## ABSTRACT

GIACOMINI, JONATHAN JOSEPH. Tri-Trophic Interactions Between Plants, Their Mutualist Pollinators and Natural Enemies of Pollinators: The Ecological Effects of Sunflower (*Helianthus annuus*) Pollen on Bees and Their Pathogens. (Under the direction of Dr. Rebecca E. Irwin).

Communities are comprised of many species that interact directly in a pairwise fashion, as well as indirectly in which one species mediates the strength and direction of interactions between two other species. Indirect species interactions can have important effects on how antagonistic and mutualistic interactions shape the evolution of phenotypic traits, population and community dynamics. Many plants provide key resources for both herbivores and pollinators, and thus play a major role in shaping multi-trophic interactions for both antagonists and mutualists. Pollinators, such as bees, are critically important for the preservation of plant biodiversity, provide billions of dollars in crop pollination services annually, and are essential for the production of hundreds of crops. Although a variety of factors are involved, pathogens have been strongly implicated in the decline of many bee species. Thus, one of the most pressing concerns in the management of bee pathogens is the identification of factors that could reduce pathogens and diseases in natural and managed landscapes.

Recent work demonstrates that floral traits (namely nectar) can mediate interactions between pollinators and their natural enemies, yet the role of pollen in such a context is poorly understood. Bees rely on pollen as the primary source of nutrition for growth and development; yet, pollen can contain high concentrations of secondary compounds, and varies tremendously in macronutrient and morphological properties. While links between bee nutrition and pathogen resistance are already established, the role of particular pollen species in mediating bee-pathogen dynamics is largely unknown.

My dissertation addresses the ecological costs and benefits of pollen species identity for pollinators and interactions with their natural enemies. I used a series of controlled laboratory experiments and field studies focused primarily on the bumble bee *Bombus impatiens*, its protozoan pathogen *Crithidia bombi*, and sunflower pollen (*Helianthus annuus*). I began by demonstrating that sunflower pollen dramatically and consistently reduced *C. bombi* infection in individual bumble bees. Additionally, in a field survey, bumble bees from farms with a greater area of sunflower plantings had lower infection rates. I then demonstrated that a 1:1 mixture of sunflower and wildflower pollen reduced *C. bombi* infection prevalence and intensity at the bumble bee colony level, relative to a control wildflower pollen diet, with no significant nutritional tradeoffs for colony worker production and most aspects of colony reproduction. Planting sunflower in agroecosystems and native habitat may thus provide a simple solution to reduce disease and improve the health of economically and ecologically important pollinators.

Given consistent effects of sunflower pollen in reducing bee pathogens, I then focused on controlled laboratory experiments to identify the mechanism(s) underlying the medicinal effect of sunflower pollen in bumble bees. I first demonstrated that ingestion of sunflower pollen stimulates rapid excretion in bumble bees, which could reduce *C. bombi* infection directly by flushing infectious cells from the host digestive tract, or may be a pleiotropic consequence of other changes in host bee physiology. Finally, I use a whole-transcriptomic approach to demonstrate that consumption of sunflower pollen stimulates an immune response in infected bumble bees dissimilar to that of wildflower-fed bees, as well as a strong detoxification response, and a response to physical damage to gut epithelial cells. Taken together, my dissertation provides a new perspective on the role of pollen in influencing pollinator enemies through changes in pollinator traits via trait-mediated indirect interactions. I propose that optimizing bee

health management requires consideration of key plant species that play disproportionate roles in protecting pollinators against stressors such as pathogens.

# Tri-Trophic Interactions Between Plants, Their Mutualist Pollinators and Natural Enemies of Pollinators: The Ecological Effects of Sunflower (*Helianthus annuus*) Pollen on Bees and Their Pathogens.

by
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APPROVED BY:

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## DEDICATION

To my dear sweet son Everest Skeldal Giacomini, who in an instant taught me the meaning of life without uttering a single word. You are my everything. And to my beautiful and wise mother Robin Anne Giacomini. Your strength and wisdom have been a guiding light that I appreciate more than you will ever know.

## BIOGRAPHY

It is difficult to describe the feelings I experience when observing some plant or animal, but it is certainly ethereal or supernatural in some sense. Whatever it is, it brings me great joy. I imagine it is the same feeling felt by many brave and magnificent ecologists that came before me and will undoubtedly come after me.

