TITLE

Sunflower spines and beyond: mechanisms and breadth of pollen that reduce gut pathogen infection in the common eastern bumble bee

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DATA, CODE AND MATERIALS

All data and R scripts can be found at https://github.com/llf44/Asteraceae-pollen.

COMPETING INTERESTS

The authors have no competing interests.

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TRANSLATED ABSTRACT (SPANISH)

1. Las plantas tienen rasgos químicos y físicos únicos que pueden reducir infecciones en un amplio rango de animales desde los primates hasta las orugas. Los girasoles (*Helianthus annuus*; Asteraceae) son un ejemplo de este fenómeno, al tener polen que inhibe infecciones causadas por el patógeno tripanosoma *Crithidia bombi* en el abejorro *Bombus impatiens*. Sin embargo, el mecanismo que explica este fenómeno aún no ha sido determinado, y no se sabe si el polen de otras especies de Asteraceae tiene efectos similares.
2. Nosotros evaluamos si los mecanismos que median el efecto antipatogénico del polen de girasol son físicos (por su exina espinosa), químicos (por sus metabolitos), o ambos. También evaluamos el grado mediante el cual otras siete especies de Asteraceae reducen las infecciones de *C. bombi* en comparación con el polen de girasol y otras dos especies no-Asteraceae, y si el largo de las espinas del polen predice su efecto.
3. Encontramos que las exinas del girasol por si solas redujeron la infección de manera comparable con el efecto ejercido por el polen completo de girasol, mientras que los metabolitos del polen de girasol por si solos no lo hicieron. Por otra parte, los abejorros que consumieron polen de cuatro de las otras siete especies de Asteraceae obtuvieron infecciones de *C. bombi* 62 – 92% más bajas que aquellas que consumieron polen de no-

Asteraceae. Sin embargo, el largo de las espinas no predijo la variación en las infecciones de los abejorros.

4. Nuestro estudio indica que la capacidad del polen de girasol para inhibir *C. bombi* está guiada por su exina espinosa, y que este fenómeno se extiende a varias especies de Asteraceae. Nuestros resultados indican que las exinas del polen de girasol son tan efectivas en reducir infecciones como el polen completo, lo cual implica que futuros estudios deben expandir la evaluación del efecto de otras especies con polen espinado en la dinámica polinizador-patógeno.

ABSTRACT

1) Plants have unique chemical and physical traits that can reduce infections in animals ranging from primates to caterpillars. Sunflowers (*Helianthus annuus*; Asteraceae) are one striking example, with pollen that suppresses infections by the trypanosomatid gut pathogen *Crithidia bombi* in the common eastern bumble bee (*Bombus impatiens*). However, the mechanism underlying this effect has remained elusive, and we do not know whether pollens from other Asteraceae species have similar effects.
2) We evaluated whether mechanisms mediating sunflower pollen's antipathogenic effects are physical (due to its spiny exine), chemical (due to metabolites), or both. We also evaluated the degree to which pollen from seven other Asteraceae species reduced *C. bombi* infection relative to pollen from sunflower and two non-Asteraceae species, and whether pollen spine length predicted pathogen suppression.
3) We found that sunflower exines alone reduced infection as effectively as whole sunflower pollen, while sunflower pollen metabolites did not. Furthermore, bees fed pollen from four of seven other Asteraceae had 62 – 92% lower *C. bombi* infections than those fed non-Asteraceae pollen. Spine length, however, did not explain variation in bumble bee infection.
4) Our study indicates that sunflower pollen's capacity to suppress *C. bombi* is driven by its spiny exine, and that this phenomenon extends to several other Asteraceae species. Our results indicate that sunflower pollen exines are as effective as whole pollen in reducing infection, suggesting that future studies should expand to assess effects of other species with spiny pollen on pollinator-pathogen dynamics.

Key words: *Ambrosia artemisiifolia*; bee disease; commercial bumble bees; *Eupatorium capillifolium*; medicinal plants; pollinator health; *Taraxacum officinale*; *Xanthium strumarium*

INTRODUCTION

Pathogens are ubiquitous in all living systems, resulting in a constant ecological and evolutionary interplay between pathogens, hosts, and their environments (Brown, 2022; Schmid-Hempel, 2011). Infectious diseases can have profound impacts on ecological communities, the severity of which is often exacerbated by anthropogenic forces such as habitat destruction, introduction of invasive species, climate change, and pollution (Brearley et al., 2013; Gibbons et al., 2000; Marcogliese & Pietrock, 2011). Plants have evolved a myriad of chemical and physical defenses to mitigate pressure from pathogens, and many animals exploit these plant defenses to reduce their own infections (Abbott, 2014; de Roode et al., 2013). Understanding the mechanisms underlying plant antipathogenic properties may inform management strategies that reduce disease in vulnerable animal populations.

Plant secondary metabolites, including phenolics, alkaloids and terpenoids, are associated with plant defense against herbivores, phytopathogens and parasites. Secondary metabolites can be present in both vegetative tissues and floral rewards (nectar and pollen), with composition and concentration varying within individuals and across species (Bennett & Walsgrove, 1994; Palmer-Young et al., 2019; Rivest & Forrest, 2020). Some of these compounds are also active against animal pathogens (reviewed in Palmer-Young et al., 2016) and thus may benefit certain herbivores by reducing infection when consumed. For example, woolly bear caterpillars (*Grammia incorrupta*) parasitized by tachinid flies (*Exorista mella*) will consume pyrrolizidine alkaloids that reduce mortality of infected hosts, even though the toxins increase mortality in unparasitized individuals (Singer et al., 2009). Diet can also shape infection in pollinators. For example, when buff-tailed bumble bees (*Bombus terrestris*) consume the secondary metabolite callunene from heather (*Calluna vulgaris*) nectar, the gut pathogen *Crithidia bombi* loses its ability to anchor into the bee gut and infect the host (Koch et al., 2019). Many insect taxa can self-medicate using plant phytochemicals in response to infection by pathogens (reviewed in de Roode & Hunter, 2019). Chemistry, however, is not the only mechanism by which plants suppress infections in animals. For example, great apes infected with certain parasitic nematodes or tapeworms consume bristly leaves, which physically irritate their gut and increase the expulsion of the pathogens, demonstrating a mechanical mechanism of dietary disease suppression (Huffman, 2003; Huffman & Caton, 2001). Pollen is consumed by many flower-visiting insects, and the exine (outermost physical structure) can vary in morphology, including

presence of spines of varying lengths in some plant species. There are many more known examples of infection suppression due to chemical rather than mechanical means, especially for insects (Bernardo & Singer, 2017).

