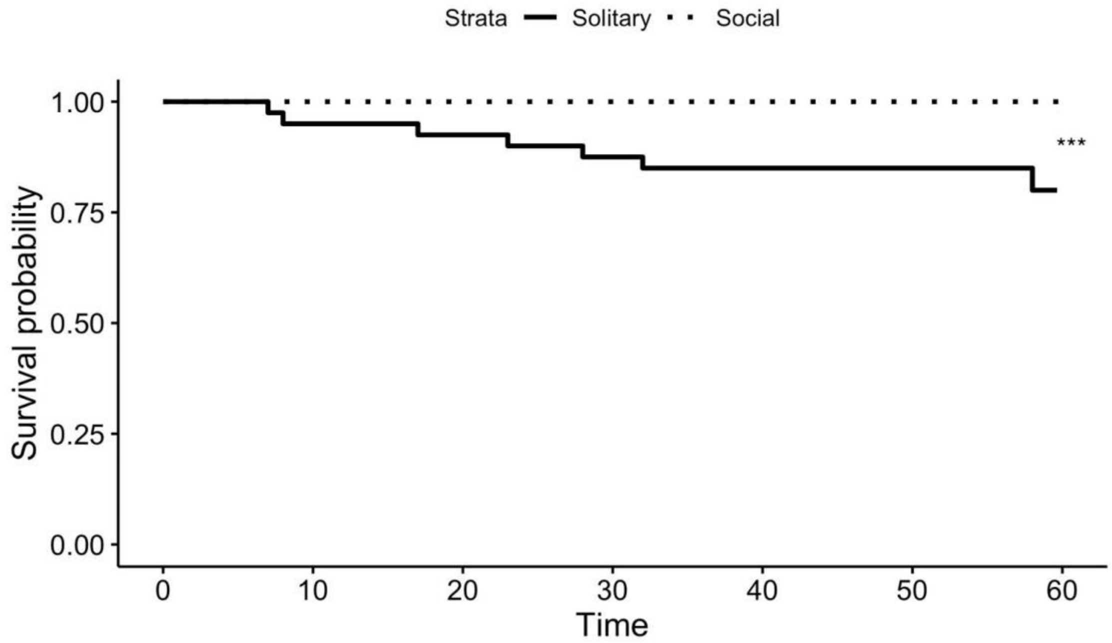

Figure 2-7. Queen mortality in Experiment 2. X-axis: number of days from the start of the nest. Y-axis: proportion of queens alive at the given time point. First and second nests are shown together on a single graph comparing solitary versus social queen survival. Only one queen from a second nest died (on day 58, queen was 101 days old). Age at death for early nest queens = Time + 13 (i.e., number of days in the first nest + age at the start of the first nest). Asterisks represent p-values (*** $p < 0.001$, n = 36-40) for Cox regression model.

Supporting Information

Experiment 1

Bee rearing and experimental design

Queens (n = 83) were sourced from seven out of the 13 total natal colonies with equal distribution of bees from each natal colony across social treatment groups. At this stage, queens were placed in small individual plastic containers (approximately W7 x D7 x H5 cm) until they were transferred to larger plastic nesting boxes (approximately W15 x D15 x H10 cm) within 24 hours of the second CO_2 treatment. In both the *early-social* and *late-social* groups, workers (n = 130) were sourced from seven out of the 13 total natal colonies, with equal distribution of bees from each natal colony across social treatment groups. Workers added to a given nest box were sourced from a single natal colony that differed from that of the queen. Late-stage queens were between 48-97 days old at the time of first offspring eclosion and were randomly assigned to each treatment group. Late-stage queens were between 50-104 days old at the time of collection.

Juvenile hormone quantification methods

To determine the concentration of JH-III and its intermediates in samples, JH-III (Toronto Research Chemicals E589400, North York, ON, CA), its intermediates farnesol (Sigma-Aldrich F203) and methyl farnesoate (Echelon Biosciences S-0153, Salt Lake City, UT, USA), and the internal standard citronellol, were used to make four standard mixes

(Table 2-S1). The retention time for each compound was determined by running dilutions of pure standards in scan (Acquisition General) mode on a Thermo Scientific Trace 1310 gas chromatograph coupled with an AI 1310 autosampler and a TSQ Duo triple quadrupole mass spectrometer running in single quadrupole mode with data acquisition and processing controlled by Chromeleon 7 software. Following this, detection and quantification of standards using selected reaction monitoring (SRM) at the specified retention times was validated using the quantitation and confirmation ion transitions validated in Kai et al. 2018 (Table 2-S2).

To determine retention times, 2 µl of sample was injected at 230°C (inlet temperature) in "splitless with surge" mode with constant helium carrier gas (purity = 99.999% UHP200) flow rate of 1.2 ml min^{-1}, split flow rate of 25 ml min^{-1}, and splitless time of 1 min. Purge flow rate was set to 50 ml min^{-1}, and constant septum purge (surge pressure 20 psi and surge duration 1 minute) and vacuum compensation were selected. Gas saver mode was enabled with a gas saver flow rate of 25 ml min^{-1} and gas saver time of 2 minutes. Compounds were condensed onto, and eluted from, a Thermo Scientific TG-5MS columns (0.25 mm i.d. × 28.33 m, 0.25 µm film thickness). The column oven was initially held at 60°C for 1 minute, then increased to 160°C at a rate of 25°C min^{-1}, followed by a 12°C min^{-1} ramp to 280°C. The run was stopped at 20 minutes. The autosampler syringe was washed for 3 cycles in acetone (Fisher Scientific A949-4) and hexanes before and after each injection. For triple quadrupole MS parameters, the temperatures of the transfer line and ion source were both held at 280°C. For determining retention times, the mass spectrometer was operated in electron ionization mode using general acquisition parameters (scan mode). Scanning within a mass range of 30-500 began at 3 minutes with dwell time set to 0.2.

Following identification of retention times, quantification of compounds in both standards and biological samples using SRM methods was performed according to ion transitions and operating parameters specified in Kai *et al.* 2018 and Table 2-S2, with GC parameters as described above and the mass spectrometer operating as a triple quadrupole instrument. In between each standard or hemolymph sample we ran a blank injection of hexanes using a quick-ramp method with a higher maximum temperature to ensure sample contents were fully eluted from the column (splitless mode with inlet temperature 250°C, column oven initially held at 60°C for 1 minute, then increased to 300°C at a rate of 35°C min^{-1}). JH intermediates (farnesol, methyl farnesoate) were detected as standards using the SRM method but were not subsequently detected in the hemolymph samples. A standard curve of JH-III was generated using the four standard mixes by correlating the spiked JH-III concentration (0, 10, 50, or 400 ng ml^{-1}, Table 2-S1) with the measured peak area ratio of JH-III / citronellol. JH-III concentration (ng ml^{-1}) in each sample hexane extraction was calculated by scaling the peak area ratio of sample JH-III / citronellol to the standard curve. JH-III concentration (ng ml^{-1}) in the original hemolymph was calculated as JH-III concentration (ng ml^{-1}) in hexane extraction times 200 divided by hemolymph volume in µl (200 divided by hemolymph volume is the dilution factor in the hexane extraction process).

Statistical analyses

The log transformation of JH titers was analyzed with a gamma distribution (family = gamma, link = log) and included social treatment (solitary or social), oocyte length (averaged across the eight terminal oocytes for each bee), queen body size (average marginal wing cell length), nest stage (early or late), and collection day (1, 4, or 7) as possible predictors. Nest stage was included as a categorical variable (rather than using sampling date alone to model time), because individual queens did not all produce their first adult offspring at the same age or on the same timeline. Thus, the collection date is relative to offspring eclosion in the late-stage nests, and is not an absolute date. Oocyte length was analyzed with a gaussian distribution (family = gaussian, link = identity) and included social treatment, JH titer, queen body size, nest stage, and collection day as possible predictors. Number of eggs in the nest was analyzed with a binomial distribution (family = binomial) and included social treatment, average oocyte length, JH titer, queen body size, nest stage, and collection day as possible predictors. Queen natal colony was included as a random effect in all analyses, and individual queen was also included as a random effect in analyzing oocyte length, where there were eight measurements per individual (corresponding to the eight terminal oocytes).

Experiment 2

Bee rearing and experimental design

Callow queens used in this experiment were sourced from five out of the 14 natal colonies with equal distribution of each natal colony across treatment groups. Workers were sourced from 13 out of the 14 natal colonies with representation from at least three worker natal colonies (all different from that of the queen) in each experimental nest. Queens were between 39-90 days old at the start of their second nest and between 62-115 days old at the time of final collection. Any workers that died during the course of the experiment (n = 20 bees in 8 nests) were immediately replaced with callow workers from a source colony different from that of the queen.

Statistical analyses

The number of days until the first eggs were detected was log transformed and analyzed with a gaussian distribution (family = gaussian, link = "log") and the statistical model included social treatment (social or solitary), social history (was previously social, was previously solitary, or NA), and nest (first or second) as possible predictors. Number of brood items was analyzed with a gaussian distribution (family = gaussian, link = "identity") and included social treatment, social history, and nest as possible predictors. It is possible that the presence of workers impacted whether or not a queen produced brood at all, but had no impact on the number of brood in those nests that did produce brood. Thus, we analyzed the number of brood for only those nests that produced brood, to be more conservative (i.e., minimize our risk of Type I error) and to increase the specificity of our results. Queen natal colony and individual queen were included as random effects in all analyses. Best fit models were identified according to the methods in Experiment 1.

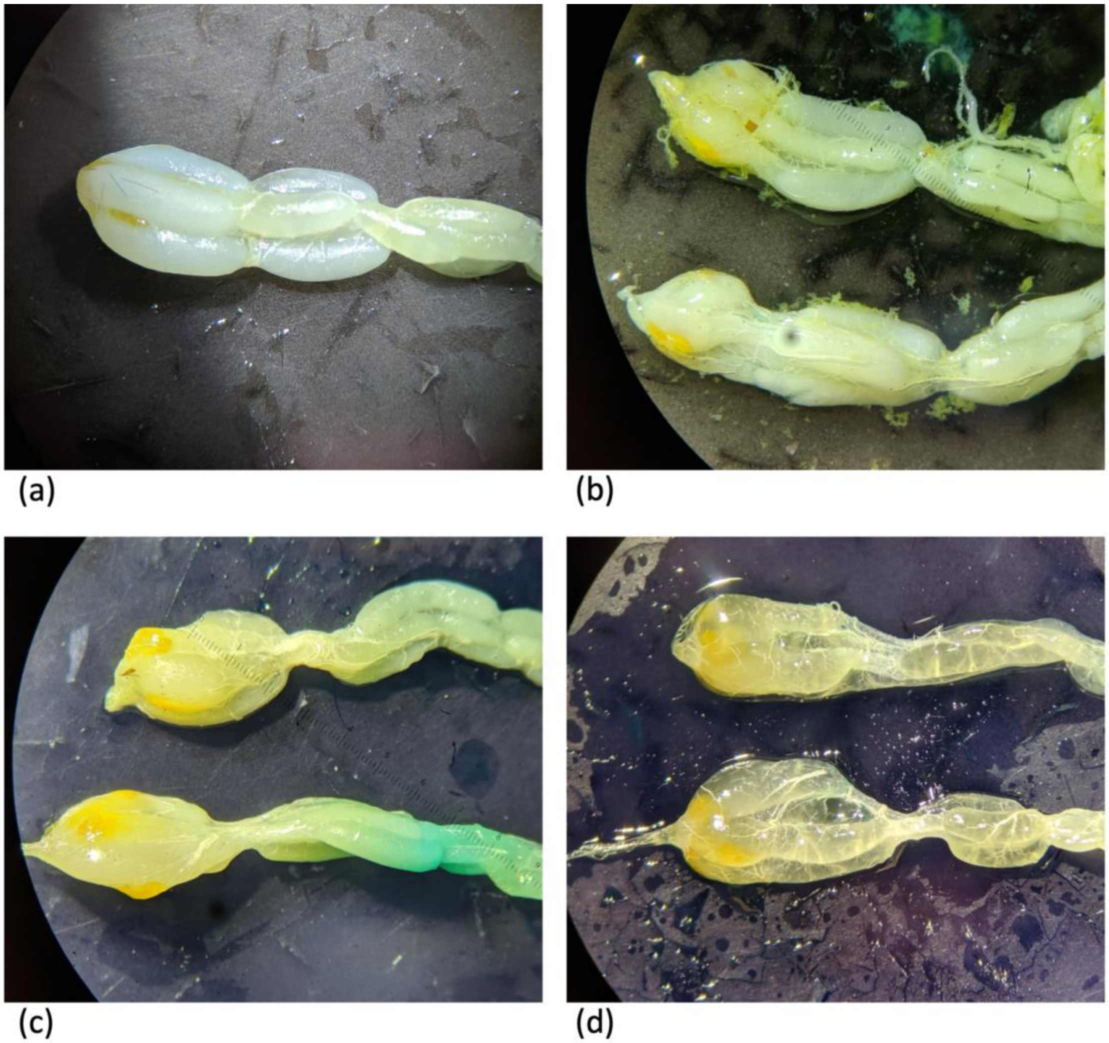

Figure 2-S1. Resorbed versus unresorbed oocytes. Mature, unresorbed oocytes (a) are uniformly shaped and white in color. Terminal ocytes in various states of resorption (b, c, d) can be identified by their yellow coloration and misshapen oocytes lacking a trophocyte.

