October 2018

Plants, Parasites, and Pollinators: The Effects of Medicinal Pollens on a Common Gut Parasite in Bumble Bees

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PLANTS, PARASITES, AND POLLINATORS: THE EFFECT OF MEDICINAL POLLENS ON A COMMON GUT PARASITE IN BUMBLE BEES

A Thesis Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2018

Wildlife, Fish, and Conservation Biology

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DEDICATION

To the women and men breathing heavy smoke and digging fireline, we are still tied in.

ACKNOWLEDGMENTS

To all of those who supported me when support was needed. Thank you very much.

ABSTRACT

PLANTS, PARASITES, AND POLLINATORS: THE EFFECT OF MEDICINAL POLLENS ON A COMMON GUT PARASITE IN BUMBLE BEES

SEPTEMBER 2018

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Declines in several pollinator species are due to a variety of factors, including pathogens. Incorporating pollinator-friendly plant species into wild and agricultural habitats could reduce the stress of pathogens if food sources act medicinally against pathogens. Previous research demonstrated two domesticated sunflower cultivars (*Helianthus annuus*) can dramatically reduce a gut pathogen (*Crithidia bombi*) of the common eastern bumble bee, *Bombus impatiens*. To ascertain the breadth of this medicinal trait, we tested whether pollen from several *H. annuus* cultivars and four relatives could also reduce *C. bombi* infections in *B. impatiens*. We also investigated whether timing of exposure to sunflower pollen relative to time of infection affected the strength of this medicinal trait. In all experiments, bees were infected and then fed their respective pollen diets for a week before they were dissected to assess infection. In our first experiment, all pollen from *Helianthus* species and relatives reduced *C. bombi* cell counts compared to our single species control of buckwheat (*Fagopyrum esculentum*). In our timing of exposure experiments, a one-time exposure to sunflower pollen present at the time of infection did not lower infection levels. In longer exposure trials, sunflower pollen suppressed *C. bombi* infection with a strength inversely proportional to the time

between treatment and infection. Our results suggest that medicinal pollen may be widespread in the *Helianthus* genus and potentially throughout the Asteraceae family. Thus, these results provide insights into how strategic plantings of certain floral resources can help mediate and influences pollinator disease dynamics.

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CHAPTER 1

MEDICINAL EFFECT OF MULTIPLE SUNFLOWER POLLEN CULTIVARS AND SPECIES ON A BUMBLE BEE GUT PATHOGEN

1.1 Introduction

Pollination services are critical in ecological and agricultural systems. In the United States, up to 90 crops are pollinated by bees (Kremen et al. 2002) and worldwide, pollinators pollinate about one third of food crops (Gallai et al. 2009). Pollinators also fill important ecological niches by aiding wild plant reproduction, contributing to the maintenance of a diverse landscape (Biesmeijer et al. 2006, Ollerton et al. 2011). But since the turn of the 21st century, several pollinator taxa have declined, including some bee species (Cameron et al., 2011; Colla & Packer, 2008; Potts et al., 2010). With mounting concerns about these declines (Goulson, Nicholls, Botías, & Rotheray, 2015; Vanbergen, 2013), research on pollinator diseases and their potential mitigation has become a pressing need (National Research Council, 2007).

Most bees rely solely on nectar and pollen as food sources, obtaining lipids and proteins from pollen and sugars from nectar (Nicolson, 2011). Wildflower gardens and pollinator strips along agricultural lands are receiving increased attention as mechanisms to provide foraging habitat and nesting sites for pollinators (Carvell, Meek, Pywell, Goulson, & Nowakowski, 2007). Flowers can provide not only nutritional benefits, but also play a role in mediating bee disease dynamics. Some floral rewards have medicinal properties that can suppress parasites (Baracchi et al. 2015), suggesting potential benefits if these species are planted in wildflower gardens or pollinator strips. Thus, identifying

plants with floral rewards that suppress pathogens could provide non-chemical options to improve pollinator health by incorporating target plant species into agroecosystems and natural habitats.

Studies of sunflower floral rewards (*Helianthus annuus* L.; Asteraceae) indicate that they may play a significant role in pathogen suppression. When compared to other monofloral pollen diets and a pollen mix, two varieties of cultivated sunflower (*Helianthus annuus* L.; Asteraceae) pollen dramatically suppressed *Crithidia bombi* (Kinetoplastea, Trypanosomatida) in *Bombus impatiens* (Cresson), and had less dramatic but still significant effects reducing the pathogen *Nosema ceranae* in honey bees, *Apis mellifera* (Giacomini et al., in review). This discovery is consistent with limited other research suggesting that floral rewards from sunflower and related taxa have medicinal properties for bees. For example, ingestion of sunflower honey, which is made of primarily nectar with some pollen, reduced the fungal parasite *Nosema ceranae* and increased survival in honey bees (Gherman et al. 2014). Additionally, some solitary bees are specialists on Asteraceae pollen (Praz et al. 2008) and it has been suggested that in *Osmia*, this may be due to pollen reducing parasitoid larval growth (Spear et al. 2016). These discoveries suggest that sunflower and possibly broader Asteraceae pollen have medicinal effects that could help bees resist pathogens or parasites, but the extent of this effect across plant taxa is unknown.

Bumble bees (*Bombus* spp.) are widespread in temperate regions, and several species have undergone dramatic declines in recent decades (Cameron et al., 2011). The causes of pollinator declines are complex and multifaceted (Goulson et al. 2015), and some declines might be associated with pathogen pressure (Schmid-Hempel et al. 2014,

Goulson et al. 2015). One pathogen, *Crithidia bombi* can reduce *Bombus terrestris* early colony growth rate and successful emergence of hibernating queens (Brown et al. 2003, Fauser et al. 2017), reduce the production of new daughter queens (Goulson et al. 2017), and interacts with starvation to increase mortality by 50%(Brown et al. 2000). Furthermore, *C. bombi* reduced *Bombus impatiens'* motor learning rates of flower handling, and foraging rates (Otterstatter et al. 2005), potentially reducing pollination and foraging efficiency. The effects of *C. bombi* on bumble bee physiology and behavior suggest this pathogen may contribute to some bumble bee declines.

The goal of our study was to assess whether pollen from multiple cultivars and wild populations of *H. annuus,* its congeners, and Asteraceae relatives significantly reduced *C. bombi* in *B. impatiens*. Additionally, because previous studies have relied on honey bee-collected pollen we compared the effects of hand- and honey bee-collected pollen. Honey bees use nectar to collect and transport pollen (Thorp 1979, Roulston and Cane 2000) and honey bee collected pollen also contains salivary enzymes (Standifer et al. 1980, Mărgăoan et al. 2010). Since pollen collected by honey bees includes some nectar while hand-collected pollen does not, comparing hand- and honey bee-collected pollen allowed us to ascertain whether medicinal properties are due to pollen, nectar, or both.

1.2 Methods

1.2.1 Study system

The common eastern bumble bee (*Bombus impatiens*) is a eusocial generalist pollinator with an annual colony life cycle (Wilson 1971) that can produce up to 400 workers (Cnaani et al. 2002). *B. impatiens* is commonly found in eastern North America (Williams et al. 2014) and colonies are commercially available. The intestinal parasite *Crithidia bombi* is found in wild *B. impatiens* populations in eastern North America and in up to 80% of bumble bee workers in western Massachusetts populations (Gillespie 2010). *Crithidia bombi* is transmitted horizontally during floral visitation (Durrer and Schmid-Hempel 1994), and in the hive from one generation of workers to the next via contact with fecal deposits (Imhoof and Schmid-Hempel 1999). Sunflower (*Helianthus annuus)* is a common early successional, self-compatible annual forb native to central North America (Reagon and Snow 2006) that is grown commercially for its oilseed and as a cover crop (USDA, 2015).

1.2.2 Plant sources and cultivation

We used pollen from nine *H. annuus* cultivars, four populations of wild *H. annuus*, two *Helianthus* congeners, two *Solidago* species and two controls (buckwheat, *Fagopyrum esculentum,* and a honey-bee collected wildflower mixed pollen). Hereafter, all 19 pollen treatments will be referred to as 'taxa' for simplicity. Pollen from most taxa was collected from plants grown from seed obtained from the USDA Agricultural Research Service through the North Central Regional Plant Introduction Station, which is part of the U.S. National Plant Germplasm System program. The seeds were sown at the

College of Natural Science's greenhouses at the University of Massachusetts-Amherst, and were grown at the Crop and Animal Research and Education Center in South Deerfield, Massachusetts. For detailed propagation and sowing information, see Appendix 1. We also collected pollen from three taxa outside our field site: *H. annuus* 'Cobalt II' cultivar and *H. annuus* 'Black Oil Seed' cultivar from farms and *Solidago* spp., which grew wild (Appendix 1, Table S1). For the species we didn't grow, we used DNA barcoding following established protocols (Bell et al. 2017) to confirm identity. Both yellow and orange-colored *Solidago* pollen had 96% and 97% matches with *Solidago rugosa* and *Solidago canadensis*. Because both pollen colors had high potential matches with both *Solidago* species, we will refer to these taxa as '*Solidago* yellow' and '*Solidago* orange' and both taxa will be considered a mix of both potential species. Both the yellow and orange colored pollen from *H. annuus* 'Cobalt II' and *H. annuus* 'Black Oil Seed' were 96-100% matches with *H. annuus.* We tested yellow and orange pollen separately for both taxa and will refer to them by their cultivar name and their color, yellow or orange. Buckwheat and one source of sunflower pollen used in our original research (Giacomini et al., in review) were obtained from Changge Hauding Wax Industry, China, and the wildflower mix pollen was obtained from Koppert Biological Systems (Linden Apiaries, Howell, Minnesota USA). We used single species of multiple sunflower taxa and relatives compared to buckwheat because buckwheat has a similar 'low quality' protein content and have a similar amino acid percentage (Yang et al. 2013).

