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Research

Why Are Queens Broodless? Failed Nest Initiation Not Linked to Parasites, Mating Status, or Ovary Development in Two Bumble Bee Species of *Pyrobombus* (Hymenoptera: Apidae: *Bombus*)

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Abstract

Bumble bees (*Bombus* [Hymenoptera: Apidae]) are important pollinators for agricultural crops, which has led to their commercial domestication. Despite their importance, little is known about the reproductive biology of bumble bees native to North America. The Hunt bumble bee (*Bombus huntii* Greene [Hymenoptera: Apidae]) and the Vosnesensky bumble bee (*Bombus vosnesenskii* Radoszkowski [Hymenoptera: Apidae] are native candidates for commercial production in western North America due to their efficacy in providing commercial pollination services. Availability of pollinators native to the region in which services would be provided would minimize the likelihood of introducing exotic species and spreading novel disease. Some parasites are known to affect bumble bee reproduction, but little is known about their prevalence in North America or how they affect queen success. Only 38% of wild-caught *B. huntii* and 51% wild-caught *B. vosnesenskii* queens collected between 2015 and 2017 initiated nests in the laboratory. Our objective was to identify causal factors leading to a queen's inability to oviposit. To address this, we dissected each broodless queen and diagnosed diseases, assessed mating status, and characterized ovary development. Nematodes, arthropods, and microorganisms were detected in both species. Overall, 20% of queens were infected by parasites, with higher rates in *B. vosnesenskii*. Over 95% of both species were mated, and over 88% had developed ovaries. This suggests that parasitism and mating status were not primary causes of broodlessness. Although some failure to nest can be attributed to assessed factors, additional research is needed to fully understand the challenges presented by captive rearing.

Key words: bumble bee, nest initiation, domestication, commercial pollinator, pathogen

Interest in domesticating bumble bees (Hymenoptera: Apidae: *Bombus*) to provide pollination services was first noted in 1912 in Sladen's book *The Humble-Bee*, and by 1987, commercial rearing of bumble bees had begun on an industrial scale (Velthuis and van Doorn 2006). Over the last 32 yr, five species have been successfully domesticated, but the two most commonly produced are *Bombus terrestris* (L.) (Hymenoptera: Apidae) in Europe and *Bombus impatiens* Cresson (Hymenoptera: Apidae) in North America. In 2005, one million of these colonies were produced annually and the industry is still rapidly growing (Velthuis and van Doorn 2006). Bumble bees are the primary pollinators of greenhouse tomatoes, a growing industry valued at over US$690 million per year in North America (Thornberry and Jerardo 2012).

Due to the ease of domestication and pollinating abilities, bumble bees have been commercially reared and transported to crops and greenhouses well outside of their native range. Introducing bumble bees to novel environments raises a number of concerns for local species, these can include hybridization with native species, competition for food and nesting sites, and introduction of parasites (including pathogens; Goka et al. 2001, Kanbe et al. 2008, Williams and Osborne 2009, Cameron et al. 2011, Evans 2017). The western bumble bee, *Bombus occidentalis* Greene (Hymenoptera: Apidae), has seen an average decline of 40.32% in its wild population after the initiation of commercial rearing of this species (Hatfield et al. 2015). The decline has been anecdotally linked to the introduction of infected, commercially reared bees from Europe

(Cameron et al. 2011, Colla et al. 2012), although there is no evidence to suggest that novel strains of *Nosema bombi* Fantham & Porter (Dissociodihaplophasida: Nosematidae) were imported to the United States (Cordes et al. 2012, Cameron et al. 2016). The decline of *B. occidentalis* has prompted research in identifying other candidate bumble bee species native to western North America, where bumble bee pollination services are currently provided by *B. impatiens*. *Bombus impatiens* is not native west of the Rocky Mountains, although it has been introduced to the west through importation for commercial pollination (Looney et al. 2019).