Table 2-S1. Standard mix compositions used in JH-III quantification.

Standard Mix	Citronellol ($ng\ ml^{-1}$)	JH-III ($ng\ ml^{-1}$)	Methyl farsenoate ($ng\ ml^{-1}$)	Farsenol ($ng\ ml^{-1}$)
1	100	0	0	0
2	100	10	10	20
3	100	50	50	100
4	100	400	400	800

Table 2-S2. MS Settings to quantify each compound in Acquisition-Timed mode on the GC-MS machine.

Compound	Retention Time	Ion Polarity	Window (min)	Pre-width (min)	Post-Width (min)	Parent Mass	Product Mass	Collision Energy
Citronellol	5.37	Positive	0.1	0	0	81	79.1	10
Farnesol Peak 1	8.77	Positive	0.2	0	0	93	77.1	15
Farnesol Peak 2	8.96	Positive	0.1	0	0	93	77.1	15
Methyl farnesoate	9.45	Positive	0.1	0	0	114.1	83.1	10
JH-III main peak	10.33	Positive	0.1	0	0	85.1	59.1	10

CHAPTER 3: BEHAVIORAL ONTOGENY OF MATERNAL CARE IN AN ANNUALLY EUSOCIAL INSECT

(developed with co-authors Kaleigh Fisher and S Hollis Woodard)

Abstract

Understanding how parents balance their time and energy under changing social conditions can directly impact group fitness and is a major goal in biological research. Bumble bees are annually eusocial insects in which queens undergo a stark transition from living a solitary, to subsocial, to eusocial lifestyle over the course of several weeks. We examined how performance of five brood care-related tasks differs for bumble bee (*Bombus impatiens*) queens living either subsocially (with no adult workers) or eusocially (with three or five workers). With three workers in the nest, queens exhibited an approximately 60% reduction in brood feeding and 30% reduction in incubation frequency, relative to subsocial queens. The addition of just two additional workers resulted in a nearly complete cessation of pollen and nectar collection behaviors. Thus, for bumble bee queens, maternal care behavior appears to be tightly regulated by the number of helpers in the nest during the incipient stage of nest development.

Introduction

Many organisms have to balance competing demands for their time, including for tasks that are essential to their survival and reproduction. Each of these tasks can have its own risks, rewards, and level of necessity, and can require different inputs of time, energy, or other resources. For the many animals that exhibit parental care, rearing offspring presents an additional challenge, in which parents must balance their time and energy budget to not only meet their own needs, but also the needs of their offspring (Trivers, 1974; Royle *et al.*, 2014). Identifying how parents prioritize their time, how investment changes under dynamic environmental conditions, and how this ultimately influences offspring survival and phenotype, are major goals in biological research (Royle *et al.*, 2012; Winkler, 2016).

The bumble bees (Apidae: *Bombus*) are a lineage of annually eusocial insects that undergo a striking life history transition from a solitary, to subsocial, to eusocial lifestyle, over the course of a few weeks during the nest-founding stage of their life cycle (Alford, 1970, 1975). After overwintering solitarily in a diapause state, queen bumble bees initiate nests independently in the spring. At this time, when brood are present in the nest, the now subsocial queens must independently carry out all tasks related to rearing offspring. These tasks include feeding and incubating brood, as well as foraging to collect nectar and pollen food resources for the nest. Feeding and incubation take place within the nest, are required for the early growth and development of offspring (B. Heinrich, 1972; Vogt, 1986), and are positively associated with offspring growth rates and body size (Plowright and Jay, 1977; Pereboom *et al.*, 2003). Pollen and nectar are collected from the surrounding floral resource environment, which requires leaving the safety of the (typically enclosed) nest and engaging in energetically costly flight (Bernd Heinrich, 1972; Harrison and Roberts, 2000). It is only after the first cohort of female offspring emerge as adult workers in the nest that the social group transitions to eusociality. At this stage, queens cease performing most brood care behaviors (here defined as both direct interaction with brood and foraging) as workers take over these tasks.

Relatively little is known about the timescale and flexibility of this ontogeny of eusociality in early bumble bee social groups. Broadly, the emergence of workers causes queens to cease feeding brood and invest more heavily in reproduction (Shpigler *et al.*, 2013; Woodard *et al.*, 2013; Sarro *et al.*, 2021). The cessation of brood feeding by queens occurs gradually over a period of a few weeks (Shpigler *et al.*, 2013; Woodard *et al.*, 2013) and leads to a shift towards the production of larger-bodied worker offspring (Shpigler *et al.*, 2013; Costa *et al.*, 2021). Workers eventually take over all brood feeding and food collection for the nest (Cameron, 1989; Jandt *et al.*, 2009), and in these initial stages of nest development they organize tasks such that the majority of workers feed brood and a smaller subset of workers also collect food (Fisher *et al.*, 2022). How queens dynamically balance their time and prioritize tasks as they transition away from parental care are currently unknown.

We recorded a comprehensive set of brood care behaviors in queens of the bumble bee *B. impatiens*, focusing on incipient colonies across the emergence of sociality, to investigate the ontogeny of brood care behaviors in this system. Specifically, we continuously recorded in-nest and food collection behaviors of queens that were either subsocial (with brood but no adult workers in the nest) or incipiently eusocial (with either three or five adult workers), to investigate two main phenomena. First, we explored how the emergence of helpers in the nest affects the task repertoire of queens. This analysis was done to more fully characterize the behavioral changes that queens undergo during this stage, given that previous studies have only examined brood feeding behaviors (Shpigler *et al.*, 2013; Woodard *et al.*, 2013). Next, we examined whether queens cease performing brood care behaviors in a particular order. Here, we predicted that queens would reduce food collection behaviors more quickly than other brood care behaviors. This was predicated on the idea that foraging outside the nest is an inherently risky behavior (Rueppell *et al.*, 2007), and survival of the queen is paramount to nesting success. Thus, we expected that the emergence of workers would cause the queen to cease food collection first, as it may reduce the chances of queen death and subsequent nest failure.

Materials and Methods

Bee rearing and experimental design

Bees were sourced from one of 15 mature (consisting of an egg-laying queen and > 50 workers) *B. impatiens* colonies obtained from Koppert Biological Systems, Inc. (Howell, MI). We created small, artificial nests with one of the following three social configurations: queen alone (subsocial; n = 10), queen with three workers [eusocial (3W); n = 9], and queen with five workers [eusocial (5W); n = 12]. These configurations were chosen to represent nest founding by an individual queen (subsocial) and the successive ontogeny of sociality as workers emerge in the nest [eusocial (3W) and eusocial (5W)]. The first cohort of *B. impatiens* typically contains five workers(Leza *et al.*, 2018; Watrous *et al.*, 2019); however, there is variation in the size of the first cohort, and incipient workers may be lost to predation, exposure, or other factors, and thus our eusocial three-worker group also represents a realistic group size at this stage. All queens in the study were sourced as callow adults (newly eclosed, < 24 hours old), were unmated, and were treated with CO_2 at adult ages 12 and 13 days (30 min per day) to cause them to bypass diapause and initiate egg laying (Röseler, 1985). On day 13, callow workers (newly eclosed, < 24 hours old) were prematurely added to nests to create the two eusocial nest types [eusocial (3W) and eusocial (5W)]. Workers within a single experimental nest all originated from the same natal colony (which differed from that of the queen) with equal representation from source colonies across each of the three social configurations. All bees were fed *ad libitum* pollen (Brushy Mountain Bee Farm, Moravian Falls, NC) and artificial nectar(Boyle *et al.*, 2018) for the duration of the experiment. All nests were maintained in the dark at 25 °C and 60% relative humidity and were minimally disturbed with the exception of replacing pollen, nectar, or deceased workers. Additional details about how bees were sourced, reared, and maintained are provided in Fisher *et al.*, 2022.

Nests were monitored daily for the presence of new eggs and mortality of queens and/or workers. Five days after eggs were first observed in a nest (the approximate time it takes eggs to hatch into larvae; Cnaani *et al.*, 2002), food resources were removed and the nest was connected with polypropylene tubing to two small, lighted (12:12 L:D cycle) foraging chambers (translucent, 177 ml, 7 cm diameter), which each contained pollen or artificial nectar. A window in the room provided additional natural light to these foraging chambers (but not nests) during the day.

Any workers who died during the experiment were replaced within 48 hours with a callow worker (newly eclosed, < 24 hours old) from the same source colony as the deceased worker. Nests, including all bees and brood contained within, were collected and frozen within 24 hours of the first adult offspring eclosion within that nest. Nests were subsequently dissected on dry ice to preserve the integrity of the brood. Total number of eggs, larvae, pupae, and eclosed adult offspring were recorded.

Collection and processing of behavioral data

Each nest and its associated foraging chambers were video recorded with infrared security cameras (VIGICA Peashooter QD520) for the duration of the experiment. We observed and scored a total of 32.65 hours of in-nest video and 303.12 hours of food collection video across all nests, for a mean (+/- s.e.m.) of 1.05 +/- 0.08 hours of in-nest video and 9.78 +/- 1.01 hours of food collection video per nest. Details on video selection can be found in the Supplementary Information. Videos were observed in Behavioral Observation Research Interactive Software (Friard and Gamba, 2016). During each observation period, we scored all instances of five brood care related behaviors integral to successful nest development: brood feeding, nectar collection, pollen collection, incubation, and brood manipulation (Table 3-1). Brood feeding, nectar collection, and pollen collection were recorded as point events with no duration for all workers and queens, whereas incubation and brood manipulation were recorded as state events with a start- and end-point and were only scored for queens. Videos demonstrating brood feeding, brood manipulation, and incubation are provided in the Supplementary Information. The frequency and duration of all recorded behaviors were scaled to the duration of video watched per nest to enable unbiased comparisons among nests.

Statistical analyses

All statistical analyses were performed in R version 4.0.3. We used linear mixed models using the lmer() function from the lme4 package (Bates *et al.*, 2015) to compare the frequency of queen behaviors across social configurations. To reduce bias introduced from nests in which few behaviors were observed, only nests with a minimum of three recorded observations of a given behavior (summed across both workers and queens) were included in analyses for that behavior (per-behavior sample sizes shown in Table 3-S1). We analyzed five models (one per observed behavior), for which the response variables included number of brood feeding events per hour, number of pollen collection events per hour, number of nectar collection events per hour, proportion of time spent incubating, or proportion of time spent manipulating brood. Social configuration [subsocial, eusocial (3W), and eusocial (5W)] was included as a fixed effect and queen natal colony as a random effect in all models. No model selection was implemented, because we only examined a single response variable (i.e., social configuration). No variables were transformed in these analyses, because all model residuals were approximately normally distributed. For all statistically significant models, we performed post-hoc pairwise comparisons among social configurations with a Tukey adjustment using the glht() function from the multcomp package (Hothorn *et al.*, 2022). We also repeated the above analyses on the frequency of behaviors scaled to the number of mature offspring in the nest at the time of collection. Details on these analyses and associated results can be found in the Supplementary Information (Fig 3-S2).