Bumble bees (*Bombus* spp.) are common pollinators in many ecosystems and include some of the world's most economically important wild bee species (Kleijn et al., 2015). Concern over bumble bee populations has grown in recent decades with reports of declines for many species; these declines are often linked, at least in part, to pathogens (Cameron et al., 2011; Goulson et al., 2015; Schmid-Hempel et al., 2014). Furthermore, there is potential for pathogen spillover from managed honey bees and bumble bees to wild bumble bee species through shared used of floral resources, though we currently do not know the full impact of the movement of managed species within and across countries on wild bee disease dynamics (reviewed in Figueroa et al., 2023). Moreover, recent studies expanding the use of molecular screenings have found widespread pathogen prevalence in wild bumble bee communities (Averill et al., 2021; Figueroa et al., 2020; Jones et al., 2021; Plischuk et al., 2017), underscoring the need to understand the impacts of pathogens and potentially reduce infections in these ecologically important species.

One globally important pathogen that frequently infects bumble bees is *Crithidia bombi*, a trypanosomatid gut pathogen that can reduce learning, survival, and reproduction, especially for overwintering queens and nutritionally stressed individuals (Brown et al., 2000; Gegear et al., 2006; Goulson et al., 2018). Prevalence of this pathogen can vary dramatically by location and year, ranging from 0 – 82% in western Massachusetts, USA, across two years of sampling in 15 sites (Gillespie, 2010). Numerous nectar phytochemicals can suppress *C. bombi in vitro*, *in vivo*, or both (Koch et al., 2019; Palmer-Young et al., 2017; Palmer-Young et al., 2016; Richardson et al., 2015), raising the question of whether plants could serve as medicines for infected bees (Koch et al., 2017).

Sunflower (*Helianthus annuus;* Asteraceae) pollen, which has a characteristically spiny exine and is low in protein, has a potent pathogen-suppressive effect against *C. bombi* when tested *in vivo* in the common eastern bumble bee (*Bombus impatiens*). Bees fed sunflower pollen had 20- to 50-fold lower *C. bombi* infection levels than those fed pollen from rapeseed (*Brassica napus;* Brassicaceae) or buckwheat (*Fagopyrum esculentum;* Polygonaceae) (Giacomini et al., 2018). Furthermore, sunflower pollen reduced *C. bombi* infection in *B. impatiens* queens as well

as workers (Fowler et al., 2020), which is particularly important because infected queens are less likely to survive overwintering and establish new colonies than uninfected queens (Brown et al., 2003). Moreover, sunflower has the potential to benefit these pollinators by reducing gut pathogen infections in the field. Specifically, Giacomini et al. (2018) found that *C. bombi* infection intensity in wild *B. impatiens* workers collected on farms was lower in areas planted with more sunflower. Similarly, Malfi et al. (in press) found that experimentally deployed *B. impatiens* colonies had lower prevalence of *C. bombi* and higher queen reproduction at farms with more sunflowers, highlighting implications for bumble bee health and reproduction under natural conditions.

While the mechanism underlying how sunflower pollen reduces *C. bombi* infection in bumble bees is unknown, several non-mutually exclusive hypotheses have been posited. These include pollen acting as a laxative (Giacomini et al., 2022), influencing immune function (Giacomini et al., 2021a, but see Fowler et al., 2022), and/or physically scraping the hindgut with the spiny exine to impede *C. bombi* attachment (Giacomini et al., 2021a; Giacomini et al., 2018). Given that protein content can strongly increase resistance and tolerance to infections and improve immune function (Brown et al., 2000; Conroy et al., 2016; Lee et al., 2006; Logan et al., 2005, but see Alaux et al., 2010), the difference in effects between sunflower and buckwheat pollen is especially startling, as these two pollen types have similarly low protein levels (Yang et al., 2013). This suggests that protein is not a significant factor mediating sunflower pollen's pathogen-suppressive effect. Assessments of sunflower pollen chemistry to date have not uncovered any compounds responsible for pathogen suppression (Adler et al., 2020), and sunflower methanolic extracts *increased C. bombi* replication *in vitro* (Palmer-Young & Thursfield, 2017). However, the role of sunflower pollen metabolites in driving effects within the host are not well explored. This raises the question of whether the physical structure of the pollen (spiny exines), the chemistry (secondary as well as nutritional metabolites), or both contribute to pathogen suppression.

Most Asteraceae produce echinate (spiny) pollen, presenting an opportunity to test whether echinate pollen from other Asteraceae species also suppresses *C. bombi* compared to non-Asteraceae species that lack spines. Furthermore, pollen spine length varies considerably within the Asteraceae (Tomb et al., 1974), yet it is unknown whether spine length variation affects the degree of pathogen suppression in bumble bees. Compared to wildflower and

buckwheat control pollens, pathogen suppression has been found across nine *H. annuus* cultivars, four wild *H. annuus* populations, two congeners and two species in a different genus of the same family (*Solidago* spp.) (LoCascio et al., 2019). These results suggest that the pathogen-suppressive effects of pollen may be more widespread within the Asteraceae.

Here we ask: (a) Do sunflower exines and/or sunflower metabolites reduce *C. bombi* infection as effectively as whole sunflower pollen? (b) Does pollen from other Asteraceae species reduce *C. bombi* infection as effectively as sunflower pollen, and (c) Does Asteraceae pollen spine length explain the degree to which pathogen infection is reduced?

MATERIALS AND METHODS

Overview

For each question we conducted paired experiments at University of Massachusetts, Amherst (Lab1) and North Carolina State University (Lab2). The experiments assessing sunflower exines and metabolites (question a) were replicated across the two institutions (same treatments), while the experiments assessing other Asteraceae pollens and spine lengths (questions b and c) were divided between the two institutions (different Asteraceae species, same controls). All experiments employed the same protocols for making inoculum and for counting *C. bombi*, described below. The *C. bombi* used was originally sourced from *B. impatiens* workers collected in Hadley, MA, USA (42°21′51.93”N, 72°33′55.88”W) and maintained in commercial *B. impatiens* colonies in both laboratories that were fed a wildflower mix pollen diet (low to no Asteraceae present, assessed via microscopy). During experiments, worker bees were housed in individual containers (plastic 16 oz. deli cups with mesh bottoms and perforated lids; Figure S1) and fed 10 mL of 30% sucrose solution along with 0.15 g of their pollen treatments, replaced every other day, and housed in the dark at 27°C and 55-60% humidity. We employed *B. impatiens* workers from commercial colonies (Koppert Biological Systems, Howell, MI, USA) in all experiments.

(a) Effects of sunflower pollen exines, metabolites and whole pollen

To determine the role of pollen exine structure and metabolites in driving the effect of sunflower pollen on *C. bombi*, we compared *C. bombi* counts in bees fed different pollen diets. We used pollen from three sources: sunflower pollen (Henan Mingshengfeng Bio-Technology

Co., LTD; Henan province, China), buckwheat pollen (Fuyang Import and Export Ltd, China), and wildflower pollen (CC Pollen; Phoenix, Arizona). We verified that the wildflower pollen had less than 5% Asteraceae (echinate) pollen via visual inspection of a subset of the mixture stained with basic fuchsin dye under a compound microscope (Kearns & Inouye, 1993). In addition to these three control diet treatments (sunflower pollen, buckwheat pollen, and wildflower pollen), we also included sunflower or buckwheat metabolites mixed with wildflower pollen, and sunflower or buckwheat exines mixed with wildflower pollen (mixed by weight; ratios in Table S1). We included buckwheat whole pollen because it has similar (low) protein concentrations (Yang et al., 2013) but results in much higher *C. bombi* infections than sunflower pollen (Giacomini et al., 2018), and buckwheat metabolites and exines mixed with wildflower pollen as methods controls (so we could ascertain whether effects were due to adding any metabolite or exine, or were specific to sunflower metabolites or exines). We included wildflower pollen as a more ecologically relevant multispecies control and used it as the substrate to mix with sunflower and buckwheat exines and metabolites. The complete experimental design is visually represented in Figure S2.