Bombus vosnesenskii, native to coastal areas of western North America, and *Bombus huntii* native to intermountain regions of western North America (Koch and Strange 2012), have proven effective greenhouse tomato pollinators (Strange 2015). Both species belong to the *Pyrobombus* subgenus, such as *B. impatiens*, and are candidates for domestication (Strange 2015, Bobiwash et al. 2018), but to date, no studies have compared factors that might influence their amenability to domestication. *Bombus huntii* has been noted for its wide distribution in the West (Koch et al. 2018), large colony sizes, and low parasite loads (Baur et al. 2019), and *B. vosnesenskii* has large colonies and is one of the most common bumble bees in the heavily agricultural region of the west coast. It is possible to capture *B. vosnesenskii* and *B. huntii* queens in the wild and rear them in a laboratory setting (Strange 2015), but a large proportion of these wild-caught queens fail to initiate a nest. Stimulating oviposition in captivity has been an active area of bumble bee domestication research (e.g., Ono et al. 1994, Kwon et al. 2006, Strange 2010), but the factors that prevent nest initiation in captivity are still largely unknown (Tripodi and Strange 2019).

Bumble bees have evolved with a number of parasites from a variety of taxonomic groups (Alford 1975, Macfarlane et al. 1995, Schmid-Hempel 1998), and parasites might play a role in preventing nest initiation. Some parasitic organisms are directly responsible for halting oviposition. For example, *Sphaerularia bombi* Dufour (Tylenchida: Sphaerulariidae), the 'queen-castrating' nematode, exclusively parasitizes bumble bee queens and affects the queen's corpus allatum, altering gene expression and in turn behavior, completely inhibiting ovary development (Alford 1975, Macfarlane and Griffin 1990, Kubo et al. 2016, Colgan et al. 2019). Although *S. bombi* has an obvious effect on nest initiation, other parasites might influence colony founding by lowering queen fitness or killing queens outright before they can initiate a nest.

The effects of many parasites on queen health are largely unknown, but some parasites are lethal to their hosts. Mermithid (Mermithida: Mermithidae) nematodes are lethal parasites that are obligate endoparasites of both aquatic and terrestrial arthropods (Poinar 1975). They are rare in bumble bees and have only been recorded parasitizing bumble bees 12 times (Tripodi and Strange 2018). In other hosts, mermithids deplete the energy reserves of infected adults, which can sterilize them, and on maturation, mermithids exit and kill their hosts (Poinar 1975). Endoparasitic flies such as Conopids (Diptera: Conopidae) and *Apocephalus borealis* Brues (Diptera: Phoridae) attack bumble bees and eventually kill them. Both flies can shorten the lifespan of infected workers (Schmid-Hempel and Schmid-Hempel 1988, Otterstatter et al. 2002), but little is known about their impacts on queen fitness. Similarly, the wasp *Syntretus splendidus* Marshall (Hymenoptera: Braconidae) is an obligate endoparasite of bumble bees infecting foraging adults of all castes by ovipositing in the thorax and developing in the abdomen (Alford 1968). Parasitism by this wasp reduces the lifespan of queens without influencing egg-laying behavior; however, foundress queens in captivity die quickly (Alford 1968, Goldblatt and Fell 1984, Rutrecht and Brown 2008b).

There are three distantly related pathogenic microparasites specializing in bumble bees that are passed horizontally including *Crithidia* spp. [*Crithidia bombi* Lipa & Triggiani (Trypanosomatida: Trypanosomatidae) and *Crithidia expoeki* Schmid-Hempel and Tognazzo], *Nosema* spp. [*Nosema bombi* and *Nosema ceranae* Fries et al.], (Dissociodihaplophasida: Nosematidae) and *Apicystis bombi* (Liu et al.) (Neogregarinorida: Lipotrophidae). All three microparasites are found in all bumble bee castes, but queens have received far less attention (McIvor and Malone 1995, Schmid-Hempel 2001, Van der Steen 2008). Given that bumble bees have an annual life cycle, these specialist microparasites must overwinter in queens, making spring queens the primary source of infection (Otti and Schmid-Hempel 2008), yet it is unclear what effects these organisms might have on queen success. *Crithidia bombi* has the most dramatic effect on colonies facing limited food resources (Brown et al. 2003), and it has a negative impact on the success of overwintering queens. Parasitized queens lose 11% body mass, leading to a significant reduction in colony-founding success (Rutrecht and Brown 2008a). *Nosema bombi* can cause distended abdomens in queens and inhibit mating (Otti and Schmid-Hempel 2007). This microparasite has received considerable attention for its prevalence in declining species such as *B. occidentalis* (Cameron et al. 2011, 2016), yet lower infection rates have been reported in many North American species in the subgenus *Pyrobombus* (Cordes et al. 2012, Malfi and Roulston 2014, Tripodi et al. 2014). *Nosema bombi* did not affect nest initiation or performance of *B. terrestris* queens in captivity (Fisher and Pomeroy 1989), and Imhoof and Schmid-Hempel (1999) found infected colonies yielded more reproductive individuals than uninfected colonies. *Apicystis bombi* resides in the fat body of its host and has been shown to deplete these reservoirs in bumble bee queens, potentially affecting overwintering survival (Schmid-Hempel 2001). In captivity, wild-caught queens with *A. bombi* infections do not survive long, suggesting that this microparasite might reduce the number of queens that initiate nests in the spring (Rutrecht and Brown 2008a); however, field data are absent. This microparasite is rarely reported and has received relatively little attention, despite its physiological impact on queen health.