To identify correlations between observed behaviors, we conducted pairwise Spearman's rank correlation tests using the cor.test() function from base R with a Bonferroni p-value adjustment. We also conducted a nonmetric multidimensional scaling (NMDS) analysis using the metaMDS() function from the vegan package (Oksanen *et al.*, 2020) to visualize the distribution of queens based on their behavioral repertoires in two-dimensional space. We performed a Euclidean analysis of similarity (ANOSIM) using the anosim() function from the vegan package to determine whether social configuration influenced NMDS clustering.

To compare the relative number of brood in nests across social configurations, we used linear mixed models with brood number as the response variable, social configuration as a fixed effect, and queen natal colony as a random effect.

Animal welfare statement

We took every effort to minimize suffering and uphold a high standard of animal welfare throughout this study. We followed all institutional and legal guidelines for the rearing of *B. impatiens* and housed all bees in the University of California Riverside's Insectary and Quarantine Facility under permit number 3182 from the California Department of Food and Agriculture. Bees were maintained in standard, climate-controlled environments with constant access to food. At the end of the study, all bees were euthanized with dry ice, which is among the most humane methods of euthanasia. Sample sizes were chosen to maximize statistical power while minimizing the number of bees and research colonies required for the study. All bees were sourced from lab-reared colonies (provided by Koppert Biological Systems, Inc; Howell, MI); thus, our study did not impact wild populations.

Results

Addition of workers to the nests resulted in a significant decrease in the frequency of queen task performance for all observed behaviors, with the exception of brood manipulation behavior (Fig 3-1). Overall, the addition of three workers to the nest was sufficient to cause queens to perform fewer brood feeding and incubation tasks (Table 3-2; Fig 3-1). By contrast, the addition of three workers did not significantly change the frequency of food collection tasks by queens, whereas the presence of five workers caused a sharp decrease in food collection by queens. Most eusocial queens with five workers appeared to cease collecting both pollen and nectar entirely (Table 3-2; Fig 3-1). We observed only one and two eusocial queens with five workers collecting pollen and nectar, respectively, whereas five fed brood, and all incubated and manipulated brood (Fig 3-S1).

There was no statistical difference in offspring development time among nest configurations in our study; all nests had approximately the same amount of time to grow (Table 3-S2). At the time of collection, however, eusocial nests with five workers contained more pupae (late-stage brood) than other nest configurations (Table 3-S2), indicating a higher reproductive output in these nests at the beginning of the study. All nest types contained statistically indistinguishable numbers of larvae and eggs at the time of collection (Table 3-S2).

We performed correlation and clustering analyses to explore associations between brood care behaviors, which revealed associations between brood care behaviors and between the behavioral patterns expressed within social configurations, respectively. Brood feeding and brood manipulation behaviors were strongly, positively correlated across all queens (Bonferroni-corrected Spearman's $p < 0.0001$, rho = 0.81), as were pollen collection and nectar collection (Bonferroni-corrected Spearman's $p = 0.0001$, rho = 0.88). Brood feeding and nectar collection were also positively correlated (Bonferroni-corrected Spearman's $p = 0.019$, rho = 0.64). No other behaviors were significantly correlated (Bonferroni-corrected Spearman's $p > 0.1$). NMDS plots revealed two distinct behavioral clusters, with no overlap between subsocial queens and eusocial queens with five workers (ANOSIM R = 0.29, $p = 0.0007$; Fig 3-2). Subsocial queens were more similar to each other than to the eusocial queens, and this appeared to be driven by frequency of brood feeding and food collection behavior. Eusocial queens with five workers also clustered together, which appeared to be driven by their expression of incubation and brood manipulation behaviors. Eusocial queens with three workers were placed in one or the other of these two clusters.

Discussion

In the face of changing ecological or social environmental conditions, parental care strategies can shift as time and energy budgets are reallocated (Royle *et al.*, 2014). In annually eusocial insects, the individuals who carry out offspring care change over time, from queen (maternal) to worker (sibling) care, as social conditions change in the nest. We manipulated the social environment of incipient bumble bee nests to examine the ontogeny of queen brood-care-related task performance across the emergence of eusociality. We show that queens undergo dramatic changes in brood care behavior as they transition from living subsocially to eusocially during the nest initiation stage. With the addition of three workers to the nest, queens exhibited an approximately 60% reduction in brood feeding and 30% reduction in incubation frequency, relative to subsocial queens; yet there was almost no effect on food collection behaviors. Strikingly, the addition of just two additional workers (for a total of five) resulted in a nearly complete cessation of pollen and nectar collection behaviors. These results support our prediction that food collection would be the first behavior queens ceased performing entirely, although it was not the first behavior to be impacted by the emergence of workers, and it appears to require a threshold of workers (five in our study) for cessation to occur.

Like many parents, bumble bee queens must balance the benefits of investing in offspring with the risks associated with acquiring the required resources for doing so. Bumble bee larvae require continuous feeding on pollen and nectar for growth and development (Plowright and Pendrel, 1977; Sutcliffe and Plowright, 1990). Although these floral resources are stored within the nest, the total amount of these stores is only sufficient to survive a few days before they must be replenished (Cartar and Dill, 1990; Heinrich, 2004; Couvillon and Dornhaus, 2010). Bumble bee queens directly benefit from accelerating the emergence of workers in the nest. As we show here, worker emergence allows queens to reduce their task repertoire, and previous studies have shown that it also allows queens to increase their reproductive output (Shpigler *et al.*, 2013; Woodard *et al.*, 2013; Sarro *et al.*, 2021), and even has positive effects on their survival at the early nesting stage (Sarro *et al.*, 2021). However, foraging to collect food resources is an inherently risky activity. Foraging queens risk exposure to environmental toxicants, such as pollutants (Sivakoff *et al.*, 2020) and pesticides(Baron *et al.*, 2017; Leza *et al.*, 2018), as well as predation (Dukas, 2005; Goulson *et al.*, 2018) and parasite infection (Rutrecht and Brown, 2008; Sarro *et al.*, 2022). Nests that are left behind unguarded are also sensitive to parasitism (Rutrecht and Brown, 2008; Goulson *et al.*, 2018) and nest usurpation by congeneric queens (Elliott, 2009; Koch *et al.*, 2021). Moreover, the stakes are extraordinarily high at the early nesting stage relative to later in the season. This is because the loss of a queen results in death of the entire colony, whereas the loss of a non-reproductive worker appears to have little impact on nest-level social organization at this stage (Fisher *et al.*, 2022) and has a lesser overall impact on nest survival.

Theory and empirical evidence suggest that solitary animals are more risk-averse in their foraging behavior than non-reproductive animals that live socially (Clark and Dukas, 1994; Evans and Raine, 2014). Given this, and the aforementioned risks of foraging, we propose that there is intense selective pressure for queens to cease foraging and remain in the nest, once helpers are available to forage in their stead. Basing the cessation of queen foraging on a distinct threshold that is based on worker number might provide insurance for the nest, so that queens can remain in the nest whilst workers leave to forage, without leaving the nest unguarded by workers. Moreover, given that pollen collection appears to be a relatively cognitively demanding (Menzel, 2012; Muth *et al.*, 2016) and specialized (Fisher *et al.*, 2022) behavior in bumble bees, it might be particularly important to have a "critical mass" of helpers who can perform this more specialized task before the queen can cease doing so.

As workers emerge in the nest, queens are released from the requirement of caretaking, which can be energetically costly and time-intensive (Dunbar *et al.*, 2009; Marshall *et al.*, 2012). This may enable queens to specialize on reproduction. Bumble bee queens increase their reproductive output for several weeks after laying their first eggs (Woodard *et al.*, 2013; Sarro *et al.*, 2021) and sustain this high level of reproduction for the life of the colony (Roseler and Van Honk, 1990; Alaux *et al.*, 2004). In our study, eusocial nests with five workers contained more total brood than subsocial and eusocial nests with three workers. Number of eggs in the nests at the end of the experiment, however, was not different across social configurations. Together, this suggests that having five workers in the nest causes an early, temporary increase in queen reproductive output or brood survival, but the egg laying rate of eusocial queens is ultimately matched by subsocial queens as they too eventually accelerate their reproductive output, a finding that is consistent with previous studies (Sarro *et al.*, 2021).

As numbers of offspring increase, caretaker efforts should increase to match this growing need (Kilner and Johnstone, 1997; Rauter and Moore, 2004). In bumble bees at the incipient nesting stage, caretaking can come in the form of both parental care and sibling care. As queens ceased performing certain tasks in our study, workers carried out these behaviors in their stead (see Supplementary Information). In nests that contained more offspring, we observed more frequent brood feeding and food collection behaviors summed across all caretakers (queens and workers). This increase in frequency of behaviors scaled proportionally to the number of offspring in the nest. Thus, in our study, the total amount of brood care in each nest was modulated both by the amount of brood and the number of helpers, such that each larva received approximately the same amount of care, irrespective of the number of offspring or the number and composition of caretakers. Wild bumble bee colonies can vary dramatically in the number of offspring and caretakers in the nest at any given time. Their ability to respond dynamically to both offspring need and caretaker number, even in small, incipient nests, is likely paramount to colony fitness.

The expression of certain behaviors may be correlated because they are released by shared stimuli, regulated by similar underlying neural circuits, or other factors. Nectar collection and pollen collection were highly correlated behaviors in our study. Both can be characterized as “food seeking” behaviors, which are often regulated by shared feeding-related circuitry (Wright, 2016). In the wild, individual bumble bees often collect both nectar and pollen, including within a single foraging trip (Goulson *et* *al.*, 2002), and all foraging individuals must be both positively phototactic (Porath *et al.*, 2019) and motivated to fly in order to leave the nest in pursuit of food. We also observed a correlation between brood feeding and brood manipulation behavior, the latter of which we propose involves inspection for larval hunger signals, which have been identified in bumble bees (Boer and Duchateau, 2006). Queens did not reduce their brood manipulation behavior with the addition of workers to the nest but did feed brood less frequently. This suggests that queens maintain their affinity for brood as they transition to eusociality, but their threshold for responding to larval hunger cues is either raised, or their need to feed offspring is reduced because workers more readily complete this task (Fisher *et al.*, 2022).

Our clustering analyses revealed two distinct clusters, whereby subsocial queens were most strongly characterized by brood feeding and food collection behaviors, whereas eusocial queens with five workers were focused primarily on brood manipulation and incubation. Interestingly, individual eusocial queens with three workers were found clustered within both of these categories, rather than existing along a gradient in between. This, together with the near-complete cession of food collection by queens with five workers, suggests that there might be a binary axis between the expression of food-related behaviors (food collection, brood feeding) and other brood care-related behaviors (incubation, brood manipulation) in bumble bee queens. We propose that queens switch between these two ends of the axis with the ontogenetic, socially regulated shift from subsociality to eusociality. In our study, the threshold number of workers that prompt this transition varied somewhat among individual queens. Given that all bees experienced nearly identical, lab-buffered environments, the source of this variation remains unknown, and presents an interesting avenue for future research. Many animals undergo a similar pattern wherein they switch between alternative life history stages, with the transition mediated by the social environment (e.g., locusts; Simpson *et al.*, 2001), as well as intrinsic sensitivity (Walton and Toth, 2016).

In addition to the social environment, ecological conditions may also influence life history transitions in early nesting bumble bee queens. The limited available evidence suggests that bumble bee queens are highly plastic at this life stage and have the capacity to change dynamically in response to environmental fluctuations. Alpine species, which have an extremely limited nesting season (Vogt *et al.*, 1994) relative to more temperate species like *B. impatiens*, appear to have evolved particularly unique strategies for using heat (produced in the thorax) to accelerate the onset of ovary development, and thus nest initiation (Heinrich and Vogt, 1993; Vogt *et al.*, 1994, 1998). Temperate queens of the bumble bee *B. vosnesenskii* often develop their ovaries before they locate nest sites, during periods of intensive flight, which might allow them to more rapidly initiate nests upon site selection (Sarro *et al.*, 2022). Thus, it appears that initiating nests earlier in the season is advantageous for bumble bees (Williams *et al.*, 2009; Sarro *et al.*, 2021, 2022), in particular when the overall nesting period is short. However, if workers are lost from the nest, queens can also reinitiate new nests and revert back to caring for brood once again, if necessary (Tripodi and Strange, 2019; Sarro *et al.*, 2021). Decision-making related to offspring investment and care may be further impacted by the abundance and quality of surrounding floral resources, which may impact when queens cease foraging, and ambient temperature conditions might influence the need for brood incubation in the nest (Vogt, 1986; Heinrich, 2004). For early nesting queen bumble bees, investment in offspring is likely mediated a complex interplay between social and ecological conditions.