1.2.3 Pollen collection and preparation

We collected pollen by hand and with honey bees and brought it to the lab every day (Monday – Friday) for storage at -20˚C until trials began. For most cultivars, we were only able to use one method or the other due to logistical constraints. However, we collected three *H. annuus* taxa (Black Oil Seed, Germany, and wild California) using both methods, allowing direct comparisons between collection methods within taxa. Hand-collected pollen was collected using paint brushes to brush pollen into aluminum foil envelopes and then stored in the freezer inside 5 ml plastic vials. Honey bee-collected pollen was collected using pollen traps (Mann Lake Ltd. Hackensack, Minnesota, USA) in honey bee hives from the UMass-Amherst Apiary. Traps were checked and pollen was collected on weekdays from July to October 2016. The pollen from all the wild *H. annuus* and the 'Dwarf' cultivar were honey bee-collected inside 3.6m x 9.7m x 3m tents (Appendix 1, Figure S1A, B) made of polyester fabric (Osgood Textile, West Springfield, Massachusetts, USA) and fine mesh cloth (Phifer Incorperated, AL, USA) with a metal frame (Delta Canopies, McKinney, TX, USA), which excluded other pollinators. Due to logistics, we did not use tents for all taxa grown at the field station. *Solidago* spp., *H. annuus* 'Cobalt II', and *H. annuus* 'Black Oil Seed' were present at natural sites or commercial farms, and were honey bee-collected without tents. Pollen collected from *Solidago* spp. and *H. annuus* 'Cobalt II' sites also contained pollen from other species. We sorted and excluded pollen from other species, and separated the yellow and orange pollen from our taxa in case colors indicated chemical differences that could affect *C. bombi* infection. We also note that *H. annuus* 'White' had white pollen, while all other taxa had yellow or orange pollen.

Before starting the trials, pollen was mixed with a 30% 1:1 glucose:fructose sugar solution, reflecting the concentration and sugar ratios in *H. annuus* nectar (Neff and Simpson 1990, Mateo and Bosch-Reig 1997). The ratio of sugar solution to pollen was different between honey bee- and hand-collected pollen because hand-collected pollen contained no nectar, and thus needed more liquid to reach the same consistency. The ratio of solution to pollen was adjusted for each pollen type to accomplish a dough-like consistency similar across all taxa. For hand-collected pollen, we added 43-47% sugar solution by weight, compared to 7-24% sugar solution added to the honey bee-collected pollen. Honey bee-collected pollen can contain up to 40% more sugars by weight than hand-collected pollen (Todd and Bretherick 1942, Roulston and Cane 2000), which roughly corresponds to the 20-40% more sugar solution added to hand compared to honey bee-collected pollen in our experiment.

1.2.4 Inoculum preparation

Crithidia bombi were maintained in commercial *B. impatiens* 'source' colonies (Biobest Canada, Leamington, Ontario, Canada) originally infected with *C. bombi* from wild *B. impatiens* workers collected at Stone Soup Farm in Hadley, MA (42°21'51.93"N 72°33'55.88"W). Every day that we inoculated bees, we prepared fresh *C. bombi* inoculum from 5-10 source colony workers. Inoculum was prepared by grinding mid- and hindguts in 1.5 mL Eppendorf tubes with 300 μL of ¼ strength Ringer's solution (Fluka 96724, Sigma-Aldrich, St. Louis Missouri, USA) with a plastic rod. The solution was then vortexed for five seconds, and allowed to settle for 4-5 hours at room temperature. After the solution settled, 10 μL samples of the supernatant were placed on a

hemocytometer. Using a compound light microscope at 40x magnification, we counted and summed moving *C. bombi* cells from the corner and central squares of the hemocytometer grid, a total of 0.02 μL volume. We then used 150 μL samples from 1-3 bees to make a mixture diluted with Ringer's solution to achieve 1200 *C. bombi* cells/ μl. This solution was mixed with an equal amount of 50% sucrose solution to prepare an inoculum with 600 *C. bombi* cells/μl in 25% sucrose, as described in Richardson et al (2015).

1.2.5 Laboratory trials

During the spring and summer of 2017, workers were isolated from commercially reared laboratory colonies that were confirmed to be free of *C. bombi* via biweekly subsamples of five bees. In total, 17 colonies were used. Before inoculation, worker bees were isolated in small vials and starved for 2-3 hours. Bees were inoculated individually with 10μl of fresh *C. bombi* inoculum made according to the protocol described above. Bees were randomly assigned to one of the 19 different pollen treatments and housed individually in plastic 500 mL deli cups with approximately 50 mg (range 40 – 70 mg) of their treatment pollen and 10 mL of the 30% sugar solution, made available by a cotton wick through a hole cut into the top of a 95 mm petri dish. Experimental bees were stored in the dark at 27˚C in an incubator. Pollen and sugar solutions were replaced every other day. After seven days, bees were dissected and *C. bombi* was counted as described in '*Inoculum preparation*' above. Additionally, radial cell length from the right forewing was measured as a proxy for bee size (Harder 1982). A total of 650 worker bees were used in the experiment (Appendix A, Table S1, for treatment sample sizes). In addition,

253 bees died before the end of the 7-day trials, 37 escaped and 13 had damaged wings. Bees that died, escaped or had damaged wings were not used in our analysis.

1.2.6 Statistical analysis

To examine the effects of pollen treatment on *C. bombi* raw cell counts (cells per 0.02 μL) in each bee after a seven-day pollen diet, we used the statistical computing and graphical environment R version 3.3.1 (R Core Team, 2017). We used generalized mixed linear models. Due to the nature of our zero bounded data, we first tested the residuals with a Poisson distribution and checked for over-dispersion. Finding that the data were over-dispersed, we analyzed data with a negative binomial error distribution with a log link function using the package *lme4* (Bates, Maechler, Bolker, & Walker, 2015), and calculated least-squares means and standard errors with the package *lsmeans* (Lenth, 2016). In addition to the predictor of pollen treatment and response of *C. bombi* cell count we used the fixed covariate of bee size (estimated by radial cell length) and random effect covariates of date of inoculation and colony of origin. We tested the significance of each covariate with the *drop1* function, which compares the goodness of fit between models with and without the terms under consideration. Upon finding a significant overall effect of pollen treatment, we compared differences between pollen treatments using a Tukey's HSD post hoc test. In a separate analysis, we asked whether species differed in ability to reduce *C. bombi* by pooling pollen treatments into their respective species (*H. annuus*, *H. petiolarus*, *H. argophyllus*, *Solidago* spp) and using species instead of pollen taxa as a fixed predictor, with random effects of inoculation date and colony of origin and fixed effect of bee size. We also asked whether hand-collected vs.

honey bee-collected pollen differed in ability to reduce *C. bombi* cell counts using a similar analysis, but with collection method as the predictor instead of species. Finally, we used a survival analysis with the package *survminer* (Kassambara & Kosinki, 2018) to examine whether pollen treatment affected mortality rates. To evaluate if the 19 pollen treatments had an effect on the individual bee survival, we compared our model with and without pollen treatment as the predictor. We removed 50 bees that escaped or had wing damage from our survival analysis. Figures were made with *ggplot2* (Wickham, 2009) and *cowplot* (Wilke, 2016) packages.

1.3 Results

All *Helianthus* and *Solidago* pollen treatments decreased *C. bombi* cell counts in *B. impatiens* when compared to buckwheat pollen (Figure 1.1). Compared to the wildflower pollen mix, all but three taxa (*H. annuus* 'Germany' hand-collected, *H. annuus,* 'wild California' honey bee-collected and *H. petiolaris*) significantly decreased *C. bombi* cell counts. Most bees treated with *Helianthus* and *Solidago* taxa had similarly low *C. bombi* cell counts, but those reared on *Solidago* (yellow) and *H. annuus* 'Germany' (honey bee collected) had lower cell counts than some others, such as *H. annuus* 'wild California' hand collected and *H. annuus* 'Cobalt II' (orange) (Figure 1.1). In the survival analysis, neither pollen treatment ($\chi^2_{(18)} = 5.59, p = 0.997$) nor bee size ($\chi^2_{(1)} = 5.59, p = 0.44$) affected survival. In our all pollen taxa analysis, there was a negative relationship between bee size and *C. bombi* counts ($\chi^2_{(1)} = 16.49, p < 0.001$).

When we pooled taxa by species (*H. annuus*, *H. petiolaris*, *H. argophyllus*, *Solidago* spp), species did not differ in their effects on *C. bombi* counts in a post-hoc

Tukey's HSD test. We collected pollen both by hand and with honey bees for three taxa (*H. annuus* 'Black Oil Seed', 'Germany', and 'wild California'), allowing us to make direct within-species comparisons between collection methods. There were significant effects of collection method on *C. bombi* cell counts in two of the three direct comparisons, but in opposite directions. Honey-bee collection increased *C. bombi* cell counts relative to hand collection in *H. annuus* 'Black Oil Seed' ($\chi^2_{(1)} = 24.5$, $p < 0.001$, Figure 1.2A) but decreased *C. bombi* counts in *H. annuus* 'Germany' ($\chi^2_{(1)} = 6.26$, $p = 0.012$, Figure 1.2B), and collection method had no effect in *H. annuus*, 'wild California' ($\chi^2_{(1)} = 0.40$, $p = 0.5246$, Figure 1.2C). We also grouped the 17 Asteraceae pollen taxa by collection method in an overall comparison, and found no statistically significant difference between collection methods on *C. bombi* counts ($\chi^2_{(1)} = 0.95$, $p = 0.33$, Figure 1.2D).