Although parasites can potentially affect a queen's ability to finding a colony, physiological and environmental factors can play an important role. Ovary development begins when spring queens emerge from their overwintering site and begin foraging (Medler 1962). Oviposition and subsequent brooding behavior are the primary indicators of successful nest founding in captivity, and ovary development is an important factor in domestication. Based on the haplodiploid sex determination of the Hymenoptera, unmated bumble bee queens can produce all-male colonies; thus, mating is not required for egg development, but we know little of how frequently this occurs. Mating does improve queen overwintering survival (Baer and Schmid-Hempel 2005), but it is unknown whether or not oviposition is stimulated by the presence of sperm.

The objective of our research was to examine factors that could potentially inhibit oviposition in spring bumble bee queens in a captive rearing setting. We compared two bumble bee species native to western North America, *B. huntii* and *B. vosnesenskii*, due to interest in commercially rearing these species for pollination services in their native ranges. Because mating status and the presence of parasites have been demonstrated to affect colony establishment, this question was addressed by diagnosing parasite presence and identifying mating status of queens that did not initiate nests, then analyzing the effects of each factor on ovary development and survival in captivity. This study provides observational data of queens that failed to oviposit in the laboratory. A direct comparison

to successful queens could not be made as successful colonies progressed with those queens for months. As queens are long-lived, we could not guarantee that queens kept in captivity for several months did not become infected while being fed pollen in captivity; thus, we did not analyze parasites of successful queens.

Materials and Methods

We captured wild spring bumble bee queens of two species, *B. huntii* (n = 473) and *B. vosnesenskii* (n = 706), throughout the western United States (Washington, Oregon, California, Colorado, and Utah) between 2015 and 2017. We placed each queen in a 10 × 10 × 8 cm plastic nesting box provisioned with a beeswax-coated pollen ball and provided unlimited access to sugar syrup (sugar, water, citric acid, sorbic acid, Amino-B Booster [Cumberland, MD]) and additional pollen ad libitum. We collected pollen from honey bee hives in Logan, UT, and mixed the pollen into a paste with sugar syrup before feeding. We stored nesting boxes in a dark room with a constant temperature and humidity of 27 ± 1°C and of 40–60%, respectively. We gave queens 21 d to oviposit, and culled and classified them as 'broodless' if they failed in initiate a nest in this period. Additionally, queens that died before oviposition in the 21-d period were also considered broodless. We stored broodless queens at −80°C in 1.5-ml tubes until dissection. Queens that oviposited within the 21-d window were classified as 'successful' and were not analyzed further for the purposes of this study.

We analyzed each queen in two major diagnostic steps: 1) dissection to microscopically diagnose macroparasites (insects and nematodes), mating status, and ovary development, and 2) genetic analysis of the contents of the metasomal cavity, which consisted of DNA extraction followed by PCR to diagnose microparasites (*Nosema*, *Apicystis*, and *Crithidia*).

Dissection

Before dissection, we sterilized the workspace with 10% bleach solution and heat sterilized all dissection tools. We removed several frozen queens from a −80°C freezer at a time and stored them on ice until thawed. We ventrally pinned each thawed queen to a parafilm-covered foam platform through the center of the thorax. We then cut open the ventral side of the abdomen with iris scissors along the right edge and then across the first sternite to separate it from the dorsal portion and pinned it to hold it in place.