Treatment preparation

We planned to extract metabolites or exines from a set weight of sunflower or buckwheat pollen and add these extracts to wildflower pollen to create the same final diet weight. For example, we extracted metabolites from 50 g of sunflower pollen, and then added them to enough wildflower pollen to create 50 g of diet, ensuring that we had the same ratio of metabolites to total diet weight in both the original and treatment diet. By extracting metabolites or exines from a standardized known weight of whole pollen and adding them to create a standard final weight of diet treatment, we ensured that each treatment used the amount of metabolite or exine from a known quantity of pollen (regardless of volume), incorporated into the appropriate weight of diet. For exines, we ended up extracting from 100 g of pollen instead of 50 due to significant loss of material during extractions because exines remained stuck in filter paper or on glassware. Thus, while our intent was to replicate the ratio of exine:total diet found in the original pollen, instead the exine treatments are a test of whether exines added to wildflower pollen can replicate the effects of whole sunflower pollen, and not necessarily a test of the ecologically relevant ratio.

To obtain clean and intact pollen exines we used modified methods from Gonzalez Cruz et al. (2018), and to obtain pollen metabolites with a wide range of polarities, we sequentially extracted sunflower and buckwheat pollen with distilled water, methanol, ethyl acetate and hexane and retained all metabolites after removal of solvents (Gonzalez-Cruz et al., 2018); methods detailed in Appendix S1. Our goal was to ensure the extraction of the broadest possible range of metabolites (including lipids and proteins) and not simply secondary metabolites, since other components, such as fatty acids, can have antimicrobial properties (Feldlaufer et al., 1993). Pollen from the three control diet treatments was pulverized using a coffee grinder, then mixed with distilled water to create a paste with a consistency palatable for bees (detailed in Table S1). For the exine and metabolite treatments, the exines or metabolites from each species (originally extracted from 100 g of pollen for exines or 50 g of pollen for metabolites) were mixed with enough wildflower pollen to weigh 50 g. For the metabolites, this replicated the original relative amount per weight of pollen, and for exines, we extracted from twice the original weight due to loss of material during extractions. Each exine/metabolite and wildflower pollen mix was then combined with distilled water to create a paste fed to bees (5-36 mL of water; detailed in Table S1). The pollen mixture to water ratios varied between treatments because the exines and metabolites varied in moisture content, and so required different amounts of water to reach similar consistencies. At Lab1, we initially added too much water to the sunflower metabolite treatment, and so both the sunflower and buckwheat metabolite diet treatments were dried at 47 ℃ for 26 hours (including both treatments in case heat affected compounds; no treatments were dried at Lab2). Pollen diets were stored at -20 °C. To feed diets to bees, we placed the treatments in microcentrifuge tube caps inside the housing container. Since grinding pollen may increase access to chemical defenses in the pollen grain and/or increase physical defenses by creating smaller "shards" compared to the intact exine (Brochu et al., 2020), we processed treatments in a similar way. We verified via microscopy that pollen morphology was not altered after grinding; therefore, it is unlikely that pollen "shards" affected our results (Figure S3).

Crithidia bombi *inoculation*

C. bombi inoculum was prepared fresh daily with 150 µL of homogenized gut solution from an infected bee diluted with ¼ strength Ringer's Solution (Lab1) (Sigma Aldrich, St Louis, MO, USA) or distilled water (Lab2) to create a solution with 1200 cells/µL. This solution was

then added to equal parts 50% sucrose solution for a final inoculum with 25% sucrose and 600 cells/μL. On the day of inoculation, bees were deprived of pollen for 2 h, transferred to individual vials, presented with 15 μl of inoculum (~9000 pathogen cells, comparable to concentrations encountered in nature; Schmid-Hempel & Schmid-Hempel, 1993) and observed until the drop was consumed. Bees that did not consume the entire droplet of inoculum were excluded from experiments.

Each bee was inoculated once, then housed in individual containers and provided the pollen treatment for the duration of the trial (7 days). At Lab1, we used worker bees from five commercial colonies, starting trials on six dates from November 11 to December 10, 2019, for a total of 252 bees (33 bees died and 17 escaped, resulting in final sample sizes ranging from n = 23 to 30 per diet treatment). At Lab2, we used workers from three colonies started over seven dates from April 12 to May 6, 2020, for a total of 294 bees (22 bees died, resulting in final sample sizes ranging from n = 37 to 40 per diet treatment). All diet treatments were evenly distributed across dates and colonies in both institutions.

Crithidia bombi *counts*

We dissected bees and assessed *C. bombi* cell counts seven days after inoculation and exposure to the pollen diet, a realistic timeframe for the infection to reach a representative population size (Otterstatter & Thomson, 2006). To determine pathogen loads, we dissected the bee gut and placed it in a 1.5 mL microcentrifuge tube with 300 μL of ¼ strength Ringer's solution (Lab1) or distilled water (Lab2), which was then homogenized and left to settle for 4 hr. We then placed a 10 μL aliquot of the supernatant on a hemocytometer (Hausser Scientific) and counted the number of *C. bombi* cells under a compound light microscope at 400× to determine cells per 0.02 μL of gut solution. We recorded daily mortality and measured marginal cell length of the right forewing of each bee to estimate bee body size (Nooten & Rehan, 2020), which often correlates with *C. bombi* infection intensity (Van Wyk et al., 2021).

Diet treatment consumption

Given that pollen deprivation can reduce *C. bombi* infections in *B. impatiens* (Conroy et al., 2016; Logan et al., 2005), we measured the amount of pollen consumed during the treatment phase from the second to the fourth day (48 hr) at Lab1 to verify that consumption did not

explain differences in infection. Pollen was placed in the cap of a microcentrifuge tube inside each housing container and weighed before being administered to the bee and again after 48 hr. We did not measure consumption for this experiment at Lab2.