1.4 Discussion

Pollen from a wide variety of sunflowers suppressed the bumble bee gut pathogen *C. bombi* when compared to buckwheat pollen and a wildflower mixed pollen as controls. Bees fed buckwheat pollen had a 20- to 40-fold increase in *C. bombi* cells compared to those that consumed *Helianthus* spp and *Solidago* spp pollen. Our results greatly extend previous work showing that sunflower pollen from two *H. annuus* cultivars dramatically reduced *Crithidia* (Giacomini et al., in review), and are consistent with other studies showing that sunflower honey reduced *Nosema* infection in *Apis mellifera* (Gherman et al. 2014) and that Asteraceae pollen may protect *Osmia* bee larvae against brood parasites (Spear et al. 2016). The current study provides a much wider range of options for using

sunflower pollen as a food supplement for managed bees. Our previous work suggested the potential for sunflower pollen to become an important component of pollinator health management plans because, in addition to reducing *C. bombi* in bumble bees, it also reduced the fungal parasite *Nosema ceranae* in honey bees, *Apis mellifera* (Giacomini et al., in review). In addition, the intensity of *C. bombi* infection was lower in wild-caught workers when agricultural lands had more sunflower acreage. By growing most of our own pollen rather than purchasing it commercially, our study removed potential variation due to different land use practices, such as the application of fungicide or other pesticides that can affect bee health (Bernauer et al. 2015) and resistance to pathogens (Pettis et al. 2013, McArt et al. 2017).

Although a wide range of sunflower pollen dramatically reduced *C. bombi* infection in our study, sunflower pollen has low protein concentrations compared to other types of pollen (Nicolson & Human, 2013). Pollen with low protein can have multiple negative effects on bees, such as reducing hypopharyneal gland size in honey bees (Pernal and Currie 2000), larval weight in *Bombus terrestris* (Tasei and Aupinel 2008), sweat bee offspring weight (Roulston & Cane, 2002), and immune function in honey bees (Rasmont et al. 2005, Brunner et al. 2014). Although we found no differences in individual bee mortality when fed sunflower, buckwheat or wildflower mixed pollen, we recommend that future work should compare the benefits and costs of sunflower pollen on bee performance, including reproduction, and ascertain the proportion of sunflower pollen in the diet that maximizes medicinal benefits while minimizing nutritional stress.

Bumble bees are generalists that typically consume a polyfloral diet, and so the comparison of our sunflower taxa to the wildflower mix is more ecologically relevant

than the comparison to buckwheat pollen. A polyfloral diet provides varying amounts of amino acids, protein, and macronutrients compared to a monofloral diet (Schmidt et al. 1995, Alaux et al. 2010). Pollen macronutrient composition affected foraging preferences in *Bombus impatiens* (Vaudo et al. 2016) and amino acid composition affected colony growth in *Bombus terrestris* (Kämper et al. 2016). Furthermore, honey bees infected with *Nosema ceranae* had improved longevity when fed a polyfloral pollen diet compared to a monofloral diet (Di Pasquale et al., 2013). Thus, pollen resource composition is potentially important when managing lands to optimize bee health and resistance to pathogens.

In the temperate regions of North America, *Helianthus* spp. and *Solidago* spp. are common native plants (Werner et al. 1980, Reagon and Snow 2006) that are in different tribes within the family Asteraceae (Bremer 1987). Because *Solidago* is in a different tribe than *Helianthus* but was equally effective at reducing *C. bombi*, it is possible that medicinal pollen is more widespread in the Asteraceae. Because Asteraceae are common components of many habitats and often bloom in mid to late summer in temperate North America, this result could have important implications. By reducing parasite infections, these plant species could reduce one of the stressors affecting bumble bee populations. In *Bombus terrestris*, high *C. bombi* infection is negatively correlated with daughter queen emergence in wild colonies (Goulson et al. 2017) and high infections can reduce early colony development by 40% when queens emerge from hibernation in spring (Brown et al., 2003). Because *Solidago* spp. bloom in late summer and autumn, infected daughter queens could have an advantage if they forage on these floral resources before entering winter hibernation.

Because previous work examined the medicinal effect of honey bee-collected sunflower pollen or sunflower honey (Giacomini et al. in review, Gherman et al. 2014), both of which contain nectar and pollen, these previous studies could not conclusively determine whether medicinal effects were due to pollen, nectar, or both. We compared the medicinal effect of hand- vs honey bee-collected pollen to ascertain whether the likely mechanism is due to a component of pollen or nectar. Surprisingly, in comparisons of hand- and honey bee-collected pollen within taxa, we found opposite results for different taxa. Within our three comparisons we found all possible results: honey bee-collected pollen resulted in more *C. bombi* (Figure 1.2A), less *C. bombi* (Figure 1.2B), or no effect (Figure 1.2C) compared to hand-collected pollen. In a larger comparison including all taxa, most of which were collected with only one of the two methods, there was no significant difference (Figure 1.2D). Because we did not consistently find that honey bee-collected pollen (which contains nectar) reduced *C. bombi* counts relative to hand-collected pollen (which does not contain nectar), overall our results suggest that the main mechanism of reduced infection is due to some component of pollen rather than nectar.

Although most of our taxa had typical yellowish-orange pollen, some of our taxa produced pollen in distinct colors of yellow (*Solidago* spp, and *H. annuus* 'Cobalt II'), orange (*Solidago* spp, *H. annuus* 'Cobalt II', and *H. annuus* 'China') or white (*H. annuus* 'white'). *Solidago* spp and *H. annuus* 'Cobalt II' produced both yellow- and orange-colored pollen, which were separated into two treatments (Supplimental inforamtion Table 1). We hypothesized that pigments might play a role in *C. bombi* suppression, since pigments are known to be biologically active and affect herbivores and bacteria

(Gronquist et al. 2001, Kagithoju et al. 2012). For example, in *Petunia* hybrid flowers with white and blue petal sections, the white part of the petal was consumed more than the blue part by two generalist caterpillars, and larvae gained more weight feeding on white than blue tissue (Johnson et al. 2008). This suggests that the anthocyanin pigments deterred herbivory and reduced herbivore growth. We found no support for the hypothesis that pollen color affects *C. bombi* counts, suggesting pigments did not play a significant role in suppression. Yellow and orange pollen did not differ within taxa, and *H. annuus* with white pollen did not differ from taxa with yellow or orange pollen in reducing *C. bombi* (Figure 1.1).

Furthermore, while our results clearly demonstrate a substantive effect, the mechanism is unknown. Future research should address whether the medicinal quality of sunflower pollen is due to secondary chemistry, nutritional components, or another mechanism, such as physical attachment of pollen to the parasite or the gut wall, preventing *C. bombi* from adhering to the gut wall (Gorbunov 1996). Previous studies have shown that nectar secondary chemistry suppresses *C. bombi* (Thorburn et al. 2015, Richardson et al. 2015) and honey bee immunity can be stimulated by the ingestion of some honeys (Mao et al. 2013). Pollen proteins could also play a role. For example, the ragweed (*Ambrosia artemisiifolia*) pollen coat proteins trigger histamine production in humans as a defense response (Munshi 2000). Future work is needed to determine whether sunflower and goldenrod pollen contain immune stimulants that induce up-regulation of genes that reduce infection. A limitation of this study is the use of bumblebees from commercial hives bred for generations in captivity. Commercial rearing may influence how the immune system responds to pathogens, although *C. bombi* is

present in commercial hives (Colla et al. 2006). The effect of sunflower pollen should be tested on wild *B. impatiens* and other bee species to investigate whether the medicinal effects are consistent across bee taxa, given that *C. bombi* infects various *Bombus* species (Gillespie 2010, Malfi and Roulston 2014).

Plants have provided humans with medicine for centuries, and our work suggests that insights about medicinal pollen for bees may be relevant to human drug discovery. Four plant species within the Asteraceae family suppressed *C. bombi* in *B. impatiens* compared to buckwheat pollen. In addition, pollen from *Artemisia annuua,* in the same family as *Helianthus annuus* and *Solidago* spp., suppressed *Trypanosoma cruzi* and *Trypanosoma brucei* in vitro (Mishina et al. 2007). Furthermore, the plant compound artemisinin, which is also found in *A. annuua,* can be used as an anti-malarial drug (Klayman 1985). The potential to use insect model systems to guide searches for human medicines from these plant species should be considered, particularly for human diseases that are vectored by insects. These studies suggest that the plant family Asteraceae has medicinal qualities for both humans and insects that should be investigated in more depth.

Whatever the mechanism of suppression, we found that sunflower and goldenrod pollen dramatically reduced the parasite *Crithidia bombi* in *Bombus impatiens*, compared to both a single-species pollen control and wildflower pollen mix. This study suggests that in addition to using sunflower and goldenrod to manage bee health in agroecosystems, these native North American species could be incorporated into natural ecosystems to manage *C. bombi* infection in *B. impatiens*. Future work should address the

breadth of this effect for additional bee species and pathogens to make responsible recommendations for management practices.

