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Sunflower pollen reduces a gut pathogen in worker and queen but not male bumble bees

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Abstract. 1. Social insect castes and sexes differ in many ways, including morphology, behavior, and sometimes ploidy level. Recent studies have found that consuming sunflower pollen reduces the gut pathogen *Crithidia bombi* in workers of the common eastern bumble bee (*Bombus impatiens*). Here, this work is extended to the reproductive individuals that represent colony fitness – males and queens – to assess if the medicinal effects of sunflower pollen vary with bee caste and sex.

2. This study examined the effect of sunflower pollen compared to a diverse wildflower pollen mix on infection in worker, male, and daughter queen commercial *B. impatiens*. Bees were infected, fed either sunflower pollen or wildflower pollen for 7 days, and then infection levels were assessed.

3. Compared to wildflower pollen, sunflower pollen dramatically reduced *Crithidia* infection in workers and daughter queens, but not males. Infection levels were very low for both diets in males; this could be due to low pollen consumption or other mechanisms.

4. Reducing *Crithidia* infection in young queens before they undergo hibernation is important for population dynamics since infected queens are less likely to survive hibernation, and those that do are less likely to successfully establish a nest the following spring. Because sunflowers bloom in late summer when new queens are emerging, sunflowers could provide an important dietary component for queens during this critical life stage. Deepening our understanding of how diet impacts pathogens in reproductive bees, as well as workers, is crucial to maintain healthy pollinator populations.

Key words. *Bombus impatiens*, *Crithidia*, diet, pathogen resistance, pollinator, social caste.

Introduction

Individuals within a species may vary in pathogen loads due to factors such as sex, age, genotype, diet, social interactions, and experience. Females and males, specifically, may differ in traits related to pathogen defense due to differential exposure or impact of the pathogen on fitness (Rolff, 2002). In many invertebrates, including all members of the order Hymenoptera, differences between females and males are confounded by their different numbers of chromosomes; females are diploid, while males are typically haploid. Haploidy could negatively affect pathogen resistance by reducing the probability of inheriting resistance alleles (Mable & Otto, 1998; Otto & Michalakis, 1998; O'Donnell & Beshers, 2004). However, evidence for this hypothesis has been equivocal, and differences between individuals might depend more on life history (Sheridan *et al.*, 2000). For example, one study compared male and female honey bees and paper wasps (Cappa *et al.*, 2015), which are both eusocial and haplodiploid, but males of the two species differ in how much they rely on protection from the colony. They found that paper wasp males, who spend less time in the colony, had higher immunocompetence than female workers, but honey bee males, who rely more heavily on colony protection, had lower immunocompetence than workers. This suggests that patterns of immunity are species-specific.

In social Hymenoptera, females develop into either reproductive queens or non-reproductive workers. Caste identity is determined by differential gene expression, often resulting in dramatic differences in morphology, behavior, longevity, and

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physiology between queens and workers (Smith *et al.*, 2008). Given their longer lifespans, queens are expected to be equipped with higher immunocompetence than their non-reproductive sisters and daughters. This has been documented in honey bees (Chan *et al.*, 2006) and some ants (Gräff *et al.*, 2007; Koch *et al.*, 2013), but does not always result in lower infection in queens. For example, queen and worker honey bees had similar susceptibility to *Nosema ceranae* (Webster *et al.*, 2004), suggesting queens may evolve higher tolerance to certain pathogens rather than higher resistance. Understanding physiological and ecological differences between reproductive and non-reproductive females is important, especially since population dynamics in many species, such as bumble bees, are strongly influenced by queen survival and dispersal (Lepais *et al.*, 2010; Dreier *et al.*, 2014).