Once the body cavity was exposed, we added several drops of sterile phosphate-buffered saline (pH 7.4) to help float the tissues and any parasites. We then closely examined the body cavity for any visible macroparasites through a stereoscopic dissecting microscope at 6×–25× magnification (Wild M5, Heerbrugg, Switzerland) and identified the parasites by gross morphology and recorded the occurrence if present. Targeted macroparasites were nematodes (*S. bombi* and Mermithidae), dipteran larvae (Conopidae and *A. borealis*), hymenopteran larvae (*S. splendidus*), and tracheal mites [*Locustacarus buchnerii* (Stammer) (Trombidiformes: Podapolipidae)]. We classified ovaries as 'developed' if eggs of any size were present or 'undeveloped' if the ovarioles remained shriveled with no observable eggs.

We removed spermathecae and mounted them on glass slides for observation. We prepared each glass slide by dripping a small amount of lacto-acid, acid-fuchsin stain in glycerol on the glass, then placed the spermatheca in the stain, and covered it with a glass coverslip. We took representative samples of fat body, midgut, and hindgut and similarly slide-mounted these samples to verify the molecular results, as developed infections are often visible under a phase-contrast microscope. After the queen's body cavity had been carefully examined, we removed the entire contents and placed them in a 1.5-ml tube, which we stored at −20°C. After each dissection, we disposed of the parafilm and sterilized the foam platform and dissection tools. After several days, during which time the stain set, we assessed the prepared microscope slides under a phase-contrast microscope (BX51, Olympus Corporation, Center Valley, PA) at 100× and 400× magnification to check for the presence of sperm and microparasites. We diagnosed each queen as 'mated' if sperm was present and 'unmated' if sperm was absent. We recorded the presence of microparasites if present.

Development of a Diagnostic Multiplex PCR

We designed a multiplex to detect the three most common bee-infecting microparasites in a single reaction, rather than conducting a series of reactions. We used either newly designed primers or chose primers from existing literature such that in the multiplex reaction: 1) primers would be specific for the targeted taxa, 2) each amplicon could be visually differentiated by size on a common 2% agarose gel, 3) interactions between primers would be minimized, and 4) the melting temperatures of all primers would be similar. The multiplex targets the microparasite taxa microsporidia (*Nosema* spp.), neogregarines (*A. bombi*), and trypanosomatids (*Crithidia* spp.), along with a bee positive control to verify successful reactions in microparasite-negative samples. Primers used for detection and a reaction control are shown in Table 1. A detailed description of the development of these diagnostic markers can be found in Supplementary Methods.

DNA Extraction, PCR, and Gel Diagnosis

We extracted the DNA for each sample using a salting-out method, adapted for insect tissues from Protocol 6: Rapid Isolation of Mammalian DNA (Sambrook and Russel 2001). We used the

Table 1. Primers used in a multiplex PCR to diagnose common bumble bee diseases in broodless queens

Target taxon	Primer name	Primer sequence	Length
Bumble bee (reaction control)	Apid18SF	5′-GTCCTATCGGTGGGCTTAGC-3′	233
	Apid18SR	5′-ATGCCCCCATCTGTCCCTAT-3′	
Trypanosomatids including *Crithidia* spp.	CB18SR2[a]	5′-TGCTCCTTTGTTATCCCATGCT-3′	584
	CB-SSUrRNA-F2[b]	5′-CTTTTGACGAACAACTGCCCTATC-3′	
Neogregarines including *Apicystis bombi*	Apicyst357F	5′-AGCGATGGATGTCTTGGGTC-3′	357
	Apicyst357R	5′-CCTAGTTAGTTTCTTTTCCTCCGC-3′	
Microsporidia including *Nosema* spp.	MSporDegR	5′-GGTGTGTRCAAAGAACAGGG-3′	270–316
	MSporF2	5′-AGTGGTGCATGGCCGTTTTC-3′	

Length is in base pairs of amplified product (including primers) as viewed in a diagnostic gel. All primers were developed as a part of this work except 1) Tognazzo et al. (2012) and 2) Tripodi and Strange (2018). Details on the development of this panel can be found in Supp Material (online only).