(b) Effects of pollen from other Asteraceae species

Pollen species and experimental methods

We compared the effect of pollen from ten species on *C. bombi* infections, including seven Asteraceae that had not been tested previously and three control species. The seven new Asteraceae were cocklebur (*Xanthium strumarium*), common sagebrush (*Artemisia tridentata*), dandelion (*Taraxacum officinale*), dog fennel (*Eupatorium capillifolium*), eastern baccharis (*Baccharis halimifolia*), marsh elder (*Iva annua*), and short ragweed (*Ambrosia artemisiifolia*), selected based on their commercial availability; all were hand-collected and sourced from Stallergenes Greer (Lenoir, North Carolina, USA). Although the pollen from these species may not necessarily be regularly collected by bumble bees in nature, our goal here was to assess the generality of Asteraceae pollen effects on *C. bombi* infection. The three control treatments were sunflower (*Helianthus annuus*; Asteraceae positive control), buckwheat (*Fagopyrum esculentum*; non-Asteraceae negative control), and red maple (*Acer rubrum*; non-Asteraceae negative control; Table S2). Sunflower and buckwheat are standard positive and negative controls used in previous experiments (Fowler et al., 2020; Giacomini et al., 2018; LoCascio et al., 2019), but they were honey bee-collected and obtained from a different source (Changge Hauding Wax Industry, China Co. LTD) than the other species tested. Thus, we included red maple as a negative control that was hand-collected and from the same source as the other Asteraceae pollens but in a different family (Sapindaceae). Sunflower and buckwheat pollen pellets were first ground using a coffee grinder and then mixed with distilled water to produce a paste that could be fed to bees. The other pollen species were received in powder form and directly mixed with distilled water to produce a paste, which was then mixed with 30% sucrose solution to reach a similar consistency as the sunflower and buckwheat pollen pastes, which were honey bee-collected and thus naturally mixed with nectar (Table S2).

Because it is logistically challenging to conduct bioassays with more than 7 treatments simultaneously, experiments at Lab1 and Lab2 each assessed 3-4 of the Asteraceae pollen

species plus the same three control pollen species. Thus, we do not compare all the Asteraceae pollens to each other, but instead assess their effectiveness compared to the same control treatments. Trials took place in 2021 on five dates between January 13 – 27 at Lab1 and six dates between January 12 – February 9 at Lab2. While we began with equal sample sizes within each institution and pollen species treatment, final sample sizes differed due to bee mortality or escape (Table S2). In both institutions, bees from three commercial colonies were used, equally distributed among treatments.

Pollen consumption, C. bombi *inoculation and counts*

We measured the amount of pollen consumed as described above in (a). We also estimated evaporation in the pollen treatments by including containers with pollen but no bees for each pollen treatment (n = 14 in Lab1 and n = 5 in Lab2). We first calculated the linear regression of the final (evaporated) pollen weight predicted by initial pollen weight separately for each pollen treatment in the absence of bees (Figure S4). From these linear regressions we estimated the predicted final pollen weight for each replicate due to evaporation, based on the initial pollen weight. We then subtracted the *predicted* final weight from the *measured* final weight to estimate consumption after accounting for evaporation. *Crithidia bombi* inoculation and counts were completed as described above in (a).

(c) Effect of Asteraceae pollen spine length

Measuring Asteraceae pollen spine length

To evaluate whether Asteraceae pollen spine length influenced *C. bombi* infection intensity, we generated images of each pollen species used to answer question (b) using Scanning Electron Microscopy (SEM) at the Lab1 Institute for Applied Life Sciences. For each pollen species, we measured and averaged the values from five spines on each of five pollen grains from each plant species to obtain the mean pollen spine length using ImageJ (Abràmoff et al., 2004).

Statistical analyses

General approach

Statistical analyses were conducted using R version 4.1.0 (R Core Team, 2021). Data were analyzed using mixed effects models (GLMM) using the glmmTMB package, which allowed us to account for zero-inflation (Brooks et al., 2017). The responses evaluated were *C. bombi* count (cells per 0.02 µL) and bee survival over the course of the experiments. Models varied in distribution selected and whether bee size (wing marginal cell length) was included as a covariate (based on model fit). We assessed model fit using the DHARMa package (Hartig, 2017). Significance of fixed effects was determined using Type II Wald χ^2 tests (Fox & Weisberg, 2018). We evaluated pairwise comparisons between treatments for *C. bombi* counts and pollen consumption using Tukey's honestly significant difference test from the multcomp package (Hothorn et al., 2016). Lastly, we evaluated differences in survivorship of bees fed different diet treatments using a Cox proportional hazards mixed effects model of the coxme package, including survival as the response (death/days elapsed) (Therneau & Therneau, 2015). For the survival analysis comparing different plant species, we evaluated the model with either species as the explanatory variable or spine length (not included in same model since intrinsically confounded). We evaluated pairwise differences across treatments in the survival analyses using the *emmeans* functions of the emmeans package (Lenth et al., 2018). Model details are described below.

(a) Effects of sunflower pollen exines, metabolites and whole pollen

Since the same treatments were used at Lab1 and Lab2, data for these experiments were analyzed together. To evaluate the effects of sunflower pollen exines and metabolites on *C. bombi* infection, we constructed a GLMM with a negative binomial distribution that included *C. bombi* count as the response and pollen diet, lab (Lab1 or Lab2) and their interaction as predictors. The model also included colony as a fixed effect and inoculation date as a random effect. Including bee size negatively affected model convergence and thus bee size was not included in the model. At Lab1, on November 12, 2019, 15 bees were inoculated from a colony that was later discovered to have *C. bombi*, and thus it is possible that these bees had been exposed to the pathogen before the trial. The effect of diet treatment was unchanged when bees from this colony were removed from the analyses ($\chi^2 = 63.25$, df = 6, $P < 0.001$ vs $\chi^2 = 65.25$, df

= 6, $P < 0.001$ when bees from the colony were included and excluded, respectively), and so the complete dataset was retained to maintain the larger sample size.

For the Lab1 bees (where pollen consumption was measured), we evaluated the relationship between pollen consumption and *C. bombi* counts by constructing a GLMM that included *C. bombi* count as the response, and pollen diet, pollen consumption (initial – final pollen weight), the interaction between pollen diet and pollen consumption, and bee size as fixed effects. The model included a negative binomial distribution. Variance inflation in our model was less than two, indicating low multicollinearity. We found no effect of pollen consumption on *C. bombi* counts ($\chi^2 = 0.77$, df = 1, $P = 0.380$), or survival ($\chi^2 = 0.95$, df = 1, $P = 0.330$). There was, however, a significant pollen consumption by pollen diet interaction on *C. bombi* count (see Results). Thus, we report both the interaction term results (bees from Lab1, where pollen consumption was measured), and results excluding consumption data (bees from both institutions, given that consumption was not measured at Lab2).

(b) Effects of pollen from other Asteraceae species

We analyzed the effect of pollen species separately for each institution because Lab1 and Lab2 compared different Asteraceae species (although they used the same controls). Our initial GLMM included *C. bombi* count as the response, pollen species, pollen consumed and colony as fixed effects, and inoculation date as the random effect. Including bee size negatively affected model convergence and thus bee size was not included in the model. Variance inflation in our model was less than two, indicating low multicollinearity. Given that there were no effects of pollen consumption in the initial model on *C. bombi* counts ($\chi^2 = 1.32$, df = 1, $P = 0.251$ and $\chi^2 = 0.14$, df = 1, $P = 0.709$, for Lab1 and Lab2, respectively) or bee survival ($\chi^2 = 0.22$, df = 1, $P = 0.642$ and $\chi^2 = 0$, df = 1, $P = 0.973$, for Lab1 and Lab2, respectively), and that including pollen consumption limited our sample size since we were unable to measure pollen consumption for all bees ($n = 13$ bees without consumption data), the final model excluded consumption as a covariate.