Like other social Hymenoptera, bumble bees (*Bombus* spp.) have queen, worker, and male individuals and are susceptible to a variety of pathogens, some of which have been implicated in their declines (e.g. Genersch *et al.*, 2006; Cameron *et al.*, 2011; Graystock *et al.*, 2013a; Goulson *et al.*, 2015). Worker bumble bees are the primary foragers and are recognised as important pollinators for both natural and agricultural ecosystems (e.g., Galen & Stanton, 1989; Elliott & Irwin, 2009; Garratt *et al.*, 2014). Queen and male bumble bees are also important pollinators (Li *et al.*, 2006; Ogilvie & Thomson, 2015) and represent colony fitness; yet pathogen dynamics in these individuals are poorly understood. Bumble bee queens had higher immune gene expression than males (Barribeau *et al.*, 2015) and were less likely to acquire the gut pathogen, *Crithidia* sp., from their nest than workers (Ulrich *et al.*, 2011). Males had lower infection levels than workers for several pathogens in the field (Shykoff & Schmid-Hempel, 1991) and had lower *Crithidia* infections than workers after 10 days in experimental trials (Ruiz-González & Brown, 2006), suggesting that males may have higher resistance than workers. Alternatively, adult male bumble bees do not spend as much time in the nest as workers and forage on different flowers (Roswell *et al.*, 2019), which may reduce their chances of acquiring and transmitting pathogens (and thus may make them less favorable hosts). Improving our understanding of infection dynamics in queens and males is critical to conserve populations in the face of increasing pressure from pathogens (Meeus *et al.*, 2011).

Differences in diet may also impact how individuals respond to pathogens. Host traits act within an environmental context, where factors such as food availability and intake can impact infection outcomes. Diet quantity and quality can impact host-pathogen interactions by either altering host condition or by directly feeding or inhibiting the pathogen. In the case of bumble bees infected with *Crithidia*, pollen starvation results in reduced immune gene expression (e.g., Brunner *et al.*, 2014) but can also reduce infection levels (Logan *et al.*, 2005; Conroy *et al.*, 2016). Certain diets can also impact *Crithidia* infection. For example, infections decreased in bumble bees fed nectar with secondary compounds (e.g., Manson *et al.*, 2010; Baracchi *et al.*, 2015; Richardson *et al.*, 2015), and infections were dramatically reduced in bees fed pollen from sunflowers (*Helianthus annuus*; Giacomini *et al.*, 2018; LoCascio *et al.*, 2019). These studies were all conducted on female workers; how diet and pathogens interact in male and queen bumble bees is unknown. Males primarily forage for nectar, rather than pollen, resulting in different floral preferences and potentially lower pollen consumption compared to workers (Roswell *et al.*, 2019). As pollen starvation reduced *Crithidia* infection in bumble bee workers (Logan *et al.*, 2005; Conroy *et al.*, 2016), this might result in low infection levels in males if they eat little pollen. Queens may also respond to certain diets differently than workers given that they undergo different nutrient metabolism and storage (Colgan *et al.*, 2011).

The goals of this study were to examine the effect of pollen diet on pathogen infection in bumble bee queens, workers, and males. We experimentally infected commercial *B. impatiens* individuals with *Crtihidia* and asked how a diet of sunflower pollen affected infections compared to a wildflower pollen mix. We used male and worker bees in one experiment, and daughter queens in a separate experiment. This study asked two questions: (i) Do commercial *B. impatiens* males differ from workers in infection intensity when fed wildflower mix or sunflower pollen diet? and (ii) Does sunflower pollen reduce infection in commercial *B. impatiens* daughter queens? While we did not statistically compare queens to workers or males because they were studied in separate experiments, we discuss apparent patterns. Commercial bumble bees are used as pollinators for many outdoor and greenhouse crops and are susceptible to pathogens such as *Crithidia* (Graystock *et al.*, 2013a). This work will improve our understanding of bee-diet-pathogen interactions, which may inform management strategies for bumble bee hives in agricultural settings, as well as conservation strategies for wild populations.