CHAPTER 2

THE TIMING OF DOSAGE RELATIVE TO INFECTION

2.1 Introduction

Pollinators are crucial components in agriculture and for maintaining diverse plant communities. Pollinators are important for yield of up to 90 crops in the United States, and about one third of crops globally (Gallai et al. 2009). Bumble bees are used for pollination services in greenhouses and in agricultural fields where they can, along with other native pollinators, complement and often exceed honey bee pollination services (Winfree et al. 2007, Russo et al. 2013). However, some pollinator species are in decline (Potts et al. 2010, Hallmann et al. 2017). Pollinator decline is thought to be caused by several factors (Goulson et al. 2015), including pesticides (Pettis et al. 2012), fungicides (McArt et al. 2017), habitat degradation (Goulson et al. 2008), land use change (Potts et al. 2010), and pathogens (Meeus et al. 2011). These declines underscore the importance of developing methods to effectively combat stressors such as pathogens.

Managed and wild bumble bees are both affected negatively by pathogens (Murray et al. 2013, Graystock et al. 2014). For example, newly emerged *Bombus terrestris* queens infected with the fungal pathogen *Nosema* spp. produce less sexual offspring and smaller colonies (Otti and Schmid-Hempel 2008). *Crithidia bombi* (Kinetoplastea, Trypanosomatida; (Lipa, and Triggiani 1988) is a common pathogen of multiple bumble bee species (Gillespie 2010) with a range of effects on hosts. Wild *B. terrestris* colonies infected with *C. bombi* are less likely to produce new daughter queens compared to uninfected colonies (Goulson et al. 2017). *Crithidia bombi* reduced motor learning rates of flower handling in *B. impatiens* (Gegear et al. 2005) which could

influence how effectively bumble bees pollinate and forage (Koch et al. 2017). Furthermore, mortality rates of *C. bombi* infected *B. terrestris* under food-limited conditions were 50% higher than infected bees with sufficient resources (Brown et al. 2000). Thus, diet can interact with pathogen infection to alter the severity of outcomes.

In addition to quantity, the quality of pollinator diet can be important for mediating bee resistance to pathogens. For example, a polyfloral pollen diet reduced *Nosema ceranae* (Zander) infections and improved longevity in infected honey bees (*Apis mellifera* L.; Apidae) compared to a monofloral diet (Alaux et al. 2010, Di Pasquale et al. 2013). Several nectar secondary compounds reduced *C. bombi* in *B. impatiens* (Manson et al. 2009, Richardson et al. 2015, Baracchi et al. 2015), indicating that floral chemistry may play important roles mediating bee disease, but see (Palmer-Young et al. 2017) for conflicting results. In addition, sunflower (*Helianthus annuus* L.; Asteraceae) honey reduced *Nosema ceranae* in honey bees compared to other types of honey (Gherman et al. 2014). Furthermore, sunflower pollen reduced two pathogens in two different hosts, *C. bombi* in *B. impatiens* and *N. apis* in honey bees (*Apis mellifera*) (Giacomini et al. in review). These studies suggest that floral rewards in pollinator diet affect pathogen prevalence and influence disease dynamics. However, sunflower pollen is low in protein and amino acids, and so it may be advantageous to only supply as much as necessary to effectively manage pathogens, and therefore to determine when exposure to sunflower pollen will be most effective, as well as how much is necessary to suppress infection.

In epidemiology, the time between when a subject is exposed to an infectious agent and the administration of medication can affect the outcome. For example, disease

was prevented in monkeys when medicinal treatment was administered within 36 hours of infection with human immunodeficiency virus (Otten et al. 2000) and with simian immunodeficiency virus (Tsai et al. 1995). Furthermore, mice experienced decreased lung inflammations if they were medicated within 48 hours after inhaling the irritant ragweed pollen (Sur et al. 1996). In these examples, the timing of medicinal dosage mattered, and early intervention reduced or prevented irritation or infection better than late intervention. However, with insect disease dynamics we know very little about how timing of exposure to potentially medicinal floral rewards affects resistance to pathogens. Here, we investigate how the interval between exposure relative to treatment affects a common bumble bee pathogen.

We used two experiments to ask if the time between infection and receiving a dose of medicinal sunflower pollen affected *C. bombi* prevalence in *B. impatiens*. First, we asked if a one-time exposure to sunflower pollen at the time of infection would reduce *C. bombi* prevalence in single-bout foraging trials. Second, in laboratory trials we asked if the amount and timing of sunflower pollen consumption relative to time of infection affects pathogen prevalence. We hypothesize that early exposure and longer exposure to medicinal sunflower pollen will prevent the infection more effectively than later exposure or no exposure.

2.2 Methods

2.2.1 One-time exposure to sunflower pollen at time of infection

2.2.1.1 Inoculum preparation

For both experiments we used *Crithidia bombi* from wild *Bombus impatiens*

workers collected in Massachusetts at Stone Soup Farm in Hadley (42°21'51.93"N 72°33'55.88"W) that were used to infect commercial *B. impatiens* colonies (Biobest Canada, Leamington, Ontario, Canada) in the laboratory. Infection was transferred to new commercial colonies when the previous colony began to decline. On days bees entered their experiments, fresh *C. bombi* inoculum was prepared by dissecting 5-10 workers. We placed guts into separate 1.5 mL Eppendorf tubes mixed with 300 μL of ¼ strength Ringer's solution (Fluka 96724, Sigma-Aldrich, St. Louis Missouri, USA), which we then homogenized with a plastic rod, vortexed for five seconds, and settled for 3-4 hours at room temperature. After guts settled, a clear 10 μL sample of supernatant was placed into a hemacytometer. Using a compound light microscope at 40x magnification, moving *C. bombi* cells in the corner and central squares of the hemocytometer grid were counted and summed, a total of 0.02 μL volume. We then combined multiple 150 μL samples from 1-4 bees and diluted with ¼ strength Ringer's solution to obtain a solution with 1200 *C. bombi* cells/μl. This solution was mixed with an equal amount of 50% sucrose solution to prepare an inoculum with 600 *C. bombi* cells/μl in 25% sucrose, as described in Richardson et al (2015).

2.2.1.2 Plant cultivation

We obtained *Helianthus annuus* seeds from the United States Department of Agriculture Agricultural Research Service through the North Central Regional Plant Introduction Station, part of the U.S. National Plant Germplasm System program. They were sown in SUNGRO Horticulture medium (Sun Gro Horticulture Canada Ltd., Seba Beach, AB TOE 280, Canada) in 50 plug trays in a greenhouse until transplanted to the

Crop and Animal Research and Education Center in South Deerfield, MA (42°28'51.93"N 72°34'55.88"W). We grew 100 cytoplasmic male sterile (CMS), non-pollen producing plants and 100 pollen-producing plants (non-CMS), from each of three cultivars (89 and CMS-89, 236 and CMS-236, and 404 and CMS-404; see Appendix B, Table S1 for propagation information), for a total of approximately 600 plants. The numbers 89, 236, 404 refer to the U.S. National Plant Germplasm System database ID plots where the seeds were grown. Thus, we used male-sterile (CMS) or pollen-producing (non-CMS) lines as the treatment within each of three cultivars to assess generality of our results.

2.2.1.3 Transmission trials

To determine how pollen exposure during infection affects *C. bombi* transmission, we allowed bumble bees to forage on *H. annuus* flowers that we hand-inoculated and then determined bee infection status after one week. We performed field trials comparing transmission of *C. bombi* on non-pollen producing inflorescences (CMS) to pollen-producing inflorescences of three cultivars of *H. annuus*. All inflorescences were enclosed with mesh bags for 48 hours prior to trials to prevent pathogen contamination from wild bee visitors. We harvested each inflorescence from the field immediately before the trial began and placed the stem in a florist's tube with distilled water in a Styrofoam holder. Before the beginning of each trial, we visually divided the inflorescence head (capitulum) into quarters, and a 10 μL *C. bombi* inoculum drop was added to one flower in each quarter. The location was marked with a red paint marker (UniPaint fine line PX-21, Mitsubishi Pencil, Sanford Corporation, Oak Brook, IL, USA)

on the outer edge of the open whorl. All trials were conducted between 10:00 and 16:00 during June to August 2016.

Worker bees for transmission trials were isolated from laboratory colonies that were confirmed to be *C. bombi*-free via weekly subsamples of five bees. Bees were placed in small vials and starved for 3-4 hours prior to trials. Trials were conducted in 60 x 35 x 45 cm^3 wood and mesh cages with a canvas cloth at one end to allow insertion of bees and flowers. We recorded the number of un-inoculated flowers probed, inoculum drops probed, time spent foraging, researcher conducting the trial, and the time the trial started and ended, all of which were used as potential covariates. A flower or inoculum drop was recorded as 'probed' when the bee inserted its tongue into the flower or drop. A trial was considered complete when a bee had foraged for a minimum of one minute, visited at least one inoculum drop, and visited at least five additional un-inoculated flowers. Most bees foraged for much longer than one minute; we allowed bees to complete foraging before terminating each trial, which was no longer than 20 minutes. Cages were kept in the sun to be sterilized for 30 minutes after each trial had ended; *C. bombi* is not viable after desiccation (Figueroa L. L. et al., in preparation) and in previous work using ethanol to sterilize dissolved the wood varnish (Adler L. S., pers. obs.). On days field trials were conducted, the inoculum was placed in a cooler with ice packs for transport and storage.