(c) Effect of Asteraceae pollen spine length

To assess whether pollen spine length explained variation in *C. bombi* infection, we constructed a separate model that combined data from both institutions. We standardized the

values of the Asteraceae pollen species before analyzing in a single model to account for differences in baseline infection levels at the two institutions. To standardize, we first calculated the average *C. bombi* count for each treatment at each institution and then divided the average from each Asteraceae species and red maple by the buckwheat average (negative control) from the same institution (hereafter, 'standardized *C. bombi* count'). The reason we standardized by buckwheat was that it was used in both institutions (and its relative effect on infection was expected to be the same) and did not have spines. We did not standardize by red maple because we wanted to include a non-Asteraceae treatment species with no spines that was from the same source as all the non-sunflower Asteraceae species. We then constructed a linear regression model that included standardized *C. bombi* count as the response variable, and pollen spine length as the explanatory variable (aggregated at the species level for both; $n = 9$, one for each species). Given that sunflower and red maple had measurements from both institutions, we randomly selected the lab from which we would take the measurement for each of the two species (sunflower value was from Lab1 and red maple was from Lab2) to avoid pseudoreplication.

RESULTS

(a) Effects of sunflower pollen exines, metabolites and whole pollen

Crithidia bombi counts differed with pollen diet ($\chi^2 = 63.25$, df = 6, $P < 0.001$; Figure 1). Bees fed sunflower exines or sunflower whole pollen exhibited the lowest *C. bombi* counts (81 – 94% lower counts than all other treatments; Figure 1). Furthermore, the effect of sunflower exines added to wildflower pollen did not differ from the effect of whole sunflower pollen ($z = 0.52$, $P = 0.999$), while sunflower metabolites added to wildflower pollen resulted in much higher *C. bombi* counts ($z = 6.05$, $P \leq 0.001$; Table S3; Figure 1). Consumption of whole sunflower pollen reduced *C. bombi* counts relative to all diet treatments except sunflower exines ($z \geq 4$, $P \leq 0.001$ for all except sunflower exines; Table S3; Figure 1). Similarly, bees fed sunflower exines had significantly lower *C. bombi* counts than all other treatments ($z \geq 3.07$, $P \leq 0.032$), except for buckwheat exines, with which it did not statistically differ ($z = 2.16$, $P = 0.301$; Table S3; Figure 1). Colonies significantly varied in *C. bombi* counts ($\chi^2 = 23.32$, df = 7, $P = 0.002$). Institution and institution by pollen diet interaction did not explain *C. bombi* counts

(χ^2 = 0.02, df = 1, *P* = 0.884 and χ^2 = 8.55, *P* = 0.201, respectively; Figure S5). Pollen diet did not significantly influence bee survival (χ^2 = 11.72, df = 6, *P* = 0.068 and χ^2 = 1.77, df = 6, *P* = 0.940, for Lab1 and Lab2, respectively).

Although pollen consumption did not significantly influence *C. bombi* counts (χ^2 = 0.77, df = 1, *P* = 0.380, at Lab1 where consumption was measured), there was a significant pollen consumption by pollen diet interaction (χ^2 = 24.10, df = 6, P < 0.001), whereby bees that ate more buckwheat whole pollen had significantly higher *C. bombi* counts and those that ate more sunflower exines had significantly lower *C. bombi* counts than those fed the wildflower whole pollen control (Table S4).

(b) Effects of pollen from other Asteraceae species

C. bombi counts varied significantly by pollen species (χ^2 = 76.37, df = 5, *P* < 0.001 and χ^2 = 63.25, df = 6, *P* < 0.001, for Lab1 and Lab2, respectively; Figure 2). *C. bombi* counts did not differ significantly between bees that consumed buckwheat and those fed red maple pollen (Table S5). Bees fed sunflower pollen, our positive control known to reduce *C. bombi*, had 74 – 77% lower *C. bombi* counts than those fed buckwheat and red maple, our two negative controls, in both institutions (Figure 2). Similarly, ragweed, cocklebur, dandelion, and dog fennel pollen had lower *C. bombi* counts than buckwheat and red maple (average 77% lower, ranging from 62 – 92% lower; Table S5; Figure 2). Colonies differed in *C. bombi* counts at Lab2 (χ^2 = 20.37, df = 2, *P* < 0.001), but not at Lab1 (χ^2 = 2.53, df = 2, *P* = 0.282).

For the Lab1 trials, there was 25% mortality. While pollen species explained differences in bumble bee worker survival (χ^2 = 16.18, df = 5, *P* = 0.006), there were no significant pairwise comparisons (Table S6). The highest survival was for bees fed buckwheat and the lowest for those fed marsh elder, and this was the only marginally significant pairwise comparison (*P* = 0.05; Table S6). At Lab2, there was very low mortality (4% overall; Table S2) and no effect of pollen treatment on survival (χ^2 = 0, df = 6, *P* = 1).

(c) Effect of Asteraceae pollen spine length

Spine length varied from 0.29 (sagebrush) to 5.25 µm (sunflower) across the eight Asteraceae species screened (Figure 3). However, spine length did not explain significant

variation in *C. bombi* counts ($F_{1,7} = 2.08$, $P = 0.192$; Figure 4), nor differences in bee survival ($\chi^2 = 0.12$, df = 1, $P = 0.729$ and $\chi^2 = 0$, df = 6, $P = 1$, in Lab1 and Lab2, respectively).

DISCUSSION

While pollen is an essential component of bee diets that varies widely in nutritional value, morphology and secondary chemistry (Bedinger, 1992; Goulson, 2010; Palmer-Young et al., 2019), we lack an understanding of how different aspects of this variation contribute to pathogen resistance in pollen-eating animals. Here we show that sunflower exines rather than metabolites reduced *C. bombi* infection in the common eastern bumble bee, *Bombus impatiens*. In addition, we found that bees fed four of seven Asteraceae pollen species had 62 – 92% lower *C. bombi* counts than those fed our non-Asteraceae controls. Our work suggests that the antipathogenic effect of sunflower pollen is driven by its spiny exine, and that this effect may be common in the Asteraceae family.

Although sunflower pollen strongly and consistently reduced *C. bombi* infections in previous studies with *B. impatiens* (Fowler et al., 2020; Giacomini et al., 2021b; Giacomini et al., 2018; LoCascio et al., 2019), a key question remained regarding whether the effect was a product of chemical and/or mechanical means. Our results are consistent with Adler et al. (2020) in finding no effect of sunflower secondary metabolites on *C. bombi* infections (Adler et al., 2020). A possible explanation is that certain plant secondary metabolites lose medicinal properties during passage through the insect midgut (Koch et al., 2022; Koch et al., 2019), while Asteraceae pollen exines can pass through the bee gut largely intact (Peng et al., 1985; Vanderplanck et al., 2018). Alternatively, it may be that chemistry is simply not responsible for the medicinal effect of sunflower pollen.