Materials and methods

Study system

Bombus impatiens *(Apidae).* The common eastern bumble bee is abundant in the eastern United States and currently not in decline (Cameron *et al.*, 2011). This species undergoes an annual life cycle; queens and males mate in late summer and then queens undergo solitary diapause during winter. Queens emerge in spring and initially lay worker eggs, progressing to males and daughter queens later in summer (Alford 1975). *Bombus impatiens* colonies are commercially available and are used commonly for crop pollination outdoors and in greenhouses.

Crithidia bombi *(Trypanosomatidae). Crithidia bombi* ("*Crithidia*" hereafter) is a gut pathogen of bumble bees transmitted horizontally through contact with infected feces on flowers or within the colony (Durrer & Schmid-Hempel, 1994; Otterstatter & Thomson, 2007). *Crithidia* directly reduces colony fitness; infected queens are 15% less likely to survive diapause (Fauser *et al.*, 2017), and infected queens that do survive are 40% less likely to successfully found a new colony (Brown *et al.*, 2003a). Infection is associated with decreased likelihood of reproduction in the wild (Goulson *et al.*, 2017), and infected workers have higher mortality when food-limited

(Brown *et al.*, 2000). Previous studies have shown that *Crithidia* elicits an immune response in bumble bees (Brown *et al.*, 2003b; Riddell *et al.*, 2009), although this response is highly variable (Riddell *et al.*, 2011; Brunner *et al.*, 2013). It appears that the infection outcome is at least partly determined by the immune system given that increased immune gene expression of two antimicrobial peptides reduced *Crithidia* infection (Deshwal & Mallon, 2014). The gut microbiota, on the other hand, seems to play a major role in determining *Crithidia* infection; bees treated with antibiotics or reared in the absence of microbes suffered higher *Crithidia* loads than unmanipulated bees (Koch & Schmid-Hempel, 2011). Furthermore, infection was negatively correlated with the presence of certain bacterial taxa but positively correlated with bacterial diversity (Cariveau *et al.*, 2014; Mockler *et al.*, 2018; Näpflin & Schmid-Hempel, 2018). The success of infection is complex, and influenced by host genotype × pathogen genotype × microbiota genotype interactions (e.g. Barribeau & Schmid-Hempel, 2013; Näpflin & Schmid-Hempel, 2016).

Helianthus annuus *(Asteraceae).* Sunflower is a native US wildflower (Reagon & Snow, 2006) and major oilseed crop worldwide whose yield is improved by bee visitation (Nicolson & Human, 2013). In 2018, the United States planted 1.28 million acres of sunflowers (USDA Acreage Report, 2018). The medicinal effect of sunflower pollen in reducing *Crithidia* infection in *B. impatiens* was consistent across *Crithidia* strains (Giacomini *et al.*, 2018) and sunflower cultivars (LoCascio *et al.*, 2019). In addition, infection in wild-caught *B. impatiens* workers was negatively correlated with acreage of sunflowers (Giacomini *et al.*, 2018). Sunflower pollen has relatively low protein content (Yang *et al.*, 2013) and sometimes leads to poor performance in bees that feed on it (Tasei & Aupinel, 2008; McAulay & Forrest, 2019). The low protein content is not likely responsible for reducing *Crithidia* since another similarly low-protein pollen diet (buckwheat, *Fagopyrum esculentum*) resulted in comparatively high *Crithidia* infections (Giacomini *et al.*, 2018). In addition, other chemical components of sunflower pollen, including several fatty acids and the secondary compounds rutin and triscoumaroyl spermidine derivatives, are not the mechanism by which sunflower pollen reduces *Crithidia* in the gut (Adler *et al.*, 2020). Currently, the mechanism underlying this medicinal effect is unknown.