After each trial, the bee was recaptured into a clean individual vial, placed in a cooler, and transported at the end of the day to the laboratory. In the laboratory, we placed bees in individual vials with nectar feeder lids and reared them in an incubator at 27ºC in darkness for seven days. Each day, bees were fed approximately 40-60 mg of

wildflower pollen (Koppert Biological Systems) mixed with 30% sucrose to create a pollen paste, and 500 μL of 30% sucrose solution made available in a modified Eppendorf tube through a cotton wick (nectar feeder). Vials, nectar feeders, and pollen were replaced daily. After seven days, we dissected bees and counted *C. bombi* cells as described above for *Inoculum preparation*. At the time of dissection, we collected the right forewing of each bee and measured radial cell length with a dissecting microscope to estimate bee size (Harder 1982) as a potential covariate. Final sample sizes of bees for each cultivar were: Non-CMS 236 = 38, CMS 236 = 39, Non-CMS 404 = 14, CMS 404 = 13, Non-CMS 89 = 30, and CMS 89 = 23, from six different colonies.

2.2.2 Timing of sunflower pollen consumption relative to infection

2.2.2.1 Laboratory trials

To evaluate how the timing of exposure to sunflower pollen affected *C. bombi* counts, we used inoculated laboratory bees exposed to one of four pollen treatments: 7 days of sunflower pollen (sunflower only), 7 days of buckwheat (*Fagopyrum esculentum*) pollen (buckwheat only), 3.5 days of sunflower and then 3.5 days of buckwheat pollen (sunflower first), or 3.5 days of buckwheat pollen and then 3.5 days of sunflower pollen (buckwheat first). We used single pollen species comparisons because sunflower pollen and buckwheat pollen have similar protein and amino acid concentration (Yang et al. 2013), but consuming buckwheat pollen results in much higher *C. bombi* infection than consuming sunflower pollen (Giacomini et al. in review). Worker bees were isolated from commercial colonies in the lab, placed in small vials and starved for 2-3 hours prior to inoculation. We provided bees with a 10 μl drop of inoculum, and only bees that

consumed the entire drop were included in the experiment. After inoculation, bees were placed in individual vials with nectar feeder lids and their respective pollen treatment, then reared in an incubator at 27ºC in darkness for seven days. Each day, bees were fed approximately 40-60 mg of a fresh mixture of buckwheat or sunflower pollen paste, and 500 μl of 30% sucrose solution made available in a modified Eppendorf tube through a cotton wick (nectar feeder). Vials, nectar feeders, and pollen were replaced daily. After seven days, bees were dissected and *C. bombi* cells were counted, as described in *Exposure to sunflower pollen at time of infection experiment* (above). At the time of dissection, we again collected the right forewing to measure radial cell length to estimate body size and use as a covariate in analysis. Our sample sizes were 41-45 bees per treatment, for a total of 172 bees, from four different colonies.

2.2.3 Statistical analysis

To test whether sunflower pollen presence or absence at time of infection in single bout foraging trials reduced infection, we used raw *C. bombi* cell count as the dependent variable with a model including pollen presence (yes/no), *H. annuus* line (404, 89, 236) and their interaction as independent fixed predictors. We initially included several fixed covariates, including the number of inoculum drops probed (to assess the amount of parasite each bee was exposed to), the number of un-inoculated flowers probed, and researcher overseeing the foraging trial. However, inoculum drops probed and researcher did not affect *Crithidia* counts ($\chi^2_{(1)} < 1.3, P > 0.3$ for all) and were dropped from the model. In addition, colony of origin (to account for the genetic similarities between bees), total time foraging on inflorescences, bee size (estimated by

radial cell length), and trial date (to account for variation in *C. bombi* inoculum preparation) were originally included as random effects. To analyze the effect of pollen presence on foraging behavior, we used separate responses of inoculum drops probed, flowers probed, and total time spent foraging, with fixed predictors of pollen presence, *H. annuus* line and their interaction, as well as random effects of colony of origin, bee size, and trial date.

To ask how timing of sunflower consumption after infection affected raw *C. bombi* cell counts, we used a generalized linear mixed model with a negative binomial error distribution and a log link function, including pollen diet treatment as a fixed effect and originally included random effects of colony of origin, bee size, and inoculation date. We compared our model with and without pollen treatment using the *anova* function. Finding a significant treatment effect, we used a Tukey's post hoc HSD test to compare the four treatments. Graphical displays were composed with *ggplot2* (Wickham 2009) and *cowplot* (Wilke 2016) packages.

2.3 Results

2.3.1 Exposure to sunflower pollen at time of infection

C. bombi cell count after one week was not affected by pollen presence ($\chi^2_{(1)} = 0.028$, $P = 0.866$), *H. annuus* line ($\chi^2_{(2)} = 0.808$, $P = 0.369$) or their interaction ($\chi^2_{(1)} = 0.697$, $P = 0.404$; Figure 1). Furthermore, the only covariate in the model that had significance was un-inoculated flowers probed ($\chi^2_{(1)} = 4.039$, $P = 0.045$). During the trials, bees foraged on inflorescences with pollen (non-CMS) for an average of 4 minutes, 18% more time than inflorescences without pollen (Figure 2.2A) and in the model pollen and

line interaction was significant $P = 0.043$, and increased flowers probed by 23%, and these effects were significant, and neither line or the interaction term of the model covariates were significant (Figure 2.2B). However, the presence of pollen significantly decreased the number of inoculum drops probed by 50% and in the model *H. annuus* line was significant $P < 0.001$(Figure 2.2C).

2.3.2 Timing of sunflower pollen dosage relative to infection

Our model explaining *C. bombi* cell counts included predictors of pollen treatment and bee size as fixed effects and a random effect of colony of origin. In our model, treatment had a significant affect on raw *C. bombi* cell counts ($\chi^2_{(3)} = 38.239$, $P < 0.001$) Using a Tukey's post hoc HSD test, a pure sunflower pollen diet most strongly reduced *C. bombi* counts, followed by exposure to sunflower pollen first, then exposure to buckwheat pollen first, and finally exposure to only buckwheat pollen (Figure 2.3). Bees fed buckwheat pollen first had approximately a two-fold increase in *C. bombi* compared to bees fed sunflower pollen first, but this comparison was not statistically significant ($P = 0.441$, Figure 2.3). However, bees exposed to buckwheat pollen first had significantly higher *C. bombi* counts than bees exposed to pure sunflower pollen, a 12-fold increase ($P < 0.001$, Figure 2.3), and this treatment was not statistically different from consuming buckwheat pollen only ($P = 0.525$, Figure 2.3). Furthermore, exposure to sunflower pollen first was different from consuming only sunflower pollen ($P < 0.001$, Figure 2.3) and also from consuming buckwheat pollen only ($P = 0.023$, Figure 2.3), indicating that both dose and timing of sunflower pollen affect pathogen counts.

2.4 Discussion

The severity of *C. bombi* infection depended on the duration, and to some extent the timing, of exposure to sunflower pollen relative to infection. The first experiment demonstrated that a single dose of sunflower pollen exposure at the time of infection was insufficient to prevent or suppress *C. bombi* infection. In the second experiment, we demonstrated that 7 days of sunflower pollen suppressed *C. bombi* infections to nearly untraceable levels while seven days of buckwheat pollen resulted in relatively high *C. bombi* infection, consistent with previous work (Giacomini et al., in review; LoCascio et al., in prep). Although exposure to 3.5 days of sunflower pollen immediately following infection was not as effective as exposure for 7 days, it was more effective than consuming only buckwheat pollen. This indicates that dose is important; consuming 3.5 days of sunflower pollen is not as effective as 7 days, but better than none. By contrast, exposure to 3.5 days of buckwheat pollen and then 3.5 days of sunflower pollen was not more effective than only consuming buckwheat pollen, indicating that timing also plays a role. These results suggest that duration of exposure is more important for reducing *C. bombi* infection, but earlier exposure can also play a role.

Pollen presence affected foraging behavior in our trials. There were significant trends for bees to probe more flowers and spent more time foraging when pollen was present. Conversely in the absence of pollen, bees foraged on more *C. bombi* inoculum drops. This is consistent with a previous experiment that showed *B. impatiens* preferred sunflower inflorescences with pollen over inflorescences without pollen (Mallinger and Prasifka 2017). Interestingly, although bees probed significantly more inoculum drops on inflorescences without pollen, pollen presence did not affect bee infection levels. Thus,

bees that foraged on inflorescences without pollen had greater exposure to *C. bombi*, but had similar pathogen counts to bees that foraged on inflorescences with pollen. In general, consumption of greater volumes of inoculum led to higher infection intensity in *B. impatiens* (Otterstatter and Thomson 2006). Although we do not know why sunflowers without pollen would incur greater exposure to inoculum without affecting pathogen counts, this has interesting implications for the relationship between foraging dynamics and exposure to infectious material in agricultural settings, since male-sterile sunflowers are often grown on farms (Parker 1981).