It must be cautioned that our study was conducted with unmated, commercially reared bumble bees in artificial environments. Bees did not fly or manipulate flowers when collecting food resources, nor were they exposed to threats such as predation. That we observed such a dramatic decline in food collection behaviors, even in a relatively buffered lab environment, suggests that these behavioral changes in queens derive from intrinsic programming and cues from the immediate social environment, rather than factors such as perceived risk. Moreover, we might expect to observe even stronger evidence for physiological or time-related trade-offs in the wild, where food resources can be more limited and bees are exposed to additional stressors. Queen mating status may further influence queen behavior, and presents an avenue for future investigation. Additional work on wild, free-foraging queens is essential to understanding how queens balance all of the competing demands present in real-world environments.

In the majority of eusocial insect species, queens found nests independently and provide all brood care, before transitioning to eusociality when the first worker offspring eclose in the nest (Cronin *et al.*, 2012). Yet to date, studies examining how foundress queens allocate their time given the heightened demands of nest founding are exceedingly rare. Queens may be under intense selective pressures at this time. The success of a foundress queen is compulsory to a successful colony: without the successful production workers, queens cannot produce reproductive offspring (i.e., males and new queens) later in the season. Our findings demonstrate that bumble bee queens respond to the emergence of helpers in the nest by reducing their expression of brood care and increasing their reproductive output. However, these changes are tightly regulated by worker number, with incremental changes in worker number having particularly strong effects on queen food collection for the nest.

Data availability: All data and associated code are available through GitHub (https://github.com/erica-sarro/OntogenyMaternalCare22).

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Tables and Figures

Table 3-1. Ethogram. Videos of brood feeding, incubation, and brood manipulation can be found in the Supporting Information.

Behavior	Definition
Pollen collection	Bee is fully inside the pollen chamber for >10 seconds
Nectar collection	Bee is fully inside the nectar chamber for >10 seconds
Brood feeding	Stereotypical behavior in which bee chews a hole in brood wax, places mouthparts into the hole, and contracts abdomen to regurgitate food into brood cell
Incubation	Bee is stationary and perched on brood wax in a characteristic flattened position;(Heinrich, 1974) >½ of abdomen contacting wax cells containing brood; abdomen often pumping
Brood manipulation	Bee is actively antennating wax cells containing brood, with <3 seconds between antennations

Table 3-2. Linear mixed model (LMM) output for queen behaviors. Each row represents a different LMM, with the listed behavior as the response variable, social configuration as a fixed effect, and queen natal colony as a random effect. Numbers represent p-values from Tukey-adjusted pairwise comparisons.

	Subsocial - Eusocial (3W)	Subsocial - Eusocial (5W)	Eusocial (3W) - Eusocial (5W)
Pollen collection (# per hour)	$p = 0.52$	**$p < 0.0001$**	**$p < 0.0001$**
Nectar collection (# per hour)	$p = 0.97$	**$p = 0.0090$**	**$p = 0.030$**
Brood feeding (# per hour)	**$p = 0.0058$**	**$p = 0.00064$**	$p = 0.92$
Incubation (proportion of time)	**$p = 0.020$**	**$p = 0.0060$**	$p = 0.98$
Brood manipulation (proportion of time)	$p = 0.94$	$p = 0.63$	$p = 0.84$

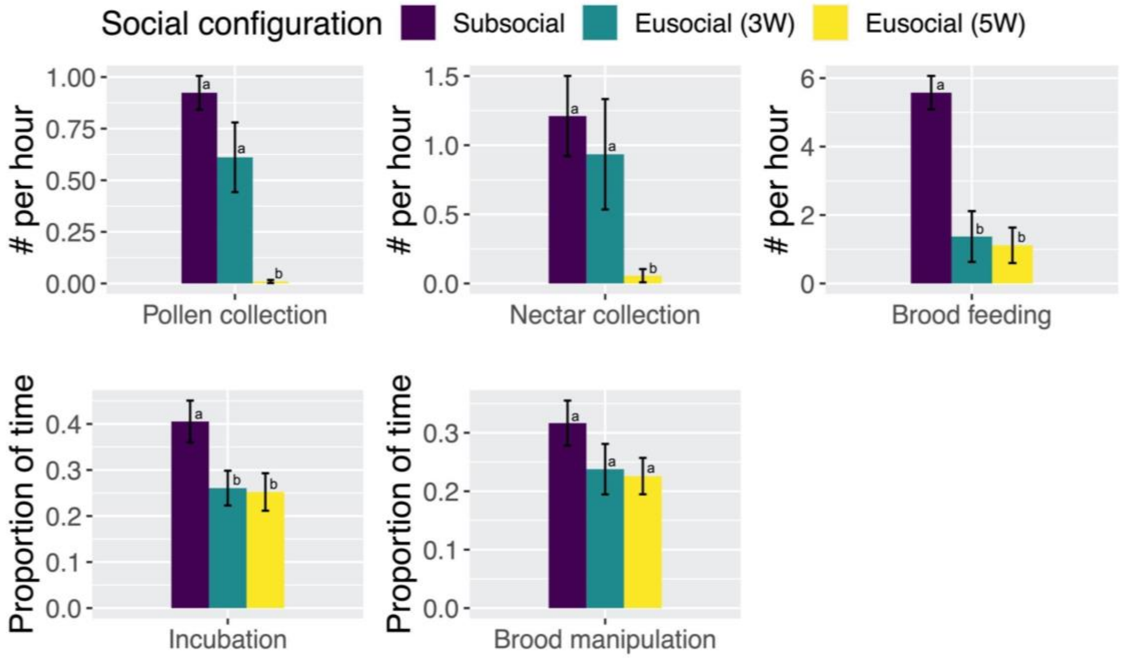

Figure 3-1. Frequency of queen task performance for all measured behaviors, scaled to duration of video watched. For each graph, data excludes nests with <3 recorded observations of the graphed behavior. Different letters within a single graph represent significant differences between groups (Linear mixed model pairwise Tukey p-value < 0.05). Model output and p-values can be found in Table 1-2.

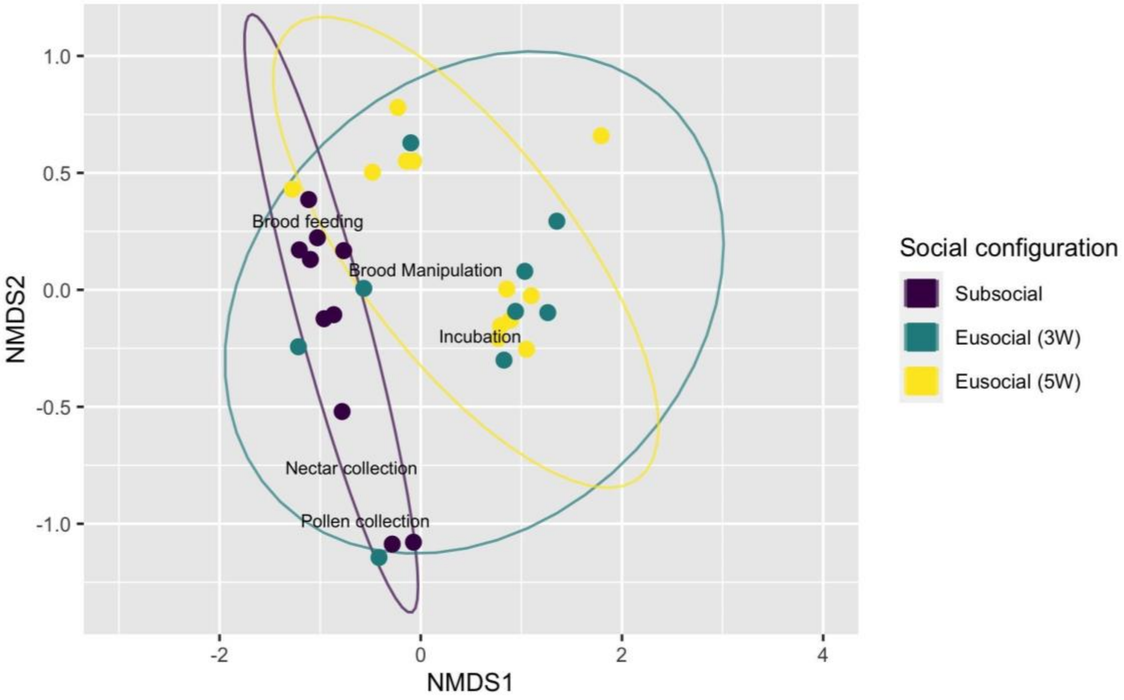

Figure 3-2. NMDS clustering of recorded behaviors, scaled to duration of video watched per queen. Ellipses represent 95% CI for each social configuration. Queens in each social configuration were more similar to one another than to queens in different social configurations (Euclidian analysis of similarity R = 0.29, p = 0.0007). Here, data includes all recorded queen behaviors, regardless of number of observations per nest.

Supporting Information

Methods

a) Experimental Design Details

For all nests, newly eclosed, callow (< 24 h old) queens were removed from their natal colonies on the day of their eclosion and maintained solitarily until adult age 13 days. Unmated queens that are subjected to CO_2 treatment as in our study develop their ovaries and lay eggs on a similar timescale and suppress worker reproduction equally as well as mated, post-diapause queens (Amsalem *et al.,* 2015; Amsalem *et al.,* 2017a; Amsalem *et al.,* 2017b; Watrous *et al.,* 2019). All queens were age matched (adult age 13 days) to minimize any age-based impacts on queen behavior. Workers were added prematurely to nests, rather than waiting for workers to emerge from nests naturally, in order to isolate the impact of the workers themselves on queen behavior and minimize bias associated with queen age or history of brood care behaviors. Wings were removed from all adult offspring and the marginal cells were measured to enable body size approximation (Medler, 1962) of adult offspring.

b) Video Selection Details

Twenty-four randomly selected hours were selected from each of two timepoints (7-9 days and 12-15 days after eggs were observed in the nest). These timepoints represent 1) when larvae are 2-4 days old and all bees in the nest have been carrying out brood care behaviors for several days, and 2) when nests contain larvae at all stages of development and may contain early-stage pupae. We chose these nonconsecutive timepoints to account for variation due to circadian rhythmicity and larval age. For each selected hour, we watched the first five minutes of in-nest video and the full hour of food collection video, because food collection behaviors occur less frequently and thus require more video data to observe rare events. Total amount of video watched per nest was less than 48 x one hour and five minutes, because some recordings skipped or were otherwise unintentionally shortened due to technical issues. Although some behaviors (e.g., food collection) were observed relatively infrequently, that all nests grew and produced adult offspring indicates that bees did carry out these behaviors, regardless of whether we observed them doing so.

c) Statistical Analyses

To investigate the frequency of task performance relative to the number of brood in the nest, we performed linear mixed models with behavioral data scaled to the number of mature offspring (pupae and adults) in the nest on the day of collection as the response variable. These offspring represent the approximate number of brood present in the nest at the time of behavioral observations (performed 15-23 days earlier) based on the development time of *B. impatiens* males (Cnaani *et al.,* 2002). This second set of analyses compares the number of observed point behaviors (brood feeding, pollen collection, and nectar collection) per hour per brood item and the duration of state behaviors (brood manipulation and incubation) per hour per brood item across social configurations. All models included social configuration as a fixed effect and queen natal colony as a random effect.

Results

We observed sharp declines in the frequency of all queen behaviors scaled to the number of offspring in the nest (Fig 3-S2). For the behaviors in which we scored both queens and workers, however, the nest-level frequency of each behavior (i.e. summed across workers and queens in each nest) scaled to the number of offspring in the nest was statistically indistinguishable among social configurations (Fig 3-S2).