Experimental design

Two experiments were conducted, the first with workers and males and the second with commercial daughter queens. In both experiments, bees were inoculated with *Crithidia*, fed a pollen treatment for 7 days, and then dissected to assess pathogen cell counts. Half of each sex (worker/male) was randomly assigned to either wildflower pollen mix (Koppert Biological Systems, Howell, Michigan) or sunflower pollen (*Helianthus annuus*; Changge Hauding Wax Industry, China). The wildflower pollen mix (>10 plant species) represents a natural bumble bee diet and is also what commercial companies feed their hives and sell to customers and is therefore relevant for both wild and commercial bees. The exact floral species composition is unknown; we purchased spring-collected pollen to avoid Asteraceae and checked the mix via microscopy for Asteraceae pollen (which is recognisable by its spiky exine) to ensure no sunflower or its relatives were in the mix. Goldenrod (*Solidago* spp.), another member of the Asteraceae family, was found to also have medicinal effects against *Crithidia* (LoCascio *et al.*, 2019). The wildflower pollen mix is assumed to have a more diverse chemical and nutritional profile than sunflower pollen, and thus allows us to compare more typical dietary conditions of a generalist pollinator to a diet of only sunflower pollen. Because sunflower is relatively low in protein (Yang *et al.*, 2013), we also suspect that the wildflower pollen mix is likely to have a higher protein concentration, although this was not tested. Both pollen types were collected by honey bees and were ground and mixed in a 7:1 ratio with 30% sucrose to make a paste that was frozen at −20 °C until use.

Upon entering the experiment, bees were inoculated with *Crithidia* originally from three wild *B. impatiens* workers from the Stone Soup Farm in 2014 (Hadley, Massachusetts: 42.363911 N, −72.567747 W) and then maintained in commercial colonies. Inoculum was prepared on each trial date with 150 µl of homogenised gut solution diluted with ¼ strength Ringers Solution (Sigma Aldrich, St Louis, Missouri) to create a solution with 1200 cells/µl, which was then added to equal parts 50% sucrose solution for a final inoculum with 25% sucrose and 600 cells/µl. Bees were starved for 2 h, transferred to individual vials, presented with a 15-µl drop of inoculum (9000 pathogen cells, comparable to what bees would encounter in nature; Schmid-Hempel & Schmid-Hempel, 1993), and observed until the drop was consumed. Bees that did not consume the whole drop were excluded from the experiment.

After inoculation, bees were transferred into individual containers (Placon plastic deli cups with mesh bottoms and lids with holes; 11.4 cm top diameter, 8.9 cm bottom diameter, and 8.25 cm height) and administered their pollen treatment for 1 week. Bees were fed 10 ml of 30% sucrose along with 0.5 g of their pollen diet, replaced every other day. Bees were housed in an incubator in darkness at 27 °C and 55–60% humidity during the experiment. *Crithidia* infection reaches a representative population size by 7 days (Otterstatter & Thomson, 2006); thus, 7 days after inoculation, we dissected bees and assessed *Crithidia* cell counts. To dissect the gut, we removed the midgut and ileum and placed them in a 1.5-ml microcentrifuge tube with 300 µl of ¼ strength Ringers Solution, homogenised, and left to settle for 4 h. We then placed a 10-µl aliquot of the supernatant on a hemocytometer (Hausser Scientific) and counted the number of moving *Crithidia* cells under a 400× compound light microscope to determine cells per 0.02 µl of gut solution. We measured marginal cell length of the right forewing of each bee to estimate bee size (Spaethe & Weidenmuller, 2002).

We did not measure pollen consumption in this experiment. Previous work has confirmed that workers and newly emerged queens consume pollen (Woodard *et al.,* 2019) and that workers consume similar quantities of wildflower pollen mix and sunflower pollen (Adler *et al.*, 2020). However, pollen consumption by male bumble bees has not been previously documented

since male bees primarily forage for nectar during adulthood. To estimate the amount of pollen consumed by males, in a separate assay, we fed uninfected males and workers wildflower pollen for 3 days, dissected their guts following the protocol above, and counted pollen grains in 0.1-µl of gut solution.