Although the mechanisms by which sunflower pollen reduces *C. bombi* in bumble bees are unknown, the results from our timing experiments lead us to speculate about possibilities. For example, if the mechanism underlying resistance was immune system-priming, we would have expected the one-time exposure to be effective. Priming with some nectar alkaloids has been suggested to help bumble bees infected with *C. bombi* (Manson et al. 2009), and warrants further studies investigating pre-exposure and post-exposure to sunflower pollen. Our results also indicate that a small dose of sunflower pollen is not directly toxic to *C. bombi*, or at least not enough to prevent bee infection. Rather, a longer-term exposure in the gut seems to be necessary for suppression, suggesting a more gradual immune response to sunflower. Alternatively, sunflower pollen may affect resistance by altering the gut microbial community, a mechanism that can only be effective post-consumption. Gut microbiota obtained from nest mates provide protection against *C. bombi* in *B. terrestris* (Koch and Schmid-Hempel 2011). Additionally, gut microbiota in honey bees interact with pollen by producing enzymes that can break down pollen walls (Engel et al. 2012) releasing internal pollen components

into the gut. Although our experiments were not designed to elucidate mechanism, the contrasting results for single foraging bout exposure vs. longer periods of consumption suggest that exploring whether immune function and the gut microbiota are affected by sunflower pollen would be important in future work.

Sunflower pollen effectively reduced *C. bombi* with a 7 day exposure, but it is relatively nutrient poor, having low protein content and less than the minimum requirement of two essential amino acids for honey bees (Nicolson and Human 2013). Sunflower pollen has been suggested to hinder honey bee development and reduce larval weight in *Bombus terrestris* when it is their sole pollen source (Tasei and Aupinel 2008), and resulted in as much mortality as pollen starvation for honey bees infected with *Nosema* (Giacomini et al, in review). Aside from the specific disadvantages of a sunflower-only diet, consuming any monofloral pollen diet may reduce bee health. For example, nurse honey bees parasitized with *Nosema ceranae* had reduced survival when fed monofloral compared to polyfloral pollen diets (Di Pasquale et al. 2013), and a polyfloral pollen mix increased immune system activity compared to monofloral diets (Alaux et al. 2010). Therefore, future plans using sunflower pollen to manage bee disease should also include other pollen sources for optimal bee health. Our data suggest that, while timing of sunflower pollen relative to infection exposure can improve beneficial medicinal effects, a 7-day exposure is optimal to reduce *C. bombi* infection.

Our findings may have important applications with managed bumble bees. Because previous studies indicate that multiple cultivars of sunflower and some relatives suppress *C. bombi* (LoCascio et al, unpublished data), future studies could directly supply bee colonies with sunflower pollen to see whether sunflower pollen supplements can

benefit bees at the whole-colony level. Sunflower supplements may also provide benefits to managed honey bee colonies. Giacomini et al. (in review) found that sunflower pollen also suppressed the widespread fungal parasite, *Nosema spp*., in honey bees. Apiary managers could investigate whether the use of sunflower pollen supplements or increased sunflower plantings improves honey bee health.

Our result that timing plays a role in disease management is consistent with studies using other trypanosomes that cause Chagas disease and African sleeping sickness in humans. With Chagas disease, early treatment suppressed acute symptoms better than when treatment was administered after chronic symptoms began (Vallejo and Reyes 2005, Jannin and Villa 2007). Early intervention is crucial for African sleeping sickness (Legros et al. 2002) and is necessary to prevent late stage progression, which requires a combination of medicines to ensure drug resistance doesn't occur (Priotto et al. 2006). In both diseases, early treatment is imperative for the disease to subside quickly or to be brought to manageable levels. Furthermore, treatment must be continued for a prolonged period of time after infection to be most effective. Although less effective than 7 days of sunflower pollen, we found that even 3.5 days of exposure to sunflower pollen could reduce *C. bombi*, but only if sunflower pollen was administered immediately after infection.

In conclusion, we found that early short-term exposure to sunflower for 3.5 days was more effective at reducing *C. bombi* compared to a buckwheat only diet. Pollen presence also affected bee foraging behavior; bees foraging on inflorescences with pollen probed more flowers but fewer inoculum drops than on inflorescences without pollen. Thus, floral resources have the potential to affect bee disease transmission both before

and after infective material is encountered. Future field studies that examine how late-season Asteraceae diets affect bee overwintering infection will help determine potential land management strategies that reduce infection harbored between seasons. This could provide insight for land managers and beekeepers about plants and diets that improve pollinator disease resistance. Our results suggest that providing *B. impatiens* with medicinal sunflower pollen at the early stages of infection of *C. bombi* and for sufficient duration is important in managing disease in bumble bees.

Figure 1.1 Mean raw *Crithidia* count per 0.02μL (± SE) for the 19 pollen taxa. Buckwheat (pink), wildflower mix (yellow), our positive control of *H. annuus* 'China' (orange), honey bee-collected taxa (grey), hand-collected taxa (blue) and *Solidago* spp (green), which were honey bee-collected. Letters a-d indicate statistically significant differences between pollen treatments after a post hoc Tukey's test. Full explanations for all taxa names are provided in Appendix A; "HC" refers to hand-collected for the three taxa where we had both honey bee and hand collection. Standard errors were calculated by back-transforming least-square means plus or minus least-square mean standard errors.

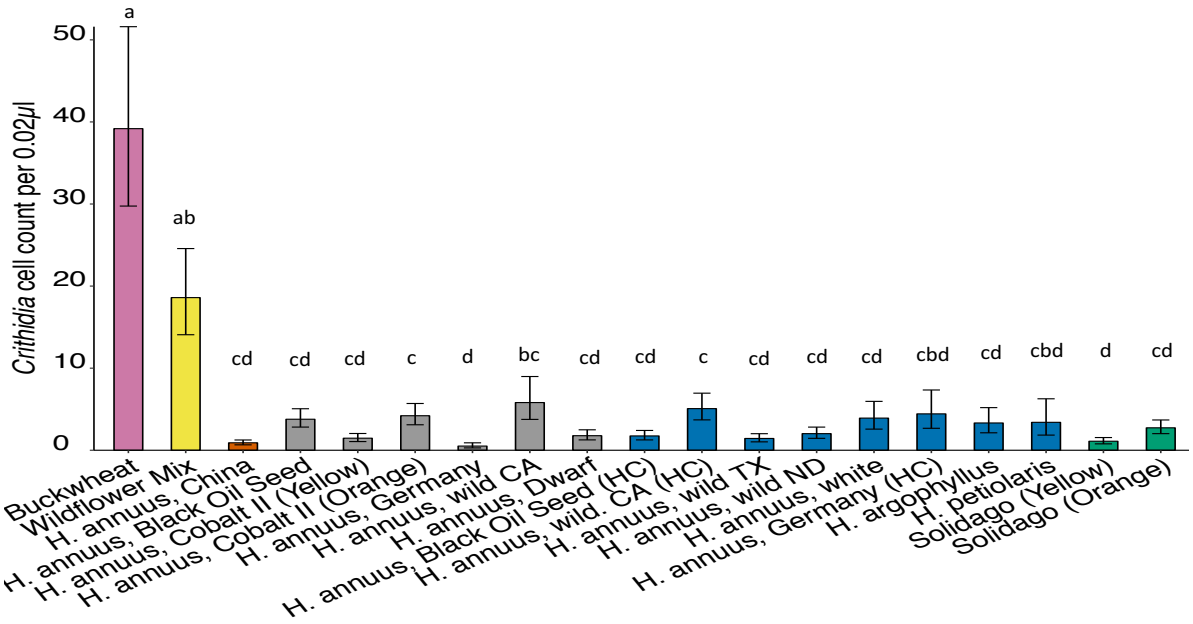


Figure 1.2 Comparison of hand vs. honey bee-collected pollen. (A) *H. annuus*, 'Black Oil Seed', (B) *H. annuus*, Germany, (C) *H. annuus*, wild California, and D) comparison pooled across all Asteraceae taxa used in the experiment (17 treatments). Asterisks (*) denotes statistically significant differences between collection method. Standard errors were calculated by back-transforming least-square means plus or minus least-square mean standard errors.

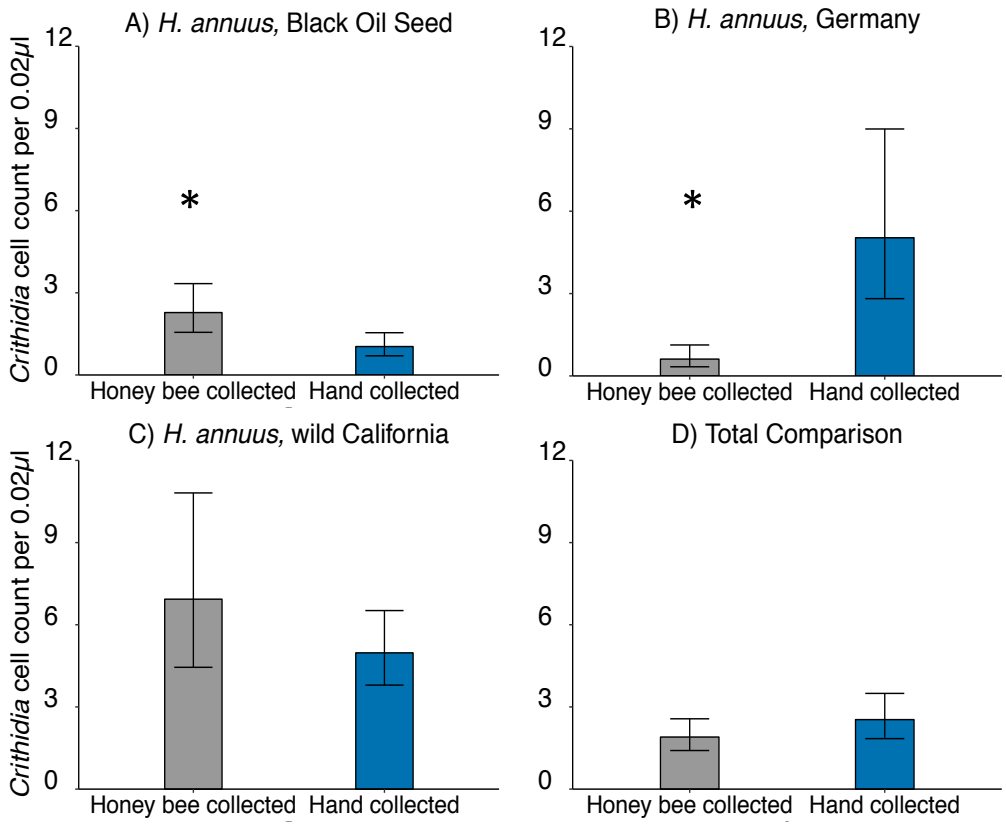


Figure 2.1 Mean *Crithidia bombi* count (± SE) within each cultivar one week following exposure in a single foraging bout. Categories on the x-axis represent cultivars with pollen (light grey) and without pollen (dark grey). Means and standard errors were calculated by back-transforming least-square means plus or minus least-square mean standard errors.