newly designed multiplex to diagnose the presence or absence of microparasites. The PCR for each sample contained 0.8 µM of each of the microparasite primers; 0.4 µM of Apid18SF-R primers, 1.3× buffer, 2.0 mM $MgCl_2$, 0.8 µM total dNTPs, 1 unit *Taq* (Apex Bioresearch Products, Waukee, IA), and 1.2 µl DNA extraction, with nuclease-free water to bring each reaction volume to 25 µl. We ran reactions in batches of 96 in a C1000 Touch (BioRad, Hercules, CA) or MultiGene Optimax (LabNet International, Edison, NJ) thermal cycler with an initial denaturation step at 94°C for 2 min, followed by 10 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 45 s then 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 45 s and finally an extension at 72°C for 5 min. Each reaction batch consisted of three positive controls with confirmed infections of each of the listed microparasites and a negative control with distilled H_2O in place of DNA template. Products were separated by electrophoresis on 2% agarose gels stained with 2.5× Gel Red (Biotum, Inc, Fremont, CA) and visualized under UV light. We estimated amplicon sizes by comparison to a 100 bp ladder (GeneRuler, ThermoScientific, Waltham, MA) and diagnosed samples based on the presence (positive) or absence (negative) of bands at ~270 bp for microsporidia, 357 bp for neogregarines, and 534 bp for trypanosomatids for each sample. We only included samples with an amplicon at 233 bp indicating the presence of bee DNA and a successful reaction in analyses.

Statistical Analysis

We recorded all factors recorded as binary outcomes (e.g., successful vs broodless, positive vs negative, mated vs unmated, developed vs undeveloped). We made comparisons using χ^2 tests of independence unless expected values were small, in which case Fisher's exact tests were used, within the R platform v.3.6.0 (R Development Core Team 2019). For each species, we ran subsequent tests to determine whether parasites had effects on nest initiation, with ovary development or early death as response variables. The size of each significant effect was estimated as an odds ratio in the package *Epi* (Carstensen et al. 2019) within R. Throughout, errors are reported as 95% confidence intervals (CI) as calculated in *Epi*.

Results

Nesting Success

Of the 473 *B. huntii* queens provided with nesting boxes, 182 (38.5%, 34.2–43.0% CI) initiated nests by ovipositing within the 21-d limit. Of the 706 *B. vosnesenskii* queens, 360 (51.0%, 47.3–54.7% CI) oviposited within the 21-d period. *Bombus vosnesenskii* initiated more nests than *B. huntii*, $\chi^2 = 17.4$, df = 1, $P = 0.00003$. With one exception, broody queens that reared adult brood produced females, which shows that they were mated. We assume that the single queen that produced exclusively male brood had never been mated. Of the broodless queens (i.e., those that did not initiate nests), $n = 210$ *B. huntii* and $n = 195$ *B. vosnesenskii* were randomly selected for further analysis. In the following analyses, deviations from the expected sample sizes (*B. huntii* $n = 210$, *B. vosnesenskii* $n = 195$) reflect that not all individuals could be confidently assessed and were scored as unknown for one or more factors.

Ovary Development

Ovary development was assessed for 193 *B. huntii* and 161 *B. vosnesenskii* broodless queens (Supp Table 1 [online only]). Both species had high rates of ovary development, with 95.3% (91.3–97.6% CI) of *B. huntii* and 88.2% (82.2–92.4% CI) of *B. vosnesenskii* exhibiting developed ovaries (Fig. 1). Of the two species, *B. huntii* exhibited higher rates of ovary development than *B. vosnesenskii*, $\chi^2 = 5.20$, df = 1, $P = 0.023$.

Mating Status

Of the 181 *B. huntii* and 167 *B. vosnesenskii* queens assessed, 95.0% (90.7–97.4% CI) and 97.6% (93.8–99.1% CI), respectively, had sperm present in the spermathecae (Supp Table 1 [online only], Fig. 1). There was no significant difference in mating status between the species, $\chi^2 = 0.97$, df = 1, $P = 0.33$.

Parasites

Out of the 405 queens analyzed for parasites, 82 (20.2%) had at least one parasite. Macroparasites observed during dissection included nematodes (*S. bombi* and one mermithid) and insects (*S. splendidus* and a dipteran maggot; Fig. 1). The microparasites, *A. bombi* and *Crithidia* spp., were detected by molecular methods (Fig. 1). Neither tracheal mites nor *Nosema* spp. were observed in our samples.