Bombus impatiens males and workers were sourced from three commercial colonies: two from BioBest LTD (Leamington, Ontario, Canada) and one from Koppert Biological Systems (Howell, Michigan). Queens were sourced from six BioBest colonies; one colony provided individuals for both experiments. Each colony was checked for the absence of *Crithidia* upon arrival and bi-weekly thereafter by screening five workers. We used 79 males and 87 workers. Sixteen males and 14 workers died and were excluded from analyses, resulting in 63 males and 73 workers. We used 25 daughter queens; all survived their 7-day trial. All bees were removed from their natal colony at least 24 h after emergence to allow inoculation of colony gut microbiota. The experiment was performed in spring and summer 2018 (File S1). For the pollen consumption trial, we used 24 males and 4 workers from one parent colony and 4 more workers from a separate parent colony. Data are available online in the Files S2–S4.

Statistical analyses

We used the open-source software R v3.3.3 (R Core Team, 2014) to analyse the number of *Crithidia* cells in a 0.02-µl gut extract as the response, with generalised linear mixed models (lme4 package, Bates *et al.*, 2015). Cell counts were over-dispersed, and thus we used a negative binomial error distribution. We ran two analyses using the same general approach: one for workers/males and one for queens. For both models, the significance of terms was tested with likelihood ratio χ^2 tests (conducted with the ANOVA function in car package, Fox & Weisberg, 2019), which compares relative goodness of fit between models with and without each term. For all models, diet was included as a fixed effect. Initially, inoculation date and natal colony were included as random effects, but both were non-significant ($P = 1$ and 0.999, respectively, in worker/male model and $P = 1$ for both effects in queen model) and thus were excluded. We included wing marginal cell length as a covariate in each model because bee size can negatively correlate with *Crithidia* count (i.e., Richardson *et al.*, 2015). For males and workers, bee sex (male vs. worker) was used as an additional fixed effect, along with bee sex by diet interaction. Although males were significantly larger than workers ($F = 82.89$, d.f. $= 134$, $P < 0.0001$), the Variance Inflation Factor indicated low multicollinearity in the model including both sex and wing marginal cell length (VIF < 2) and thus both were retained in the model. For the pollen consumption trial, bee sex (male vs. worker) was used as the fixed effect with pollen grain count as the response variable in a generalised linear model with a negative binomial error distribution. Bee wings were not collected for the pollen consumption trials, and thus, bee size was not accounted for in this analysis. Plots were made using emmeans (Lenth, 2020) and ggplot2 (Wickham, 2016). R code is available in the File S5.

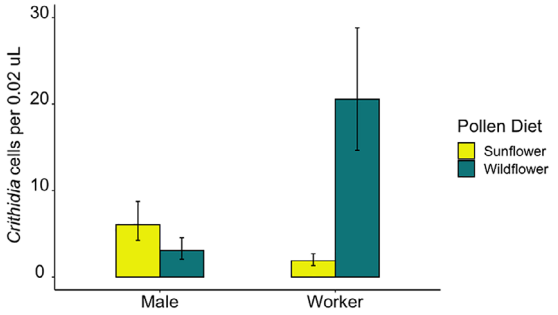

Figure 1. Effect of pollen diet on *Crithidia bombi* cell counts per 0.02 µl of gut solution in *Bombus impatiens* workers and males. Means estimated by a generalised linear model; error bars indicate standard error back-transformed by emmeans. [Colour figure can be viewed at wileyonlinelibrary.com].

Results

Crithidia cells replicated in hosts; when fed wildflower pollen, average raw counts in 0.02 µl of gut were between 2.74 and 22.08 cells, which is, on average, approximately 21 times more *Crithidia* cells than the initial inoculation. For males and workers, we found a significant interaction of diet and bee sex (male vs. worker) on *Crithidia* counts ($\chi^2 = 18.9831$, d.f. $= 1$, $P < 0.0001$; Fig. 1) and no significant relationship between wing cell length and infection ($\chi^2 = 0.6464$, d.f. $= 1$, $P = 0.4214$). After finding a significant interaction effect, we analysed each sex separately to determine responses to diet treatments. Workers fed wildflower pollen had over 10 times higher *Crithidia* counts than those fed sunflower ($\chi^2 = 23.2296$, d.f. $= 1$, $P < 0.0001$), but diet did not significantly affect *Crithidia* counts in males ($\chi^2 = 1.7909$, d.f. $= 1$, $P = 0.1808$; Fig. 1). Workers had over eight times higher *Crithidia* counts than males when fed wildflower pollen ($\chi^2 = 15.6267$, d.f. $= 1$, $P < 0.0001$), while males and workers did not differ when fed sunflower pollen ($\chi^2 = 2.2739$, d.f. $= 1$, $P = 0.1316$).