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Figures, Tables, and Videos

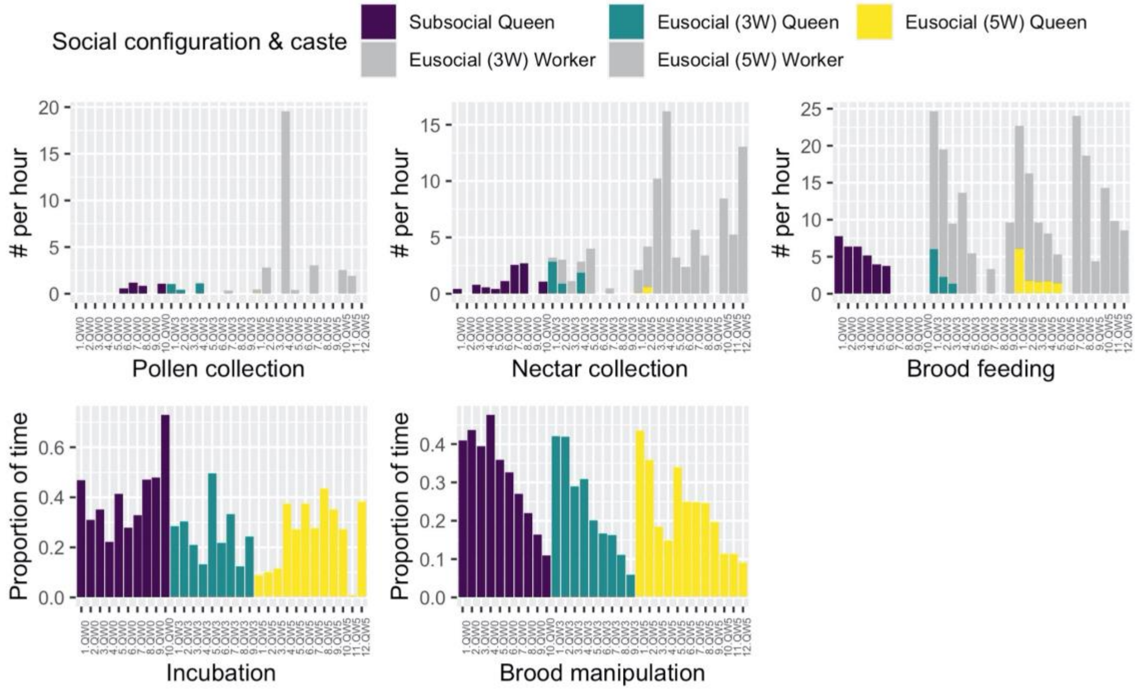


Figure 3-S1. Frequency of queen and worker task performance for all measured behaviors, scaled to duration of video watched, split out by individual nest. Nests in all panels are ordered first based on their observed frequency of queen brood feeding and then by their observed frequency of queen brood manipulation. Here, data includes nests with <3 recorded observations of the graphed behavior, although those respective nests were not included in analyses for that behavior. Incubation and brood manipulation behaviors were not recorded for workers.

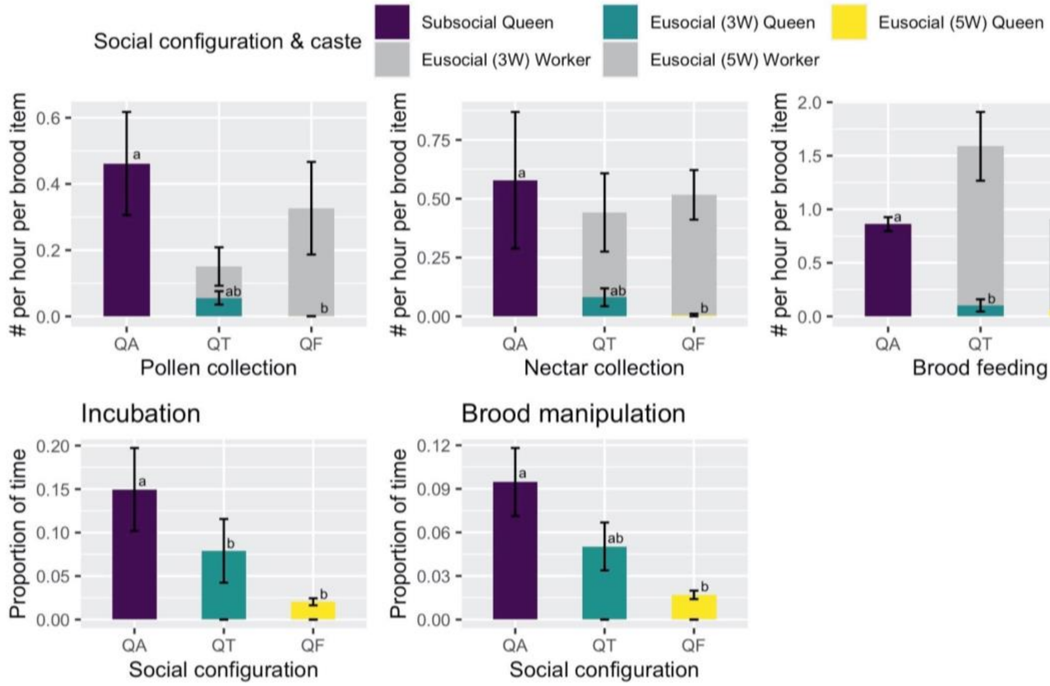


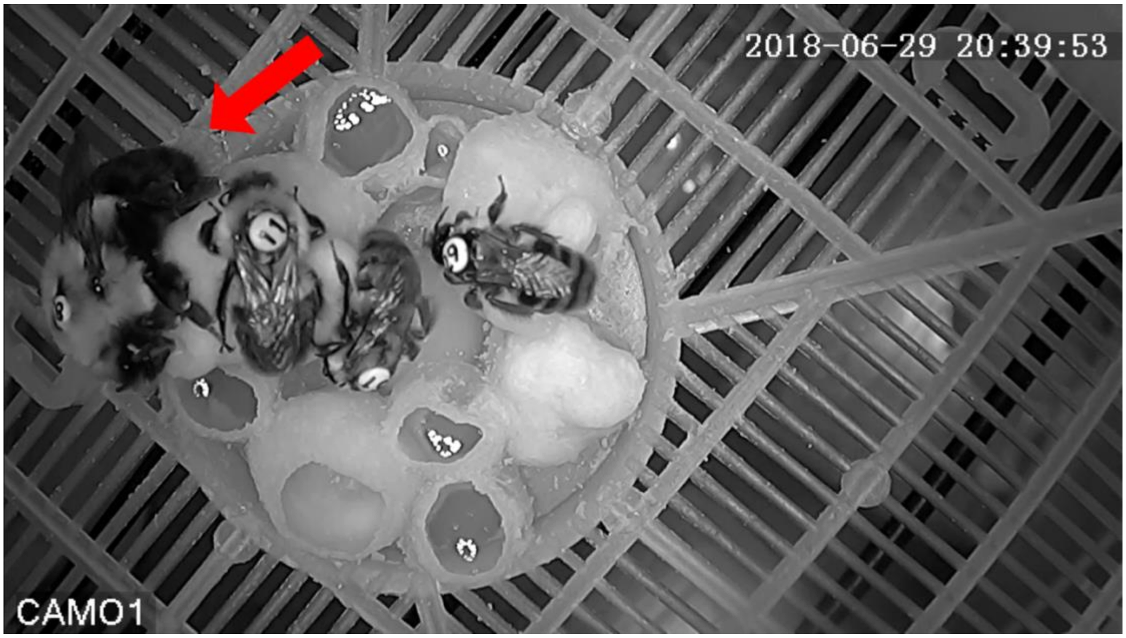
Figure 3-S2. Frequency of queen and worker behaviors, scaled to both the number of hours of observation and the number of brood items in the nest. Incubation and brood manipulation behaviors were not recorded for workers. Different letters within a single graph represent significant differences between queens (linear mixed model pairwise Tukey p-value < 0.05). There were no significant differences in the frequency of behaviors at the nest level (i.e. summed across workers and queens in each nest) among social configurations.

Table 3-S1. Sample sizes for each behavioral analysis.

	Subsocial	Eusocial (3W)	Eusocial (5W)
Pollen collection	4	7	7
Nectar collection	8	6	11
Brood feeding	6	7	11
Incubation	10	9	12
Brood manipulation	10	9	12

Table 3-S2. Mean +/- s.e.m number of brood items, offspring development time, and adult offspring size at the time of nest collection. Asterisks indicate a significant difference among groups; letters within a single column indicate which groups are different from one another, in which differing letters indicate a pairwise Tukey comparison p-value < 0.05.

	Eggs	Larvae	Pupae*	Eclosed adults	Total brood items*	Development time (days)	Adult size* (marginal wing cell mm)
Subsocial	10.4 +/- 2.37	26.60 +/- 3.10	^{a}3.5 +/- 0.76	1.5 +/- 0.22	^{a}42 +/- 5.82	30.50 +/- 1.78	^{a}2.78 +/- 0.039
Eusocial (3W)	14.78 +/- 3.29	26.22 +/- 4.06	^{b}7.78 +/- 2.45	2.33 +/- 0.66	^{a}51.11 +/- 3.77	29.00 +/- 1.59	^{b}2.92 +/- 0.045
Eusocial (5W)	17.00 +/- 3.19	36.83 +/- 3.90	^{b}12.58 +/- 1.34	1.91 +/- 0.12	^{b}68.33 +/- 4.77	29.58 +/- 1.48	^{a}2.84 +/- 0.42



Video 3-S1. Brood feeding behavior. Queen (largest bee, #85, left) opening wax cup and feeding larvae. Red arrow points at queen as she contracts her abdomen and regurgitates food into the brood cell. Full video can be found at https://github.com/erica-sarro/OntogenyMaternalCare22.

Video 3-S2. Incubation behavior. Queen (largest bee, #85, right) in putative incubation stance on brood wax, with abdomen pumping. Full video can be found at https://github.com/erica-sarro/OntogenyMaternalCare22.

Video 3-S3. Brood manipulation behavior. Queen (largest bee, #85, left) antennating brood wax. Full video can be found at https://github.com/erica-sarro/OntogenyMaternalCare22.

CHAPTER 4: DEVELOPMENT AND PILOTING OF A CUSTOM RADIO FREQUENCY IDENTIFICATION (RFID) SYSTEM TO TRACK FOUNDRESS QUEEN BUMBLE BEE FORAGING ACTIVITY

(developed with co-authors William Grover and S Hollis Woodard)

Abstract

Bumble bee queens initiate new nests independently each spring, at which time they solitarily carry out all tasks related to larval rearing and nest maintenance. This foundational stage of the bumble bee life cycle is thought to be particularly sensitive to environmental stressors, which may help explain national and global declines in bumble bee populations. The colony founding stage and the factors that limit nesting success, however, remain severely understudied. In the summer of 2021, we piloted a project to produce the first-ever continuous records of bumble bee queen foraging activity, as inferred by entrances into and exits from the nest. These data are a first step in identifying how early-nesting queens juggle the various demands on their time and energy in the earliest stages of the colony.

We developed a custom radio frequency identification (RFID) system to continuously record the comings and goings from the nest of three wild, trap-nested bumble bee queens (*Bombus appositus, B. rufocinctus,* and *B. centralis)*. Queens were successfully recorded for a majority of their flight periods (approximately three weeks). Our results suggest that queens make an average of 10 foraging trips per day, each lasting an average of 32 minutes, with the longest foraging trips occurring in the latter half of their flight periods. Queens ceased foraging within one week after workers were observed in the nests. Our results suggest that queens spend a substantial amount of their time and energy foraging for their young nests, but readily cease foraging once adult workers are present in the nest and capable of doing so in their stead.

Introduction

Bumble bees are among the most effective wild pollinators because of their highly efficient foraging strategies, and they are central players in pollination networks because they visit a wide breadth of flowering plant species (Ballantyne *et al.*, 2015). Bumble bees are also among the most economically important groups of managed native pollinators in US agriculture and are the primary pollinators for many field-grown and greenhouse crops (National Research Council, 2007). Early nesting queen bumble bees play a vital role in early season pollination because they emerge early in the season when temperatures are cool and few other pollinators are able to fly (Willmer *et al.*, 1994). The group has recently emerged as a model system for molecular research (Woodard *et al.*, 2015), with two fully-sequenced genomes and additional large-scale datasets available for answering long standing ecological and evolutionary questions

(Sadd *et al.*, 2015). However, despite the importance of these animals and the extensive ongoing research on their biology, there is a deficit of natural history information for wild, free-living bumble bees.