Our results show that *B. vosnesenskii* have a higher prevalence of parasites overall with 36.4% ($n = 71$) testing positive for any of the targeted parasites compared with only 5.2% ($n = 11$) of *B. huntii*. This finding is significant, $\chi^2 = 58.93$, df = 1, $P = 1.6 \times 10^{-14}$ (Supp Table 2 [online only]).

The presence of macroparasites was low overall. There was no significant difference in the prevalence of *S. bombi*, which was similar in the two species, with 3.3% (1.6–6.8% CI) of *B. huntii* and 3.1% (1.4–6.7% CI) of *B. vosnesenskii* infected ($\chi^2 = 5.2 \times 10^{-31}$, df = 1, $P = 1$; Fig. 1). Only one *B. huntii* queen had a mermithid, as they are relatively rare, with this being only the 13th recorded occurrence in a bumble bee host (Tripodi and Strange 2018). We verified the identity of the mermithid through sequencing using the same methods as Tripodi and Strange (2018). Although the species is unknown, the sequences (GenBank MK542854 and MK542855) matched most closely (98.5% similarity) with other mermithids extracted from bumble bee hosts from Arkansas and Japan. We found five *B. vosnesenskii* queens—all collected from the same location—parasitized by the wasp, *S. splendidus* (2.6%, 1.1–6.0% CI; Fig. 1). In one case, the *B. vosnesenskii* queen was host to more than 100 larvae. No *B. huntii* were infested with *S. splendidus*, and the difference was significant (Fisher's exact $P = 0.025$). Only one *B. vosnesenskii* queen hosted a dipteran parasite (Fig. 1).

Two common microparasites were detected, *Crithidia* spp. and *A. bombi*, but none of the 405 analyzed queens tested positive for *Nosema* spp. *Apicystis bombi* was more common in *B. vosnesenskii* (10.8%, 7.1–16.0% CI) than in *B. huntii* (1.9%, 0.07–5.0% CI; $\chi^2 = 12.23$, df = 1, $P = 0.00047$; Fig. 1). Similarly, *Crithidia* spp. was more common in *B. vosnesenskii* (23.6%, 18.2–30.1% CI) than in *B. huntii*, which had no *Crithidia* spp.-positive samples ($\chi^2 = 53.57$, df = 1, $P = 2.5 \times 10^{-13}$; Fig. 1). Coinfections with multiple parasites were rare. Seven *B. vosnesenskii* queens were positive for both *A. bombi* and *Crithidia* spp.; one had both *Crithidia* spp. and *S. bombi*. One *B. huntii* queen had *S. bombi* and *A. bombi*.

Effects on Brood Production

Mating status had no effect on ovary development for either species (*B. huntii*: Fisher's exact $P = 1$, *B. vosnesenskii*: Fisher's exact $P = 0.36$). In both species, the presence of *S. bombi* inhibited ovary development. In *B. huntii*, *S. bombi* reduced the likelihood of ovary development by 43.9% (18.2–73.8% CI; Fisher's exact $P = 0.0001$). In *B. vosnesenskii*, *S. bombi* reduced the likelihood of ovary development by 31.6% (15.2–54.0% CI; Fisher's exact $P = 0.000001$). No