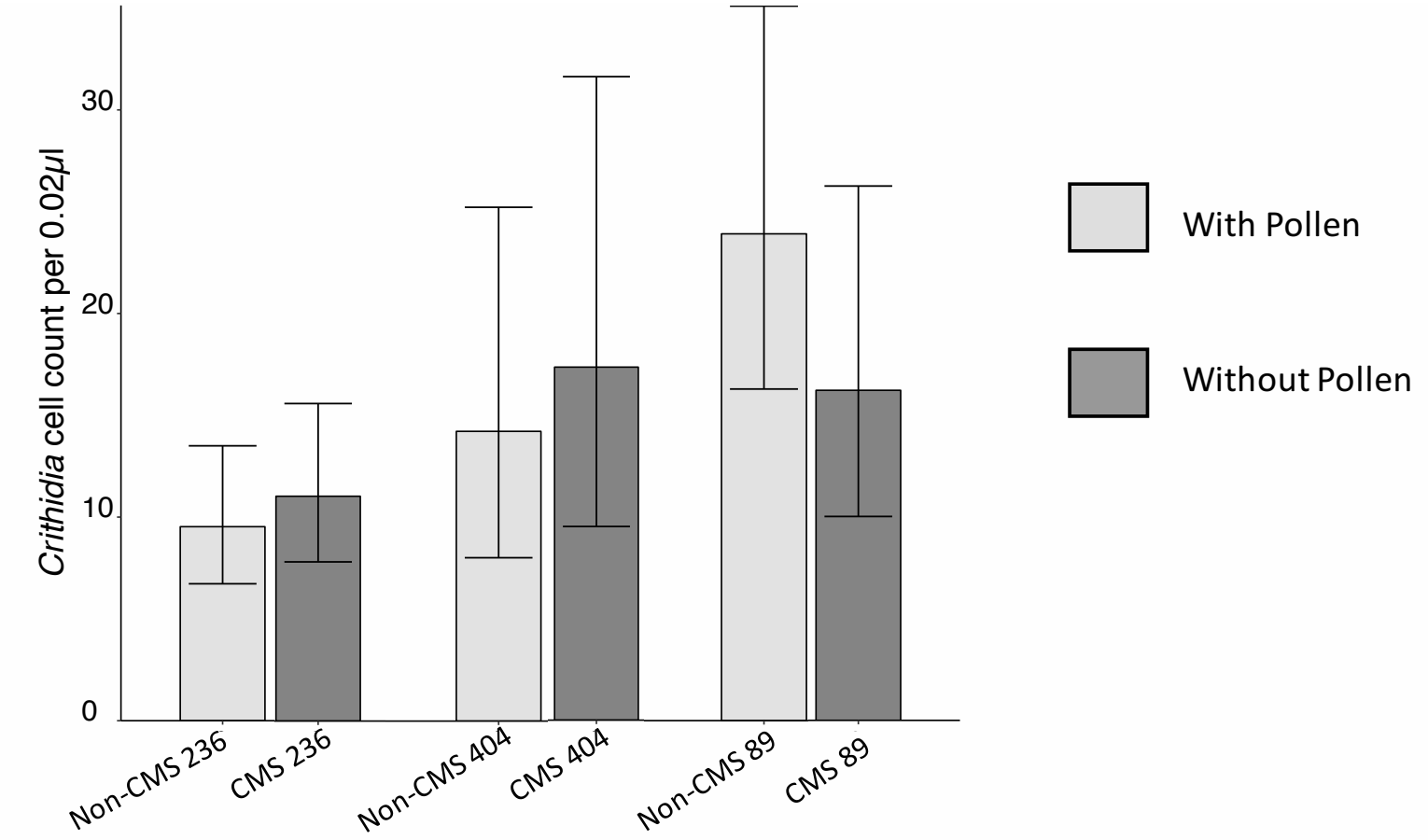


Figure 2.2 Effect of pollen presence on bee foraging behavior. In all plots, 'No' indicate inflorescences without pollen (CMS) and 'Yes' indicates inflorescences with pollen (non-CMS). A) mean (± SE) total minutes foraging on each inflorescence type. B) mean (± SE) flowers probed per inflorescence. C) mean (± SE) inoculum drops probed per inflorescence. The asterisk (*) denotes significantly different at $P < 0.05$. Means and standard errors were calculated by back-transforming least-square means plus or minus least-square mean standard errors.

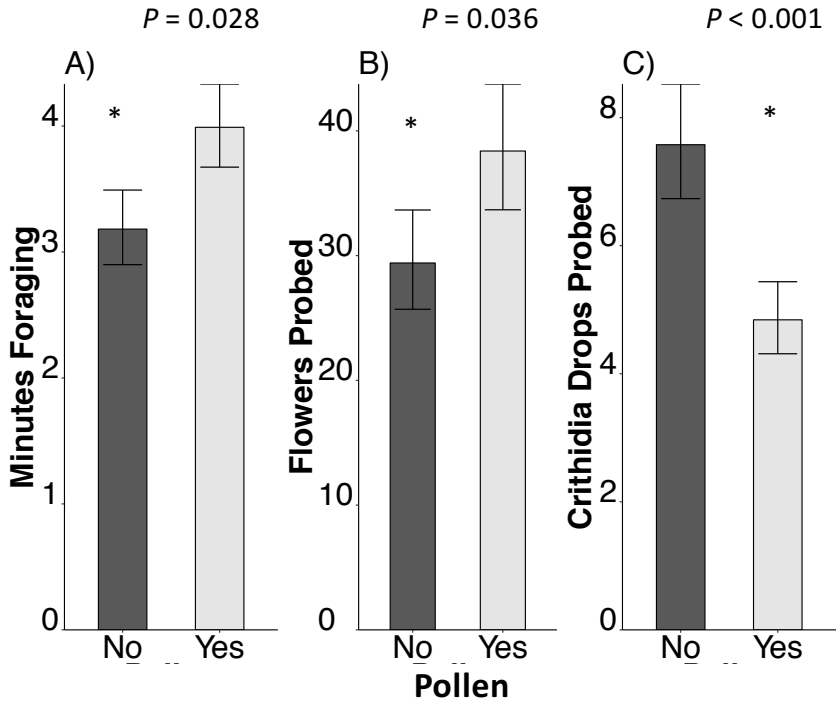
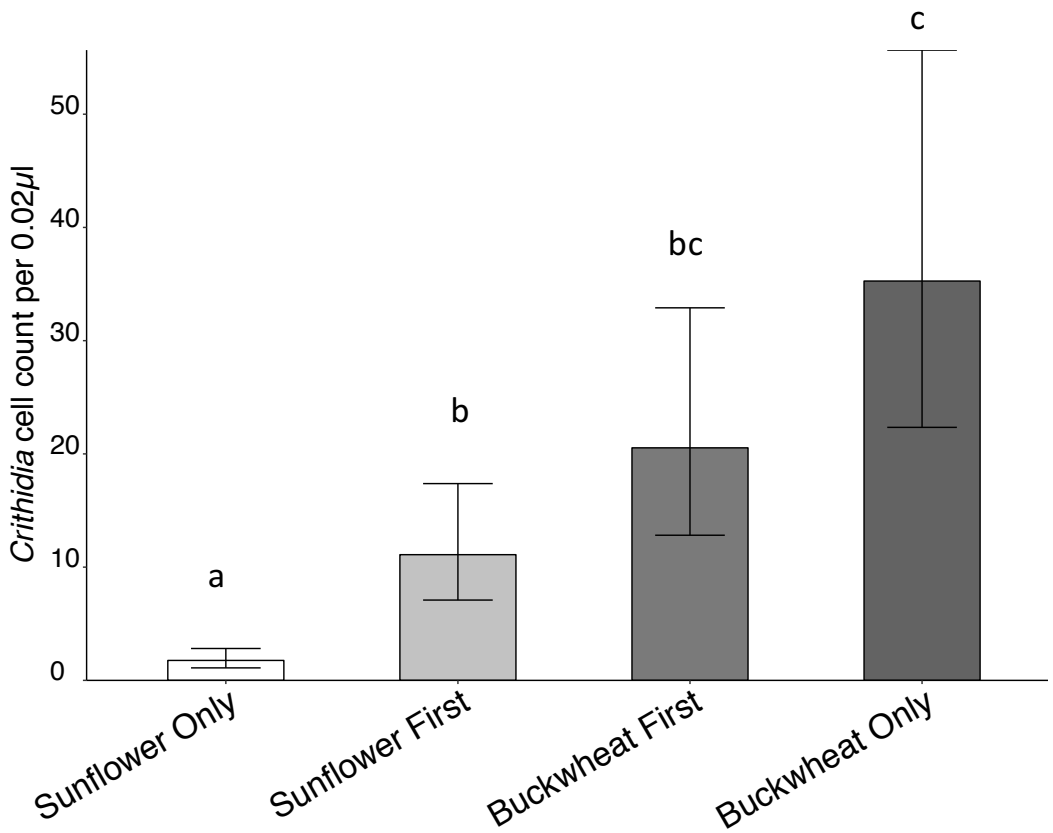


Figure 2.3 Mean (± SE) *C. bombi* counts comparing timing of dosage relative to infection. Sunflower for 7 days, sunflower for 3.5 days then 3.5 days of buckwheat pollen, buckwheat pollen of 3.5 days then 3.5 days of sunflower pollen, and buckwheat for 7 days. Different letters above bars denote significantly different treatments at $P < 0.05$ using a Tukey's post hoc HSD test. Means and standard errors were calculated by back-transforming least-square means plus or minus least-square mean standard errors.