One life stage that remains severely understudied for bumble bees is the colony founding stage. Bumble bee queens initiate new nests independently each spring. During this time, queens forage for resources, maintain the nest, and feed and incubate brood, all without the help of workers. The success of a foundress queen at this stage is compulsory to a successful colony; if a queen is unable to successfully rear worker offspring, she cannot ultimately produce reproductive offspring (i.e., males and new queens) later in the season. This fundamental stage of the bumble bee life cycle is thought to be particularly sensitive to environmental conditions. For example, recent laboratory studies have found that stressors such as a lack of pollen dietary diversity (Leza *et al.*, 2018) and pesticide exposure (Wu-Smart and Spivak, 2016; Baron *et al.*, 2017; Leza *et al.*, 2018) have strong negative effects on queen nest initiation and development. Additional work suggests that lack of dietary diversity negatively affects larval development and egg production in small, artificial, queenless colonies (Tasei and Aupinel, 2008; Moerman *et al.*, 2016), and poor resources early in the season have persistent, negative effects on late colony growth rate and queen production (Williams *et al.*, 2012; Malfi *et al.*, 2020).

During the eusocial nesting stage, when workers are present in the nest, worker bumble bees can compensate for poor resource environments by altering their foraging behavior, for example by increasing the lengths of foraging bouts (Kleijn *et al.*, 2015; Hemberger and Gratton, 2018) or the distance traveled (Jha and Kremen, 2013) during resource dearths. Early nesting queens, however, may face constraints in their ability to compensate in similar ways. This is because queens must simultaneously forage and produce eggs, two activities that are physiologically limiting to one another in many animals (the “flight-fecundity trade-off”; Zera and Harshman, 2001). Furthermore, time away from the nest foraging is time not spent incubating or feeding brood and leaves the nest vulnerable to parasitism (Lhomme and Hines, 2019). Detailed analyses of early nesting biology that focus on queen behavior and physiology are thus critical to gain a complete understanding of how queens balance the various demands on their time and energy (Dunbar *et al.*, 2009; Sarro, in review). Ultimately, this information is necessary for accurately predicting bumble bee responses to a changing world.

We piloted a new methodology to trap-nest and monitor foundress queen bumble bees with a custom-built radio frequency identification (RFID) system. We continuously recorded queen comings and goings from the nest for the majority of their spring flight period. Given that worker bumble bees typically make multiple foraging trips per day (Russell *et al.*, 2017), we predicted that queens would also make several foraging trips per day. We additionally predicted that queens would cease foraging entirely within two weeks of adult workers emerging in the nest. Foraging is an inherently risky behavior, thus we expected queens would cease performing this behavior once workers were capable of performing it in their stead (Sarro *et al.*, In prep). We present this methodology and preliminary data as an auspicious approach to studying this elusive, yet foundational life stage in bumble bees.

Materials and Methods

Study Sites

We placed 100 wooden nesting boxes (~20 x 20 x 20 cm; ½” maple sanded SoyStrong plywood) at subalpine sites in the Rocky Mountain Biological Laboratory (Gothic, Colorado) in early spring (~May 15, 2021), to encourage *Bombus* queen colonization. Boxes were placed in one of five habitat types (Table 4-1) and either placed directly on the ground or strapped to trees or other structures at a height of ~1.5 m. We recorded the habitat type, cardinal direction of nest entrance, ground slope, ground aspect, and relative canopy cover for all boxes. Boxes were filled with small animal bedding, including aspen wood shavings, sterilized sphagnum moss, and paper bedding. To the greatest extent possible, we filled boxes with these materials according to Mjelde (2020), with the exception that we omitted all cotton substrate, which can get caught on radio frequency identification tags. We lined the interior of 19 of the 100 nest boxes with materials from abandoned rodent nests found within Gothic. We also added baked clay balls (1 cm diameter) to half of all nest boxes. Lab-reared queens prefer to lay eggs on these clay balls (Sarro *et al.*, 2021 *unpublished data*), thus we included them in the case that wild queens might find them attractive, as well. We covered half of the nest boxes with clear plastic sheeting to repel rain.

Study Subjects

We inspected nest boxes weekly, at night, with an endoscopic camera (Anhendeler B315) inserted into the nest entrance, to check for evidence of queen colonization. Once a queen successfully colonized a nest (as evidenced by queen presence in the box and wax deposited within the box), we temporarily removed the queen from the nest box and placed her into a queen marking tube (unanesthetized). While the queen was immobilized in the marking tube, we identified her to species and attached a radio frequency identification (RFID) tag (3.2 x 3.2 x 0.4 mm; Murata Electronics XMS33HCNK-171) to her thorax with cyanoacrylate glue (Fig 4-1a). We then affixed a custom-built bidirectional RFID reader (described below) to the nest entrance to passively collect timestamp data as the queen entered and exited the nest (Fig 4-1b).

For several weeks after the queens were tagged, we monitored colonized nest boxes twice weekly, during the day, to replace RFID reader batteries. We removed RFID readers and ceased monitoring >3 weeks after queens ceased daily foraging at a given nest.

We returned to these sites in late September, after the nesting season had ended, to dissect colonized nest boxes and quantify reproductive output in nests. Based on previous studies, queens of most species (and specifically *B. appositus* and *B. centralis*) are on average 1.5 times the size of workers of the same species (del Castillo and Fairbairn, 2012). This body size difference is reflected in pupal case sizes (Elliott, 2009), thus we measured the number and size of intact, empty pupal cases in our study nests to quantify colony reproductive output over the course of the season.

RFID Technology

Bidirectional RFID readers consisted of a custom-made printed circuit board (PCB) connected to two antenna cards (Adafruit PN532). An onboard lithium-ion button-cell battery powered a 24-hour clock, and a microSD card slot transferred RFID read data to external storage. Each paired reader was powered by a portable 6V battery. The antenna cards were positioned over a tunnel, through which all bees had to walk to enter or exit the nest. In this way, the RFID tag on a bee’s thorax came in close proximity with the antenna cards upon every entrance and exit to and from the nest, at which point a timestamp record with the unique RFID tag identifier automatically printed onto the external microSD card.

Data Filtering and Analyses

All data filtering and analyses were conducted in R version 4.0.3 (R Core Team, 2020). It must be cautioned that our RFID system was not foolproof. It is possible that queens entered or exited the nest without the RFID system picking up on their movement (for example if they entered the nest upside down, with the RFID tag pointing away from the reader). This may have resulted in an overestimation of the duration of some foraging bouts and an underestimation of the number of foraging bouts overall. We filtered the RFID data based on several assumptions to account for much of this error. First, we defined paired reads as successive reads on opposite boards with < 10 s between reads. Next, we labeled the direction of motion for each paired read as either an “entrance” or “exit” from the nest, based on the order of reads (i.e., reads on the inner board followed by the outer board were labeled as an “exit”, and vice versa labeled as an “entrance”). We then identified lone, unpaired reads that occurred > 10 s before or after any other reads. For each unpaired read, if it was immediately preceded by and immediately followed by an entrance to the nest, it was labeled as an exit. Likewise, if it was immediately preceded by and immediately followed by an exit from the nest, it was labeled as an entrance. Then, we calculated the length of time between each entrance or exit and the subsequent read, to identify the duration of each out-of-nest (foraging) trip. Finally, we removed outlier trips of exceptionally short or long duration, which were likely the result of defecation trips (Dosselli *et al.*, 2016) or read errors, respectively. To do so, we first removed all out-of-nest trips shorter than 1 minute or longer than 24 hours. We then removed any out-of-nest trips > 3 standard deviations from the average duration.

Lack of intraspecific replication precluded statistical analyses in this pilot project. Instead, we calculated summary statistics (mean and standard error [s.e.m.]) of individual foraging bout duration, daily foraging bout duration, and number of foraging bouts per day for each nest individually. We created data visualizations using the ggplot2 package (Wickham, 2016) in R.

Results

Colonization of nest boxes

Of the 100 boxes we placed out, five boxes were colonized by bumble bee queens. All five queens laid eggs and formed at least one honeypot in their nest box (Fig 4-2). As described in Alford (1975) and Heinrich (2004), queens constructed honeypots adjacent to the brood clump and between the brood and the nest entrance (Fig 4-2). All colonized boxes were south facing on south facing slopes or flat ground, in partial shade. Four colonized boxes were located within or adjacent to aspen forest and one was adjacent to willow (Table 4-1). All boxes were ≤ 10 m from a meadow edge. Two colonized boxes were affixed to aspen trees, and the remaining three were located on the ground. Four of five colonized boxes contained clay balls, although no eggs were observed on the clay. Two of five colonized boxes were lined with rodent nest materials. No colonized nest boxes were covered with waterproof sheeting.

Queens

All five queens were first observed in their respective nest boxes between June 9 - 13 (Table 4-3). Three trap-nested queens (*B. appositus, B. centralis*, and *B. rufocinctus*) survived tagging and led to successful data collection. An additional queen (of an unknown species) absconded shortly after laying eggs and did not return to the nest. Evidence of this queen’s colonization was based purely on eggs deposited in the nest and buzzing sounds heard within the nest. Another queen (*B. rufocinctus*) died inside the nest box after tagging with an RFID tag on a particularly cold night. We have never observed mortality from this tagging process when we tagged queens in previous projects (n = > 50; Sarro *unpublished data*), and the cause of this death remains unknown. Hereafter, all information pertains to the three successfully tracked queens.

RFID readers at the nest entrances continuously recorded the comings and goings of each queen to and from the nests for the majority of their spring flight period (~3 weeks). Queens made an average of 6-15 foraging trips per day, each lasting an average of 24-36 minutes (Table 4-2; Figs 4-3 – 4-5). Queen foraging behavior, and specifically the maximum duration of foraging bouts, appeared to vary over the course of the season, with queens making their longest foraging bouts later in their flight periods. All queens ceased foraging within one day of each other, on July 3 or 4 (Table 4-3). Workers were first observed in the three nests between June 28 - July 5 (Table 4-3). We did not monitor the presence of workers in a standardized fashion, however; thus, workers may have emerged in the nests earlier than these observed dates.

At the end of the season, the *B. appositus, B. centralis,* and *B. rufocinctus* nests contained 57, 42, and 38 intact pupal casings, respectively (Fig 4-6). In our study, there was a bimodal or nearly twofold size variation in cocoon widths in all three boxes, suggesting that all colonies produced reproductive queens (gynes).

Discussion

Natural history information on wild, free-foraging bumble bee queens is exceedingly rare. This is due in part to the fact that incipient nests are difficult to locate and the spring flight period of bumble bee queens is short relative to the entire nesting period. We trap-nested bumble bee queens and used a custom-built RFID system to continuously record their foraging activity (inferred from their entering and exiting the nest) for the majority of their spring flight period. We found that queens made frequent, short foraging trips with some variation in foraging behavior over the season, and queens ultimately ceased foraging entirely within one week after workers emerged in the nest.

In annually eusocial systems, colony-founding queens must balance their time and energy to accommodate the heightened demands of solitary nest founding. Bumble bees store food resources in the nest, but these reserves are only sufficient to last a day or two in incipient nests (Cartar and Dill, 1990; Heinrich, 2004). Thus, to adequately feed and incubate developing larvae, queens must forage for pollen and nectar on a near-daily basis. In bumble bees, larvae must be regularly fed and incubated by adult caretakers in the nest (Alford, 1975). Insufficient feeding or temperature regulation can result in smaller, slower growing larvae or even larval death (Pereboom *et al.*, 2003; Heinrich, 2004). In our study, foundress queens typically foraged for less than an hour at a time. This is consistent with previous laboratory studies (albeit on other species) that have shown that individual larvae are fed approximately once per hour (Sarro *et al.*, In prep; Costa *et al.*, 2021). Thus, frequent, yet short, foraging bouts likely enable queens to both feed and incubate brood on an hourly basis.

As workers emerge in bumble bee nests, foundress queens respond to this influx of helpers by reducing parental care behaviors (Sarro *et al.*, In prep; Shpigler *et al.*, 2013; Woodard *et al.*, 2013) and readjusting their time and energy balance to focus on reproduction (Sarro *et al.*, 2021). In our study, queens abruptly ceased foraging within one week after adult workers were first observed in the nests. This is consistent with previous work showing that in the laboratory, *B. impatiens* queen food collection behavior appears to be a binary task that queens either do or do not perform, with the number of workers in the nest mediating this transition (Sarro *et al.*, In prep). Future studies examining the precise date of adult worker emergence in nests will be necessary to quantify how worker emergence impacts wild queen foraging behavior.