Interestingly, we found that bees fed sunflower exines mixed with wildflower pollen reduced *C. bombi* similarly to those fed whole sunflower pollen (Figure 1), indicating that pollen exines are a primary driver of how sunflower pollen reduces infection in *B. impatiens*. Our results raise the question of whether the spines are removing attached pathogen cells or preventing attachment of free-swimming cells by scraping the hindgut. This could occur if the spines injure and subsequently melanize the gut (Giacomini et al., 2021a), resulting in surfaces that are more difficult for the flagellated pathogens to adhere on. Furthermore, the echinate pollen could irritate the bee gut and subsequently increase expulsion of the pathogen, as previous

work has found that consuming sunflower pollen increases the rate and volume of defecation (Giacomini et al., 2022). Alternatively, the exines could directly impact pathogen cells and cause flagellar retraction or detachment (the flagellum is key for mounting successful infections; Koch et al., 2019). We note that while sunflower exines reduced *C. bombi* counts 81% more than buckwheat exines, these differences were not significant, even though buckwheat exines resulted in significantly higher *C. bombi* counts than sunflower whole pollen. These results warrant further evaluation into the mechanism by which pollen can influence disease dynamics in the host. Furthermore, sunflower and buckwheat exines differ in morphology (Figure 3), and thus they likely occupied different amounts of space in the pollen diets. Future work should elucidate how pollen surface area, structure, nutrition, and even exine thickness influence antipathogenic effects. Determining how sunflower exines interact with the host and/or the pathogen to reduce infection is the next step to increase our understanding of how diet mediates infection dynamics.

We found that pollen from multiple other species in the Asteraceae family reduced *C. bombi*, although this was not the case for all the Asteraceae species we screened. In addition to sunflower, four other Asteraceae species reduced *C. bombi* infection: ragweed, cocklebur, dandelion, and dog fennel (Figure 2). The three Asteraceae that were not significantly different from buckwheat in terms of their impact on *C. bombi* infection were marsh elder, sagebrush, and baccharis (although the mean *C. bombi* counts of both marsh elder and baccharis were much lower than for buckwheat; 61% and 58%, respectively). Interestingly, while seven of the eight species screened were in the highly speciose Asteroideae sub-family, the one species in a different sub-family (dandelion, Cichorioideae) yielded the lowest pathogen counts of all species, suggesting that the pattern may be more widespread in the family. Specifically targeting and screening species across the entire Asteraceae phylogeny would be an important future direction to determine generality and any phylogenetic signal within the family. Given that we did not find a significant relationship between spine length and relative infection in the eight Asteraceae species we screened (Figure 3 and Figure 4), expanding the number of species to include a broader range of spine lengths, and evaluating other metrics that vary among pollen, such as grain shape and size, as well as spine density, could explain differences in effects on pathogen counts. Thus, the ability to reduce *C. bombi* infection may be common in the species-rich Asteraceae family, although the specific role of spines remains to be determined.

Asteraceae plants, which have characteristically echinate pollen walls, are often considered poor quality forage for bees, in part because they have low protein content, are missing essential amino acids, and have poor digestibility (Nicolson et al., 2018; Nicolson & Human, 2013; Vanderplanck et al., 2018). For example, *B. impatiens* workers die more quickly when fed pollen from sunflower exclusively compared to broad bean (*Vicia faba*, Fabaceae), rapeseed *(Brassica napus*, Brassicaceae) or summer squash and watermelon (*Cucurbita pepo* and *Citrullus lanatus*, respectively, Cucurbitaceae) (McAulay & Forrest, 2019). Nonetheless, bumble bees are generalist foragers and seldom exclusively forage on a single species. Consuming Asteraceae pollen in combination with other types of pollen may compensate for its nutritional deficits. For example, *B. impatiens* worker mortality on a mixed pollen diet (50% as opposed to 100% sunflower), was similar to non-sunflower diets (McAulay & Forrest, 2019), and sunflower pollen reduced *C. bombi* infections even when mixed 50% with wildflower pollen (Giacomini et al., 2021b). Furthermore, recent work found that greater abundance of sunflowers on farms was associated with lower prevalence of *C. bombi* and higher queen production in experimentally deployed *B. impatiens* workers, (Malfi et al. in press). As such, the inclusion of Asteraceae pollen in diverse pollen diets has the potential to reduce disease loads in *B. impatiens* without costs in terms of survival or reproduction. Additionally, consumption of dandelion pollen strongly reduced *C. bombi* counts (Figure 2), bringing to light the importance of considering Asteraceae "weeds" as potential resources for bees, especially in otherwise ecologically depauperate environments (Campbell et al., 2017; Requier et al., 2015; Vaca-Uribe et al., 2021, but see Vanderplanck et al., 2020).

Multiple plant families beyond Asteraceae have species with echinate pollen, including Malvaceae, Caprifoliaceae, Cucurbitaceae, and Campanulaceae, and their spines can vary greatly in length (e.g., < 1 µm to > 10 µm; Konzmann et al., 2019). The effect of the pollen from these other plant families on *C. bombi* infection is unknown, and pollens from species in these families vary in how palatable they are to foraging bees. Pollen can vary greatly in the nutrition it provides bees and the presence/intensity of chemical and physical protective barriers (Konzmann et al., 2019; Palmer-Young et al., 2019; Vaudo et al., 2016); some types of pollen can even impair nutrient absorption (Brochu et al., 2020). The buff-tailed bumble bee, *B. terrestris*, which generally avoids consuming the echinate pollen from *Alcea rosea* (Malvaceae), will readily collect the pollen after the spines are bent via vortexing, illustrating how spines can inhibit pollen

consumption by bees (Lunau et al., 2015). However, in an assessment of pollen palatability across multiple plant families, pollen size, spine length, and spine density were not strong predictors of collectability by *B. terrestris* (Konzmann et al., 2019). Evaluating whether consumption of echinate pollen from species across plant families also suppresses *C. bombi* infection in bees will shed light on the generality of this medicinal effect.