In daughter queens, sunflower pollen reduced *Crithidia* by 99.5% compared to wildflower pollen ($\chi^2 = 8.6996$, d.f. $= 1$, $P = 0.0032$; Fig. 2). Wing cell length was not significantly related to infection in queens ($\chi^2 = 0.4207$, d.f. $= 1$, $P = 0.5166$).

In the pollen consumption trial, pollen grain count in the gut was significantly lower in males than workers ($\chi^2 = 5.677$, d.f. $= 1$, $P = 0.017$). Male bumble bees had an average of 14 pollen grains per 0.1 µl of gut solution after 3 days, compared to an average of 122.75 pollen grains per 0.1 µl of gut solution in workers. However, one male had 313 pollen grains in 0.1 µl of gut solution; the remaining males had fewer than 10 grains. If we remove this outlier, males had an average of one pollen grain per gut sample, and the effect size of sex is more statistically significant ($\chi^2 = 75.364$, d.f. $= 1$, $P < 0.0001$).

Discussion

We found significant differences in how sunflower pollen affected pathogen counts in female queens and workers

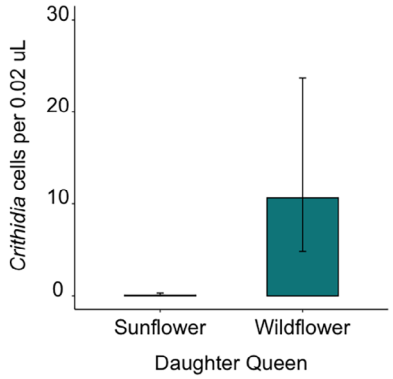

Figure 2. Effect of pollen diet on *Crithidia bombi* cell counts per 0.02 µl of gut solution in newly emerged *Bombus impatiens* daughter queens. Means estimated by a generalised linear model; error bars indicate standard error back-transformed by emmeans. [Colour figure can be viewed at wileyonlinelibrary.com].

compared to male bumble bees. Males responded differently to infection and diet than both castes of females. Although *Crithidia* grew in males relative to the initial inoculation dose, they exhibited remarkably low *Crithidia* counts compared to females regardless of diet, and there was no significant effect of diet on *Crithidia* counts. In workers and daughter queens, sunflower pollen dramatically reduced infection compared to wildflower pollen, consistent with previous findings (Giacomini *et al.*, 2018; Adler *et al.*, 2020).

Bumble bee queens are in a critical life stage when they first emerge. Before diapause, new queens have approximately 1 week in their natal nests before leaving to mate and overwinter (Goulson, 2010). It is during this critical window when queens sequester nutrients to prepare for diapause and when they are most likely to be exposed to pathogens. Prevalence of *Crithidia* increases over the season and is at its highest when daughter queens emerge (Popp *et al.*, 2012). *Crithidia* persists from 1 year to the next by infecting queens before they overwinter, and therefore, there should be strong selection on the pathogen to infect queens. Since infected queens are 15% less likely to survive diapause (Fauser *et al.*, 2017) and exhibit up to 40% lower colony-founding success than uninfected queens (Brown *et al.*, 2003a), there should also be strong selection for queens to resist infection before entering into diapause. The effect of sunflower pollen on infection in daughter queens was dramatic; only 1 of the 13 queens fed sunflower pollen had any detectable *Crithidia* infection, and that was only one cell found in 0.02 µl of gut solution. Our study also corroborates findings that daughter queens were overall less likely to become infected with *Crithidia* than workers (Ulrich *et al.*, 2011) since 7 of the 12 queens fed wildflower mix pollen had no detectable *Crithidia* infection.