Laboratory work suggests that bumble bee queens respond dynamically to their social environment (Shpigler *et al.*, 2013; Woodard *et al.*, 2013; Sarro *et al.*, 2021, *In prep*), but whether and how queens respond plastically to the environment outside the nest remains understudied. Queen foraging behavior appeared to vary over the course of the flight period in our study. Although our sample size precluded statistical analyses, the longest foraging bouts of all queens occurred in the latter half of the season. We are unable to identify the source of this variation with our study design. However, we suggest that social factors, such as larval age or the emergence of adult workers in the nest, or ecological factors, such as floral bloom phenology or seasonal weather patterns, may impact queen foraging activity. For example, as larvae age, as adults emerge in the nest, or as outside temperatures warm, queens may be released from the requirements of feeding, incubating, and/or defending offspring on an hourly basis, enabling longer foraging bouts to occur. Alternatively, or in addition to these factors, more mouths to feed in the nest or a change in floral resource composition may have necessitated that queens increase their foraging to adequately provision their nests. Bumble bee workers have been shown to regulate their foraging behavior based on the resource environment (Hemberger and Gratton, 2018) and energetic requirements of the nest (Cartar and Dill, 1990), and it is possible that queens may do so as well. Additional studies recording in-nest and foraging behaviors of wild queens are necessary to elucidate whether and how wild queens dynamically balance these maternal care and foraging behaviors in a free-foraging environment.

Despite the economic and ecological importance of early nesting queens, current conservation strategies focus primarily on supporting bumble bee colonies during the social phase of their life cycle, when workers are present (Goulson *et al.*, 2008). The needs of early nesting queens remain largely unknown and unaddressed, although this stage may represent a particularly important demographic for bumble bee populations. For example, supporting ample pesticide-free floral resources in close to nesting habitats may be disproportionately important for early spring queens relative to mid-summer colonies (eg. Malfi *et al.*, 2022). Solitary queens must both forage and perform all the tasks required for colony success and reproduction, so this stage may respond strongly to environmental stressors such as diminishing or degraded floral resources, urbanization, pesticide use, and higher temperatures, and may help explain national and global declines in bumble bee populations (Goulson *et al.*, 2008, 2015; Sydney A. Cameron *et al.*, 2011). An improved understanding of the nest-founding life stage in variable environments may help predict bumble responses to a changing world and inform conservation strategies to protect these vital pollinators. We present this trap-nesting and subsequent RFID tracking method as a promising path forward for studying this evasive, incipient life stage.

Data Availability: Raw data and all code associated with filtering and analyses are available on GitHub (https://github.com/erica-sarro/QueenRFID22).

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Tables and Figures

Table 4-1. Summary of nest box locations. Edge habitats typically bordered open meadows. Numbers represent sample sizes for each category, numbers in parentheses represent the number of colonized nests.

	Number of nest boxes (colonized)
Open Meadow	15
Aspen Forest	**18 (3)**
Aspen Edge	**15 (1)**
Conifer Forest	6
Conifer Edge	5
Willow	7
Willow Edge	**16 (1)**
Cabin Edge	18
TOTAL	**100 (5)**

Table 4-2. Summary of queen foraging trips (average +/- s.e.m), as recorded by RFID readers.

Species	Total number of recorded trips	Number of trips/day	Duration of trips
B. appositus	136	9.1 +/- 1.4	35.6 +/- 4.3 min
B. centralis	285	15.0 +/- 1.8	24.2 +/- 1.1 min
B. rufocinctus	87	6.7 +/- 1.1	36.6 +/- 3.4 min

Table 4-3. Summary of relevant dates for all observed queens over the season. We do not have foraging data or worker data for the second *B. rufocinctus* queen and an unknown queen, because they died and absconded from their nests, respectively, prior to data collection.

Species	Queen first observed in nest	Queen RFID-tagged; dates of recording	Workers first observed at nest*	Last day queen was recorded leaving nest
B. appositus	June 9	June 16 - July 26	June 28*	July 4**
B. centralis	June 13	June 14 - July 26	June 28*	July 4
B. rufocinctus	June 13	June 16 - July 26	July 5*	July 3
B. rufocinctus	June 9	NA	NA	NA
Unknown	June 9	NA	NA	NA

*Monitoring for workers was not standardized. These dates may be overestimated.
**The *B. appositus* queen stopped foraging with any regularity after July 4 but was recorded leaving the nest for 288 minutes on July 13 and 2.6 minutes on July 18.

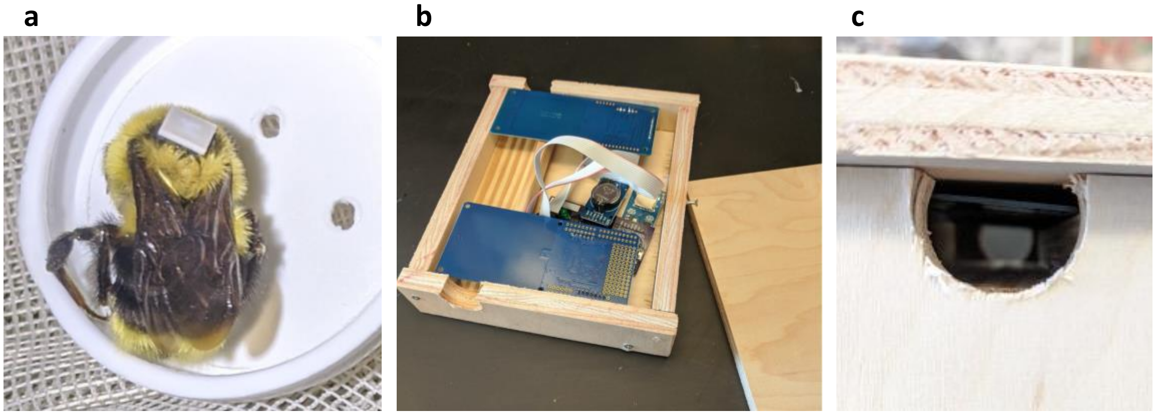

Figure 4-1. Photos of RFID system. (a) *B. rufocinctus* queen with RFID tag glued to thorax. (b) Paired RFID antenna cards (blue rectangles) connected to a central PCB with an internal clock and microSD card slot. All bees had to walk through a channel beneath the RFID antenna cards (c) in order to enter or exit their nest box.

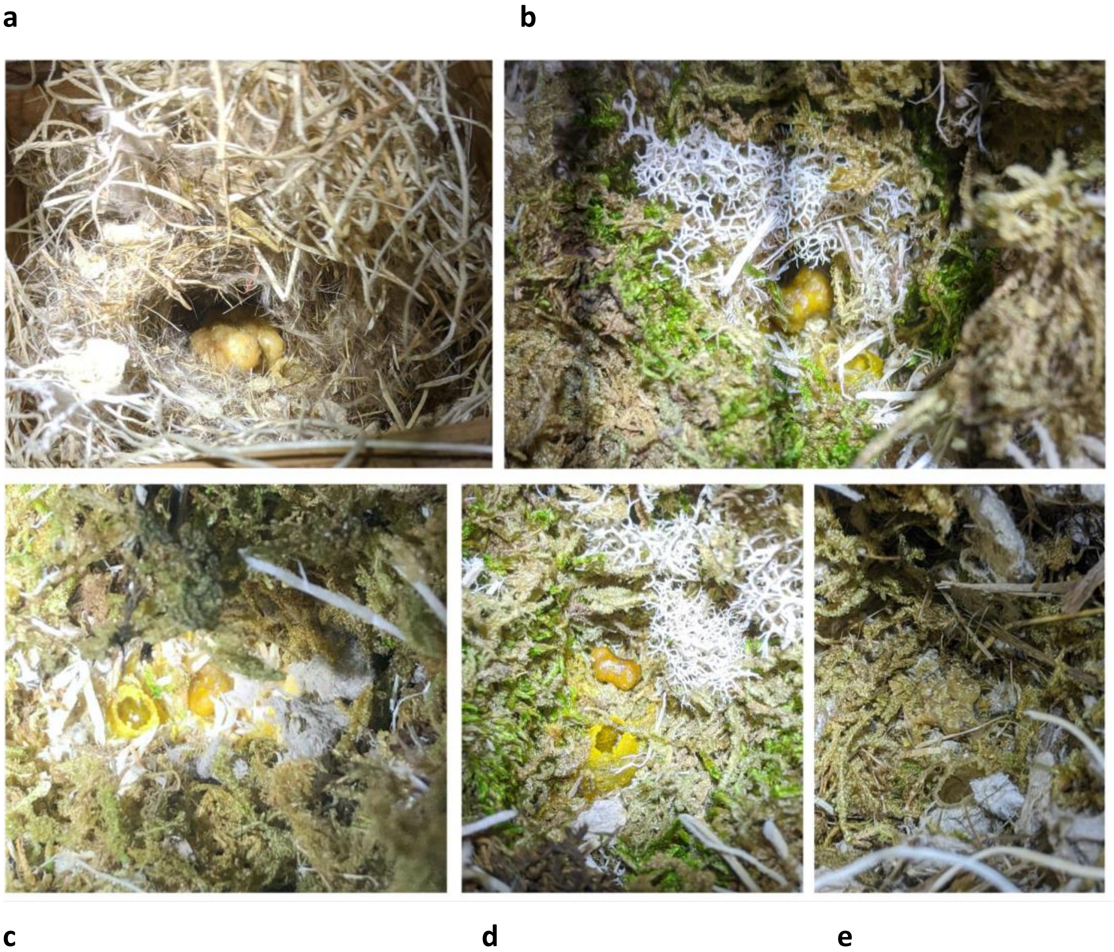

Figure 4-2. Photos of colonized nest boxes at the time of queen tagging, where applicable. (a) *B. appositus*; (b) *B. centralis*; (c) *B. rufocinctus*; (d) *B. rufocinctus*; (e) unknown species. All nests were enclosed in nesting materials, which were temporarily moved in order to take these photographs.

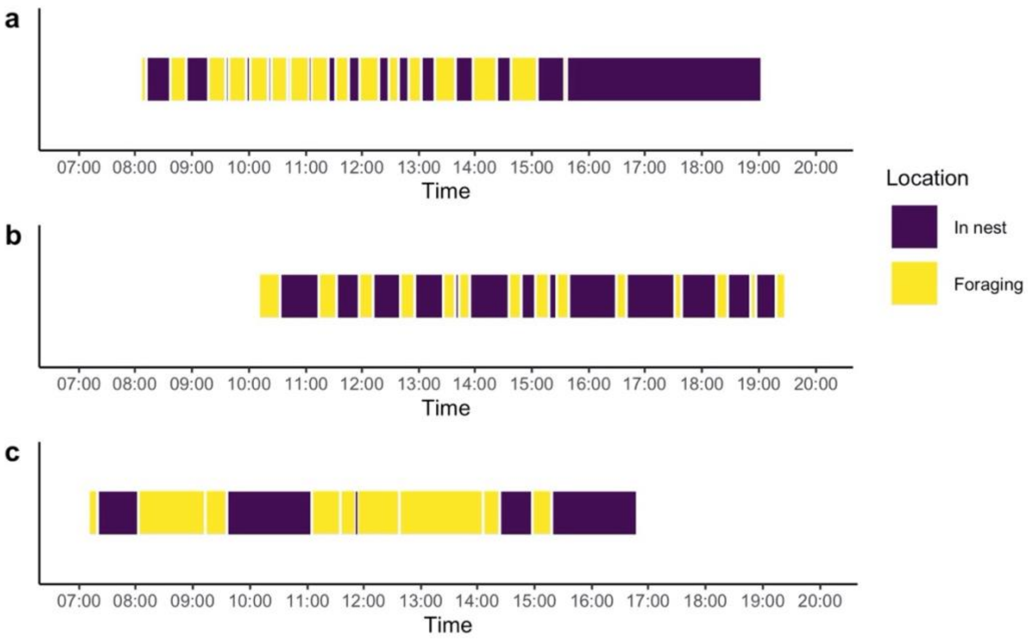

Figure 4-3. Ethogram of queen foraging behavior on June 18, 2021. *B. appositus* (a), *B. centralis* (b), and *B. rufocinctus* (c). Transitions between in-nest and foraging locations are inferred based on RFID data.