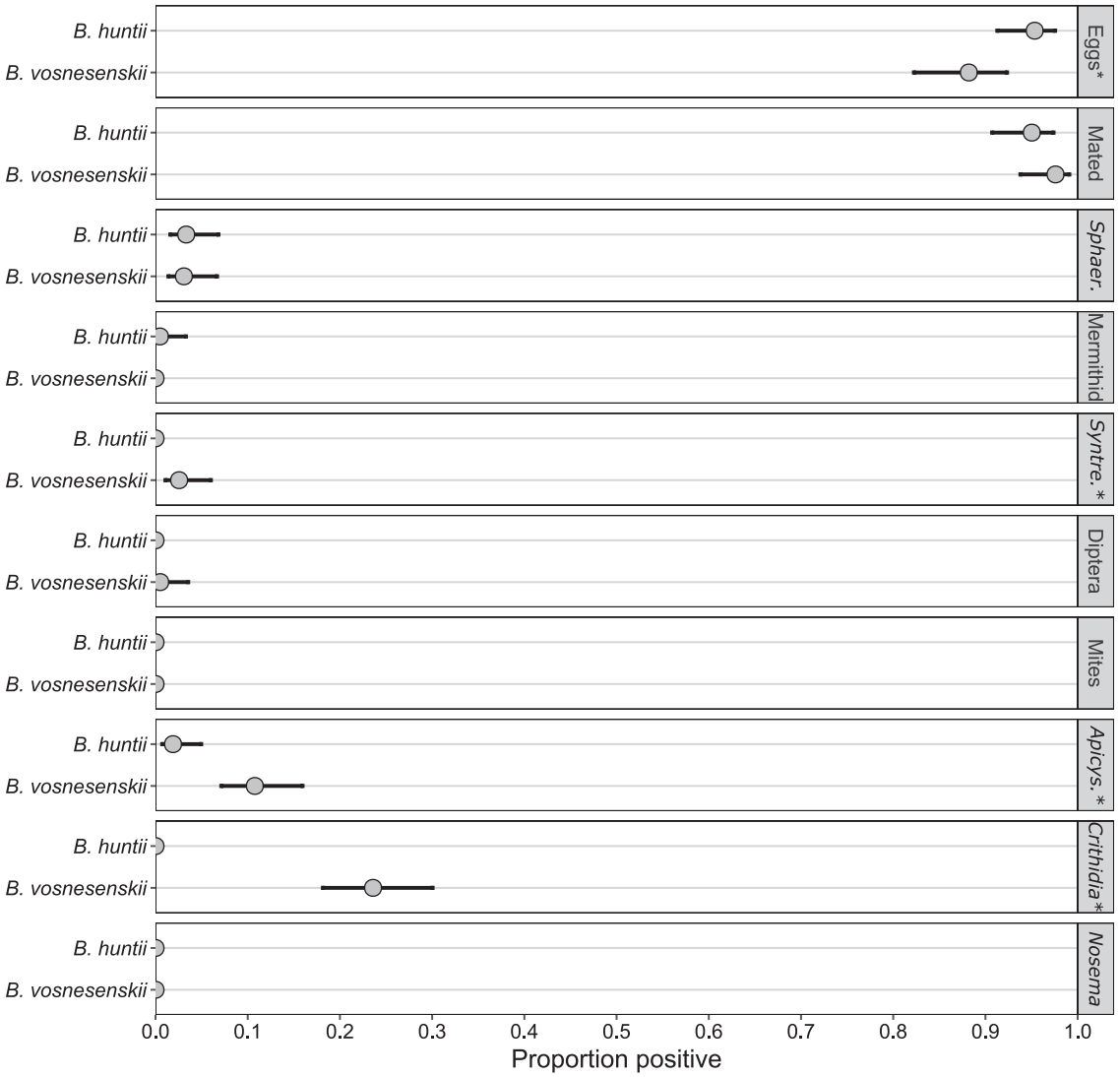

Fig. 1. Summary of all of the measured factors in this study by species. Gray dots mark the proportion of samples scored as positive (positive = eggs: ovaries developed, mated: sperm present, and present for each of the parasites) for each factor in each species (out of $n = 210$ for *Bombus huntii* or $n = 195$ for *Bombus vosnesenskii*). All factors were scored by microscopy, with the exception of the microparasites (*Apicystis, Crithidia,* and *Nosema*), which were diagnosed through molecular tests. Black bars show the 95% confidence interval around the recorded proportion. An asterisk (*) after the name of a factor indicates that the factor was significantly different between species. *Sphaer.* = *Sphaerularia bombi, Syntre.* = *Syntretus splendidus,* Mites = *Locustacarus buchnerii, Apicys.* = *Apicystis bombi.*

other parasites affected ovary development. *Apicystis bombi* presence increased the likelihood that a *B. vosnesenskii* queen would die before the end of the 21-d experiment by 15.4% (3.7–31.0% CI; Fisher's exact $P = 0.017$), but no other parasites were associated with this measure.

Discussion

This research shows that nearly all wild-caught *B. huntii* and *B. vosnesenskii* queens are mated, have developed ovaries, and harbor very few infections; thus, the cause of high rates of broodlessness in captivity is yet to be determined. Parasitized bumble bee queens still often had developed ovaries, a phenomenon noted in the British species *Bombus pratorum* L. (Hymenoptera: Apidae) by Rutrecht and Brown (2008a). Our analyses also show that most infections did not inhibit ovary development. All queens were captured in the spring after a successful hibernation. It has been shown that mated queens have greater odds of survival and increased fitness while overwintering in *B. terrestris*, perhaps suggesting that unmated, infected queens do not successfully overwinter (Baer and Schmid-Hempel 2005). Because we limited our analyses to queens that had successfully overwintered, the true prevalence of unmated and parasitized queens in the wild might be higher than indicated by our results.