As late-blooming flowers, sunflowers may be an important medicinal and nutritional food source for young queens in particular by increasing their chances of surviving diapause and founding a new colony in the spring. Recent research suggests that lipid and carbohydrate stores are essential for queen survival through diapause, whereas protein is not a critical macronutrient for storage (in contrast to its importance during larval development; Woodard *et al.,*2019). Sunflower pollen has relatively low protein and high lipid contents (Yang *et al.*, 2013; Treanore *et al.*, 2019). Therefore, sunflowers may provide new queens with important nutrients for surviving diapause, such as lipids, while also reducing *Crithidia* infections. Feeding on sunflower pollen during this life stage may therefore increase queen probability of surviving diapause and founding a colony in the spring (due to higher lipid stores and lower *Crithidia* infection). Queen survival through diapause constitutes significant population bottlenecks every year (Straub *et al.*, 2015), and thus maximising macronutrient storage and minimising infection during the pre-diapause period is crucial for colony reproduction. Interestingly, *Bombus impatiens* workers are known to preferentially forage for pollen with high protein:lipid ratios to feed developing larvae (Vaudo *et al.*, 2016); however, diet preferences in new adult queens before diapause may be different, or even reversed. Whether workers shift their foraging preferences when the colony starts to produce queens is an open area for future research.

Males had significantly lower baseline infection levels than workers (i.e. when fed the wildflower mix diet), and infection levels were not affected by diet. Previous work has found that male and worker bumble bees were similarly susceptible to *Crithidia* infection (Ruiz-González & Brown, 2006). This study also found that, for the first few days, males and workers had similar infection intensities, but over time, male infection intensities increased at a lower rate than workers, resulting in significantly lower cell counts in males by 10 days post-inoculation (Ruiz-González & Brown, 2006). These findings, along with our results, suggest that males do not suffer from haploid susceptibility and may, in fact, exhibit higher resistance to *Crithidia* than diploid workers. Alternatively, the low infections seen in males may be due to low pollen consumption since pollen starvation reduces *Crithidia* infection (Logan *et al.*, 2005; Conroy *et al.*, 2016). This hypothesis is supported by our pollen consumption trials, in which we found 10 times less pollen in the guts of male compared to worker bees. Nonetheless, studies comparing immune responses of males and workers would help clarify the mechanisms driving the observed patterns.

The bumble bee gut microbiota influences *Crithidia* infection (Koch & Schmid-Hempel, 2011; Koch & Schmid-Hempel, 2012, Cariveau *et al.*, 2014; Mockler *et al.*, 2018) and could be another mechanism driving differences between male and worker infection levels. Male and worker honey bees show differences in bacterial microbiota; males had lower bacterial diversity and higher prevalence of *Lactobacillus* "firm-5" in their guts than female workers (Kapheim *et al.*, 2015). *Lactobacillus* "firm-5" abundance in the gut is negatively correlated with *Crithidia* infection in bumble bees, potentially by lowering the pH of the gut environment (Palmer-Young *et al.*, 2018). If adult male bumble bees have higher prevalence of this bacteria than workers, that may be driving their low infection intensities.

The role of pollen nutritional content, including that of sunflower, in bee immune function is unclear. In bumble bees, immune gene expression is lower in bees starved of pollen, but immunocompetence was not affected by differences in dietary protein content in honey bees (Alaux *et al.*, 2010) or

bumble bees (Roger *et al.*, 2017). However, diet diversity may play an important role. Poly-floral diets increased immunocompetence in honey bees when compared to mono-floral diets (Alaux *et al.*, 2010). This suggests that sunflower pollen as a mono-floral diet (as used in our experiments) may reduce immune function in bees compared to a wildflower mixed diet. If sunflower pollen reduces immune function, then it likely inhibits *Crithidia* by another mechanism and may render bees susceptible to other infections that sunflower does not protect against. Future studies should focus on the specific roles that pollen diet, specifically sunflower pollen, plays in the bee immune system.