APPENDIX A

CHAPTER 1 SUPPLEMENTAL INFORMATION

Table legend. Source information and propagation protocols. All dates are from 2016. Taxa column refers to the plant species or cultivar we used in the experiment. The color indicates the color of the pollen. Sample size refers to the number of bees that went through the experiment with that pollen treatment. Bee, hand, or both indicates how the pollen was collected. 'Bee' was collected with honey bees using pollen traps, 'hand' was collected by hand using paint brushes and colleting the pollen into aluminum foil envelopes, and 'both' was when we used both methods. The source column refers to where we obtained the pollen from, and the GRIN seeds are accompanied with the plot ID number (PI) that the seeds were obtained from. In the location column, 'UMass' refers to the Crop and Animal Research and Education Center in South Deerfield, Massachusetts (42°28'39.6"N 72°34'51.1"W), operated by the University of Massachusetts, Amherst. Seed scarification, transferred to 15-cm pot, and date transferred to field refers to the propagation methods of the taxa we grew. The amount of plants refers to the total acres or the total number of plants that were grown.

Taxa	Pollen color	Sample size (n)	Collection type	Source	Location	Seed Scarification	Transferred to 15-cm pot	Date Transferred to field	Amount of plants
Solidago spp	Yellow	36	Bee	East Leverett Meadow	Massachusetts 42°43'91.80" N, -72°48'68.03" W	N/A	N/A	N/A	~5 acres

Solidago spp	Orange	48	Bee	East Leverett Meadow	Massachusetts 42°43'91.80" N, -72°48'68.03" W	N/A	N/A	N/A	~5 acres
H. annuus, Black Oil Seed	Yellowish orange	93	Both	Laurenitis Farm	Massachusetts 42°44'17.77" N, -72°55'04.64" W	N/A	N/A	N/A	~1 acres
H. annuus, Cobalt II	Yellow	41	Bee	Messa Farm	Wisconsin 44°73'16.91" N, -91°94'86.32" W	N/A	N/A	N/A	~75 acres
H. annuus, Cobalt II	Orange	47	Bee	Messa Farm	Wisconsin 44°73'16.91" N, -91°94'86.32" W	N/A	N/A	N/A	~75 acres
H. annuus, China	Orange	49	Bee	Changge Hauding Wax Industry	China	N/A	N/A	N/A	Unknown
Buckwheat	Brown	57	Bee	Changge Hauding Wax Industry	China	N/A	N/A	N/A	Unknown
H. annuus, Germany	Yellowish orange	25	Both	GRIN Seeds Pl-650375	UMass	No	No	June 17	~300 plants
H. annuus, wild California	Yellowish orange	56	Both	GRIN Seeds Pl-613732, Pl-649815, Pl-649816	UMass	Yes	No	July 12	~300 plants
H. annuus, 'Dwarf'	Yellowish orange	36	Bee	The Chas. C. Hart Seed Co Wethersfield, CT, USA	UMass	No	No	July 8 (directly sown)	~300 plants
Helianthus argophyllus	Yellowish orange	14	Hand	GRIN Seeds Pl-435630, Pl-494569	UMass	Yes	Yes	June 17	~300 plants

H. annuus, wild Texas	Yellowish orange	35	Hand	GRIN Seeds Pl-613728, Pl-649810, Pl-649811	UMass	Yes	No	June 17	~300 plants
H. annuus, wild North Dakota	Yellowish orange	35	Hand	GRIN Seeds Pl-613724, Pl-613725, Pl-613750	UMass	Yes	No	June 27	~300 plants
H. annuus, white	White	18	Hand	GRIN Seeds Pl-650655	UMass	Yes	No	June 17	~300 plants
Helianthus petiolaris	Yellowish orange	7	Hand	GRIN Seeds Pl-435825, Pl-435826, Pl-435827	UMass	Yes	Yes	N/A	~30 plants
Wildflower mix	Various	53	Bee	Koppert Biological Systems	Minnesota	N/A	N/A	N/A	Unknown

Supplemental methods

Plant propagation information

All seeds were sown in SUNGRO Horticulture medium (SunGro Horticulture Canada Ltd., Seba Beach, AB TOE 280, Canada) in 50-plug trays with natural lighting. They were germinated at 21˚C and misted every 15 minutes for 30 seconds from sunrise to sunset on a mist top bench. When 50% of the seedlings in a tray reached the four true-leaf stage, the tray was relocated to a different greenhouse with natural light plus 14 hours of artificial supplementary lighting consisting of 50% 400W high pressure sodium and 50% 400W metal halide lights. Plants were watered as needed. The plants ranged from 18-60 cm in height when transplanted to the field site. *H. petiolaris* was transplanted into 15-cm pots and remained in the greenhouse due to small sample sizes. *H. petiolaris* was fertilized twice, on April 20 and May 13, 2016, and *H. argophyllus* once on May 13, 2016 with Peters Professional 20-10-20 Peat lite (JR Peters Inc. Allentown PA, USA) at 350 ppm. Other taxa were not fertilized. *Helianthus annuus* 'Dwarf' cultivar was sown directly into soil in the field. Pollen collection methods are located in the main body of the methods section of the manuscript.

Seed scarification protocol

Seeds were sterilized with a 2% bleach (The Chlorox Co, Oakland CA, USA) and 1% Triton-X 100 (Sigma-Aldrich, St. Louis Missouri, USA) solution for 10 minutes, stirring for 10 seconds every minute. Afterwards, they were rinsed in distilled water for 5 minutes, stirring for 10 seconds every minute. After rinsing, a small section of the blunt end of each achene was removed with scissors. The seeds were then placed on moist filter

paper in 95 mm petri dishes, sealed with parafilm and placed in an incubator for 48 hours at 27˚ C. After 48 hours, the remaining seed coat was removed by hand and cotyledons were placed on a new petri dish with new moist filter paper in an incubator for an additional 24-48 hours. When the radicle grew to > 10 mm on ~50% of the seeds, petri dishes were moved to a lab bench in ambient light for 3 days, after which they were transplanted into the soil growing medium and grown in the greenhouse as described above in '*Plant propagation information*.'

Tent assembly

The metal tent frame (Delta Canopies, McKinney, Texas, USA) was assembled according to manufacturer instructions with slight modifications. We removed 12, 0.75m joint pieces, which reduced the width of the tent from 4.8m to 3.6m; length was 9.7 m and height was 3 m. The 9 joints where the legs and the roof of the frame connected were reinforced by 0.6m metal conduit pieces. The conduits were attached to roof sections spanning the joints perpendicular to the vertical legs with six to eight zip ties. The frame was also reinforced with three evenly spaced ratchet straps pulled taught, spanning the width of the frame parallel to the ground. Next, the polyester fabric and mesh was draped evenly over the frame and held in place with 10 2.54 cm PVC snap clips (Johnny's Select Seeds, Winslow, Maine, USA), one per leg. Four pieces of 0.6m factory-provided angle iron, two on each side near the tent ends, were hammered into the ground and reinforced by 2 0.6m pieces of 7.62cm rebar to provide an anchor for rope that spanned the top of the tent to secure the frame and fabric in place. Finally, a trench approximately 12cm deep and 6cm wide was dug around the perimeter of the tent, except

for a space of 2m across the one of the narrow sides, which served as an entrance. Excess fabric was rolled around lumber (4cm x 8cm, various lengths) and buried in the trenches. To secure the entrance, fabric was rolled on one piece of lumber and weighted down with concrete bricks and soil.

APPENDIX B

CHAPTER 2 SUPPLEMENTAL INFORMATION

Table legend. Plant cultivation information. All dates were in the year 2016. The cultivar column includes whether the plant had pollen or no pollen (CMS). The date and total sown describes the date each group of plant was sown at the University of Massachusetts Amherst greenhouses and the amount that was sown. Date trails completed indicates the last day we used plants from that group for foraging trials.

Cultivar	**Date and total sown**		**Date from propagation room to greenhouse**	**Date transplanted to pots**	**Date transplanted to field**	**Date trials completed**
404	April 22	100	April 29	May 19	June 16	July 11
CMS 404	April 22	100	April 29	May 19	June 16	July 11
89	May 2	100	May 9	May 20	June 15	July 22
CMS 89	May 2	100	May 9	May 20	June 15	July 22
236	May 12	100	May 19	June 13	June 17	August 15
CMS 236	May 12	100	May 19	June 13	June 17	August 15

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