Out of all of the parasites we observed, only *S. bombi* had an effect on ovary development. One *B. vosnesenskii* queen was infected with *S. bombi* yet had developed ovaries. This is a rare occurrence (Alford 1969, Röseler 2002, Jones and Brown 2014), as the queen-castrating nematode is known to inhibit the function of the corpus allatum, preventing infected queens from completing their reproductive maturation (Alford 1975). One report of infected queens successfully initiating nests suggests that if a queen is infected in spring after emerging from overwintering, the nematode cannot revert her ovarian development and she remains capable of having brood (Röseler 2002).

A foundress bumble bee queen must emerge in spring healthy enough for the task. Little is known about the microparasite *A. bombi*; however, it seems to have significant impacts on the capacity of bumble bee queens to initiate a colony (Rutrecht and Brown 2008a). The neogregarine was relatively common in broodless *B. vosnesenskii* (10.8%) and associated with early death, with nearly half (42.9%) of infected queens dying before the 21-d cull date in our study. This pattern of early mortality in *A. bombi*-infected queens was also seen in the British species *B. pratorum*, *B. hypnorum* L. (Hymenoptera: Apidae), *B. terrestris*, and *B. lucorum* L. (Hymenoptera: Apidae); Rutrecht and Brown 2008a, Jones and Brown 2014). In these studies, *Apicystis*-infected queens did not initiate colonies because they did not survive long enough to do so. With so little information on the pathology of *A. bombi*, a closer look at the association and its potential impacts on colony founding is necessary.

As discovered in other studies of queens in the *Pyrobombus* subgenus, no *Nosema* spp. infections were detected in our samples (Rutrecht and Brown 2008a, Tripodi et al. 2014). The fact that all of these queens were collected early in the spring suggests that perhaps those infected with *Nosema* spp. did not survive the winter, but current research suggests that *Nosema* spp. does not affect overwintering survival. In laboratory-reared *B. terrestris*, *Nosema bombi* infections had no effects on overwintering weight, overwintering survival, or colony founding (Van der Steen 2008). Furthermore, studies of *Nosema bombi* prevalence in North America observe low prevalence (0–11.75%) in foraging workers of most species in the subgenus *Pyrobombus* (Cordes et al. 2012, Tripodi et al. 2014), suggesting that this parasite is uncommon in these species.

This study focused on one fundamental step in nest initiation: oviposition, but there are other steps in successfully founding a colony that could potentially be affected by parasitism. A previous study by Rutrecht and Brown (2008a) found that, aside from the high mortality rates seen in *S. splendidus* and *A. bombi*-infected queens and the inhibition of ovary development in *S. bombi*-infected queens, most parasites had no effect on the probability of *B. pratorum* founding a new colony. Similarly, our results indicate that parasitism is not the leading cause of failure to initiate a nest in captivity, although cryptic microparasites such as *Nosema* spp., *A. bombi*, and *Crithidia* spp. that do not influence nest initiation may be more dangerous in commercial rearing conditions as infections could go unnoticed and proliferate.

Our study is the first to look at important nest-initiation parameters necessary for domestication of North American bumble bees. Broodless bumble bees from within the same subgenus (*Pyrobombus*) showed similar ovarian development and mating status, but *B. vosnesenskii* harbored more parasites than *B. huntii* in our samples. Most wild-caught queens of these two species are mated, free of parasites, and capable of developing eggs, yet only 38% of *B. huntii* and 51% of *B. vosnesenskii* initiated nests in captivity. Aside from the queen-castrating nematode, none of the observed parasites were associated with ovary development in either species. Obviously, there are further factors to investigate to explain cause of broodlessness. Nutrition would be an important first start, as the fat provided by pollen is a necessity for ovary development and certain pollens have been shown to assist in decreasing parasite load (LoCascio et al. 2019). In addition, traditional pathology experiments inoculating queens reared in controlled, sterile environments may provide insights beyond what our assessment of wild-caught queens has to offer.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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