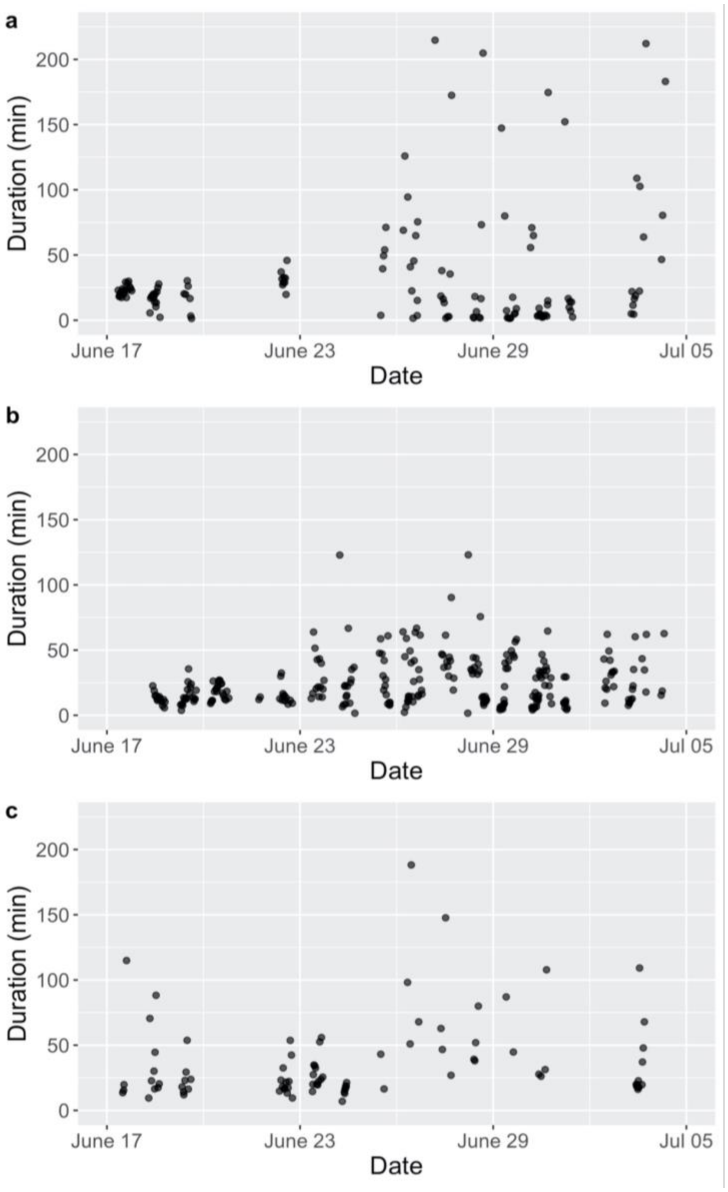

Figure 4-4. Duration of all RFID-recorded foraging bouts. Queen *B. appositus* (a), *B. centralis* (b), and *B. rufocinctus* (c). The *B. appositus* queen was also recorded leaving the nest once for 288 minutes on July 13 and once for 2.6 minutes on July 18 (not pictured on graph).

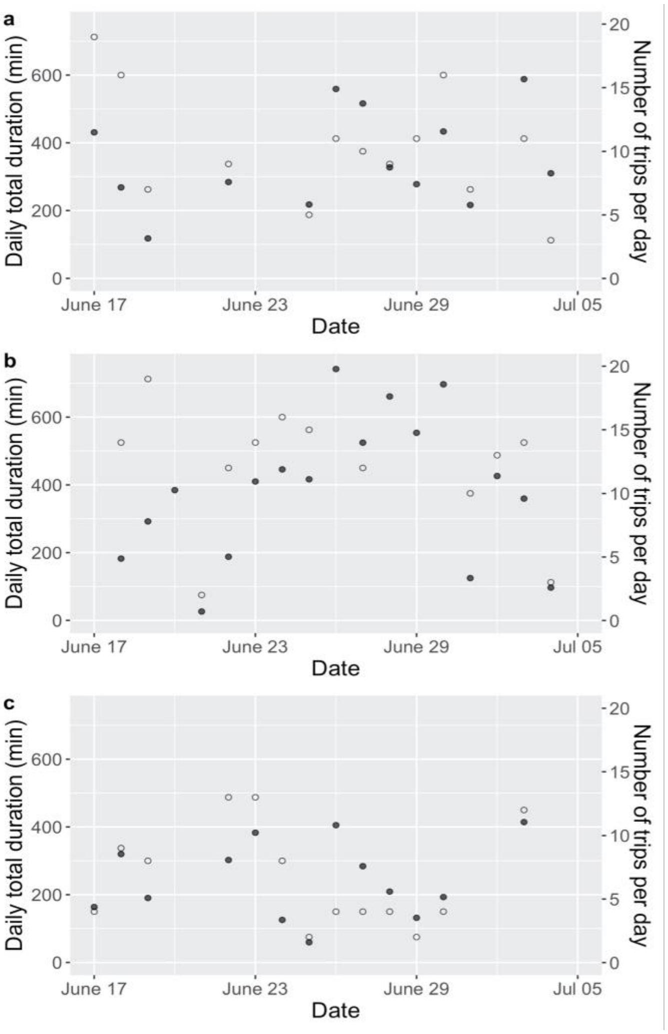

Figure 4-5. Total daily duration and frequency of all RFID-recorded foraging bouts. Queen *B. appositus* (a), *B. centralis* (b), and *B. rufocinctus* (c). Filled circles correspond to the total number of minutes each queen spent foraging per day. Open circles correspond to the number of recorded foraging trips per day. The *B. appositus* queen was also recorded leaving the nest once for 288 minutes on July 13 and once for 2.6 minutes on July 18 (not pictured on graph).

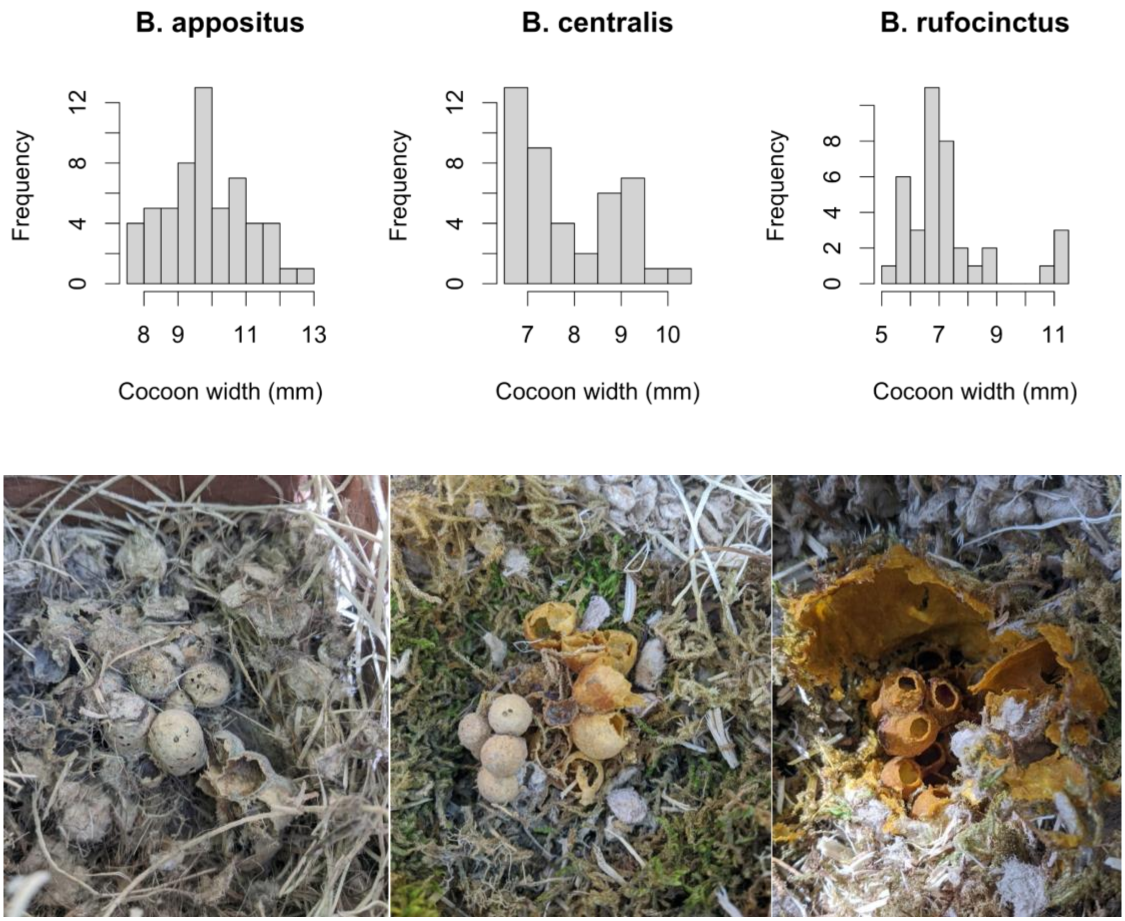

Figure 4-6. Histograms of pupal cocoon widths and photos of colonized nest boxes at the end of the season. *B. appositus* (a & d); *B. centralis* (b & e); and *B. rufocinctus* (c & f). Bimodal distributions and/or a nearly twofold size difference among workers in all nests suggest that all colonies produced reproductive queens, which are substantially larger than workers. All nests were enclosed in nesting materials, which were pulled back to expose the brood wax in these photos. The *B. rufocinctus* nest was also covered in a waxen canopy [top left of (f)], which was pulled back prior to taking this photo.

CONCLUSION

In this dissertation, I explore the understudied colony founding life stage in bumble bees, and the plasticity of bumble bee queens across the ontogeny of sociality. Overall, my work suggests that bumble bee queens are highly plastic at this life stage and have the capacity to respond dynamically to the social (Sarro *et al.*, 2021; Sarro *et al.,* In prep a) and ecological (Sarro *et al*., 2022; Sarro *et al.* In prep b) environments. The likelihood of queen survival is also linked to the presence of workers in the nest (Sarro *et al.* 2021), suggesting that queens experience physiological benefits from the social environment.

Juvenile hormone (JH) is one compound involved in bumble bee queen reproduction that is regulated by the social environment in incipient nests (Sarro *et al.* 2021). In honey bees, which share a common origin of eusociality with bumble bees (Peters, 2017), juvenile hormone titer is associated with division of labor and specifically brood care and foraging behaviors (Robinson 1987). The observed changes in bumble bee maternal care behavior (i.e., brood feeding and food collection) in Chapter 3 occurred around the same point that JH rose in Chapter 1. Future work should investigate whether this compound may play a direct role in regulating maternal care behaviors in bumble bees, in addition to its observed impact on queen reproduction, across the ontogeny of eusociality.

Subsocial queens spend the majority of their time foraging and caring for brood (Sarro *et al.,* In prep b), and it appears that rearing workers earlier in the season is advantageous for bumble bees, as it enhances reproduction and survival of bumble bee queens (Sarro *et al.,* 2021). Thus, conservation strategies may benefit from interventions that support the early nesting period and facilitate the production and maintenance of workers in incipient nests. This idea is also supported by the widescale pattern that bumble bee species that emerge from diapause earlier in the spring are less likely to be in decline relative to species that emerge later in the season (Williams *et al.*, 2009). Early season interventions could include ensuring high quality, pesticide-free floral resources within close proximity of nesting habitat in early spring. Early season food resources may be disproportionately important for bumble bee colony success (Malfi *et al*. 2022).

If workers are lost from young nests, however, queens can reinitiate new nests and revert back to caring for brood once again (Sarro *et al.,* 2021). Whether this has lasting impacts on colony fitness, however, remains to be seen. For early nesting queen bumble bees, behavior, reproduction, and parental care investment are likely mediated by a complex interplay between social and ecological conditions. Future work should investigate the limits of this plasticity and the resilience of queens at this life stage.

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