Most of what is known about bee disease dynamics comes from studies on *A. mellifera*, *B. impatiens*, and *B. terrestris* (Schmid-Hempel, 1998), though there is evidence that even within the bumble bees, there are differences in susceptibility and likelihood of pathogen transmission (Ruiz-González et al., 2012). The medicinal value of sunflower to pollinators beyond *B. impatiens* remains largely unknown but may extend to at least some other bee species. For example, sunflower pollen also markedly reduced *C. bombi* infections in *B. terrestris*, a highly abundant and commercially available European bumble bee species (Koch et al. unpublished data), although not always (Gekière et al., 2022). Furthermore, the antiparasitic effects of sunflower may extend beyond trypanosomatids, as *Nosema ceranae* infections in *A. mellifera* were reduced by consumption of sunflower pollen (Giacomini et al., 2018) and honey (Gherman et al., 2014). Similarly, three species of mason bees (*Osmia*) that are specialized on Asteraceae pollen had significantly lower brood parasitism compared to congeners in the same habitat who are generalist pollen provisioners or those specialized on Fabaceae (0% compared to 33% brood parasitism; Spear et al., 2016). However, the effects of sunflower pollen are not evident in all bee species; the patterns are less strong for *B. bimaculatus* and *B. vagans*, and nonexistent for *B. griseocollis* (Fowler et al., 2022), highlighting the need to evaluate the medicinal effect of sunflower pollen across a diversity of bee species in locations with different pathogen strains and resource availabilities (Sadd, 2011).

Here we show that multiple species from one of the most speciose plant families in the world reduced infections of the trypanosomatid gut pathogen *C. bombi* in the common eastern bumble bee and identify the pollen exine as a mechanism driving this effect. Our results suggest that sunflower exines as well as whole sunflower pollen could be effective non-chemical methods of managing *C. bombi* infection in commercial rearing facilities. Assessing the effects of spiny pollen from other plant families and evaluating the ecological consequences of plant species composition in established pollinator habitat, will further advance our understanding of bee disease dynamics and pollinator health.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Appendix S1. Pollen metabolite and exine extraction protocol.

Table S1. Ratios of pollen treatments to water.

Table S2. Pollen species, including family, spine length, collection method, and sample sizes for *C. bombi* infection and survivorship models.

Table S3. Comparisons between sunflower whole pollen and other diet treatments (buckwheat and wildflower whole pollen, as well as buckwheat and sunflower metabolites and exines added to wildlflower pollen) on *C. bombi* cell counts.

Table S4. Comparison of pollen consumption by pollen diet interaction relative to wildflower control.

Table S5. Pairwise comparisons of *C. bombi* counts between pollen species.

Table S6. Pairwise comparisons in survival between pollen species at Lab1.

Figure S1. Experimental set-up housing the bumble bees for bioassays.

Figure S2. Visual representation of the seven pollen diet treatments.

Figure S3. Pictures of the pollen treatments used in the experiment comparing effects of pollen exines, metabolites and whole pollen.

Figure S4. Differences in initial and final pollen weight for evaporation controls (no bees).

Figure S5. Effect of diet treatment on *C. bombi* counts in bees one-week post-inoculation.

Figure 1. Boxplots showing the effect of diet treatment on *C. bombi* counts in bees one-week post-inoculation for bees from both Lab1 and Lab2. The sunflower and buckwheat exines and metabolites (metab.) were added to a wildflower mix (Figure S2), and thus we also include wildflower pollen (WF) as a separate control. Whole pollen refers to pollen diets that were exclusively wildflower, sunflower or buckwheat pollen. Data from both institutions were analyzed together (as shown here) and visualized separately by institution in Figure S5 to show consistency of patterns. Letters above bars indicate significant differences (Table S3).

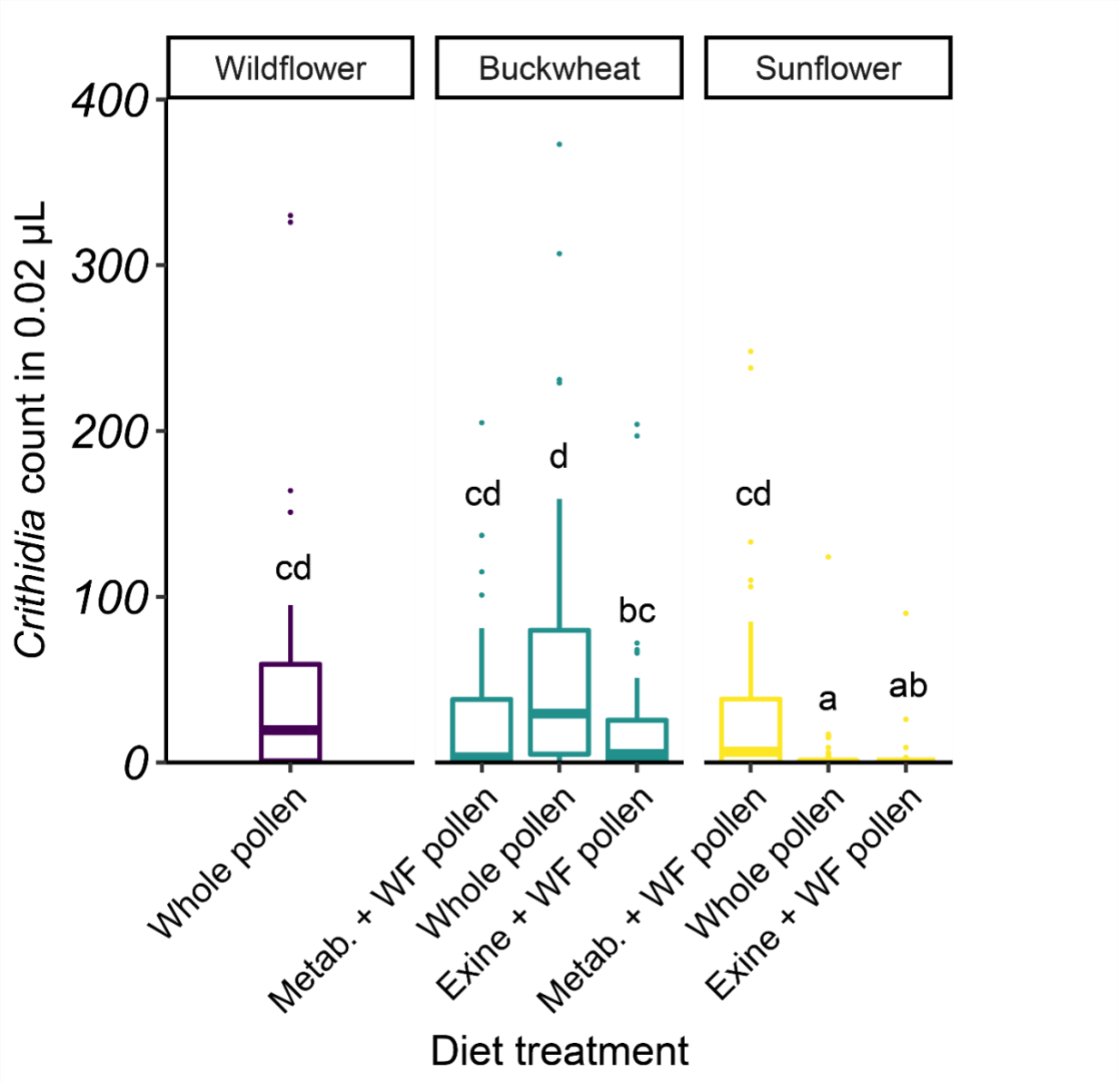
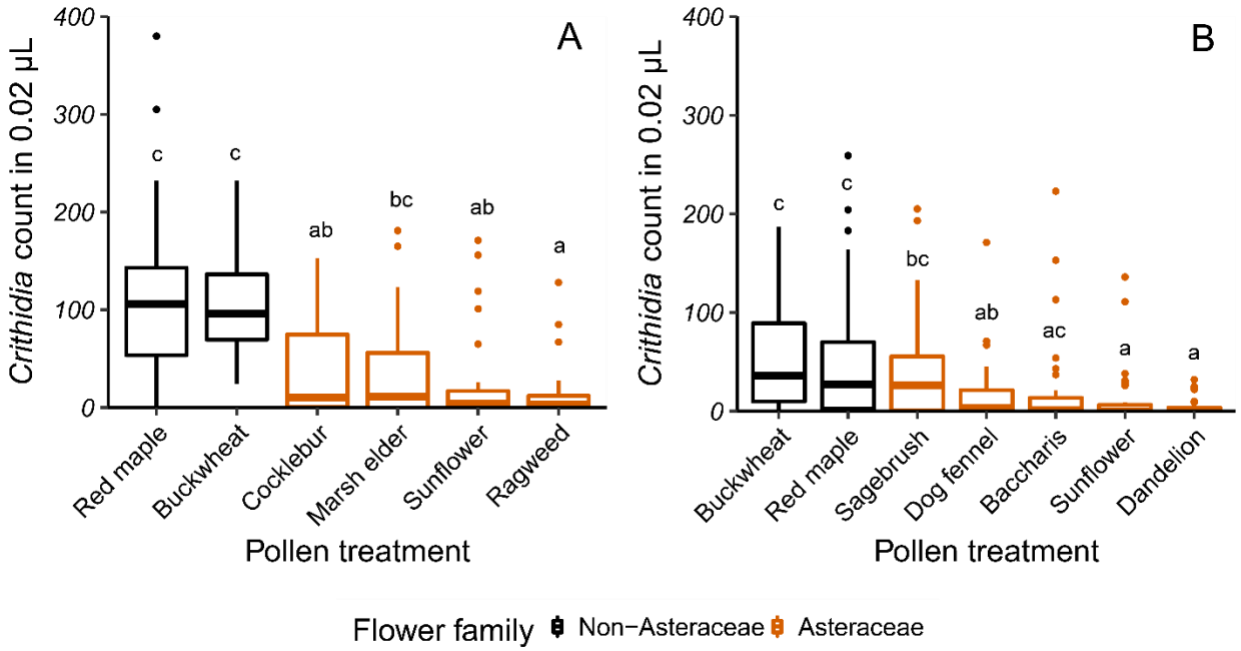