The mechanism underlying the medicinal effect of sunflower pollen is currently unknown. Chemical extracts from sunflower pollen reduced the growth of bacteria and fungi (Fatrcová-Šramková *et al.*, 2016) but increased *Crithidia* growth *in vitro* (Palmer-Young & Thursfield, 2017) and did not reduce *Crithidia* to the level of pure sunflower pollen in bee assays (Adler *et al.*, 2020). A compound in heather nectar (*Calluna vulgaris*) reduced *Crithidia* infection by removing the flagellum and preventing attachment to the gut wall (Koch *et al.*, 2019); the spiky pollen coat of sunflower could mechanically inhibit attachment via similar mechanisms. Alternatively, sunflower pollen may influence *Crithidia* infection by inducing a community shift in the gut microbiome, an altered immune response, or changes in gut passage time; all of these mechanisms are currently being assessed in our research group. It is possible that these processes differ in male, queen, and worker bees. Adult males forage for nectar rather than pollen (Roswell *et al.,* 2019), and new adult queens have different nutritional needs than developing larvae and adult workers (Vaudo *et al.*, 2016; Woodard *et al.,* 2019). These dietary differences may have large implications for the immune system and gut microbiota. Because the gut microbiota plays a role in resistance to *Crithidia* infection (e.g., Koch & Schmid-Hempel, 2011), caste and sex differences in the microbiota could affect how bees respond to both diet and pathogen infection.

Our results indicate that sunflower pollen effectively reduces a pathogen in commercial queens and workers and therefore could be used in commercial colony management. However, there are important distinctions between commercial and wild bumble bees to consider when interpreting these results in the context of wild populations. A previous study found that wild *B. impatiens* workers had higher susceptibility to *Crithidia* than commercially reared workers (Mockler *et al.*, 2018), possibly due to the acquisition of non-core gut bacteria from the environment. In addition, commercial bees may be selected for resistance to pathogens that are often present in commercial rearing facilities (Graystock *et al.*, 2013b). However, previous studies suggest that sunflower does reduce *Crithidia* in wild bumble bees (Giacomini *et al.*, 2018) and that sunflower pollen is frequently collected and consumed by bumble bees and other pollinators (Westphal *et al.*, 2003). These findings suggest that sunflower pollen, as part of a diverse diet, may be a promising natural remedy for bumble bee populations facing *Crithidia* infections. It is important to study both wild and commercial bees in order to best understand how they respond to diets and pathogens, especially since commercial and wild bees often share resources in agricultural settings (Graystock *et al.*, 2016).

Queens and males are the reproductive individuals and so represent colony fitness, yet the vast majority of studies on bumble bees focus exclusively on workers. For example, worker bumble bees have been used to understand learning and behavior (Leadbeater & Chittka, 2007), the impacts of pesticides (Laycock *et al.*, 2012), and immunity (Brown *et al.*, 2003b). Meanwhile, males and queens are more difficult to acquire in large numbers, resulting in a knowledge gap about the ecology and physiology of individuals that will pass their genes to future generations. Pathogens are a strong selective force and an important factor contributing to wild and managed bee declines (Goulson *et al.*, 2015); thus, it is crucial to understand how reproductive bees respond to pathogen infection to inform population conservation efforts. With a greater understanding of diet effects on bee pathogens, we could better manage pathogen spread by creating pollinator habitat tailored to provide bees with sufficient diet diversity and access to nutritional and medicinal food.

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AUTHOR CONTRIBUTION

LSA and REI designed the study, ECS and AEF collected the data, AEF analysed the data, and AEF wrote the paper with help from all authors.

Data availability statement

The data that supports the findings of this study are available in the Supporting Information.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1 Table 1 with inoculation dates.

File S2 Daughter queens' data file.

File S3 Workers and males' data file.

File S4 Pollen grains' data file.

File S5 R script.

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