Figure 2. Boxplots showing effect of pollen species treatment on *C. bombi* counts in bees one-week post-inoculation at A) Lab1 and B) Lab2. All pairwise comparisons between pollen species can be found in Tables S5; data analyzed separately for each institution. Letters above bars indicate significant differences (Table S5).



946 **Figure 3.** SEM images of pollen from plant species used in experiments. A) buckwheat
947 (*Fagopyrum esculentum*; Polygonaceae), B) red maple (*Acer rubrum*; Sapindaceae), C)
948 sagebrush (*Artemisia tridentata*; Anthemideae, Asteraceae), D) ragweed (*Ambrosia*
949 *artemisiifolia*; Heliantheae, Asteraceae), E) dog fennel (*Eupatorium capillifolium*; Eupatorieae,
950 Asteraceae), F) dandelion (*Taraxacum officinale*; Cichorieae, Asteraceae), G) cocklebur
951 (*Xanthium strumarium*; Heliantheae, Asteraceae), H) marsh elder (*Iva annua*; Heliantheae,
952 Asteraceae), I) baccharis (*Baccharis halimifolia*; Astereae, Asteraceae), and J) sunflower
953 (*Helianthus annuus*; Heliantheae, Asteraceae). Spine lengths in Table S2.

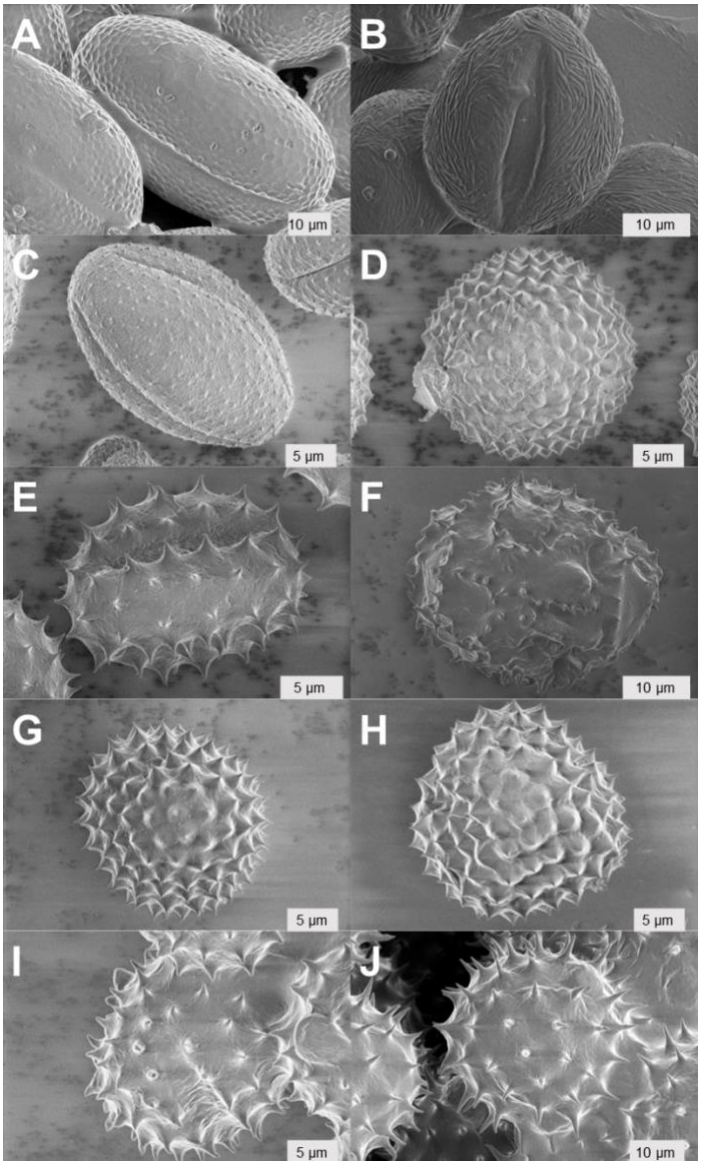


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Figure 4. Correlation between pollen spine length and *C. bombi* counts, standardized by counts in bees fed buckwheat pollen (BW). There is one data point for sunflower and for red maple even though those species were screened in both institutions (one institution randomly selected to represent each species). The confidence interval corresponds to standard error. Dashed line indicates that P > 0.05.