I graduated from North Providence High School in 2004, after which I had no clue what I wanted to do for a career. Raised by a single mother and having lost my father when I was a child, it was difficult to both find my place in the world and make ends meet at the same time. My first attempts at community college were futile. Courses were boring, disengaged, or lacking in passion. So, I dropped out of college and instead worked long hard days under the beating sun as a landscaper, and nights as a package handler for a major shipping company. Needless to say, I was tremendously bored and far out of touch with my connection to nature. Brief adventures on the weekends or short vacations simply could not satisfy me. In 2011, a new opportunity to be a part-time courier changed everything. This new job afforded me the opportunity to work part-time, maintain expensive health-insurance, pay rent and tuition and attend college full-time. The direct and indirect costs of education in the U.S.A. are astronomical. Every day I woke up at 4 am, worked hard delivering priority letters during the day and then attended Bunker Hill Community College in Boston in the afternoons and evenings. Looking back, I realize how crazy I must have been to take on such a monumental task, but the passion I had for science and biology gave me the fuel I needed; daily 20-minute naps helped. I graduated from BHCC as valedictorian and then transferred to the University of Massachusetts Amherst, where for the first time in my life I felt fully immersed in science and biology. There, I met Lynn Adler, who has since become an invaluable mentor, colleague and friend. I worked as a research technician for

Lynn for two summers, where I assisted in experiments that explored the ecology and evolution of floral rewards and pollinator health, and eventually ran an experiment of my own. Handling bees on a daily basis, I fell in love with pollinator ecology. In 2015, I graduated UMass with a B.S. in biology. Lynn then introduced me to Becky Irwin, who took me on as a research technician where I continued to assist in experimental pollinator ecology at NCSU before officially joining the lab as a PhD student in 2016. That same year I was fortunately awarded the prestigious National Science Foundation Graduate Research Fellowship. Since then, Becky and Lynn have been extraordinary mentors that I simply cannot thank enough for the amazing opportunities they have given me. I have had the benefit of experiencing what it means to work hard doing both things one does not enjoy and things that one does enjoy. As a result, I am extremely grateful to spend my career experiencing that sense of joy I get when I explore the ecology of some plant or animal. For now, the rest of my scientific career remains to be written, but I am certain I will continue exploring the many mysteries of the world as a professional scientist and hope to continue this great global effort conserving the tremendously important pollinators that play an integral role in many ecosystems.

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Thank you especially Sara June Connon. You were an invaluable part of my journey and I will always treasure our time together. Thank you most of all for being an amazing mother for our beautiful son.

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**CHAPTER 1: Medicinal value of sunflower pollen against bee pathogens.**

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## Abstract

Global declines in pollinators, including bees, can have major consequences for ecosystem services. Bees are dominant pollinators, making it imperative to mitigate declines. Pathogens are strongly implicated in the decline of native and honey bees. Diet affects bee immune responses, suggesting the potential for floral resources to provide natural resistance to pathogens. We discovered that sunflower (*Helianthus annuus*) pollen dramatically and consistently reduced a protozoan pathogen (*Crithidia bombi*) infection in bumble bees (*Bombus impatiens*) and also reduced a microsporidian pathogen (*Nosema ceranae*) of the European honey bee (*Apis mellifera*), indicating the potential for broad anti-parasitic effects. In a field survey, bumble bees from farms with more sunflower area had lower *Crithidia* infection rates. Given consistent effects of sunflower in reducing pathogens, planting sunflower in agroecosystems and native habitat may provide a simple solution to reduce disease and improve the health of economically and ecologically important pollinators.

## Introduction

Pollinators are critically important for the preservation of plant biodiversity, and provide billions of dollars in crop pollination annually[1,2]. Bees are the dominant pollinators of the majority of animal-pollinated flowering plants globally[3] and are important for the production of many crops[4]. There have been mounting concerns about increased mortality in both honey bees

and native bees[5]. Although a variety of factors are involved, pathogens have been strongly implicated in the decline of many bee species[5]. One of the most pressing concerns in the management of bee disease is the identification of factors that could reduce bee disease in natural and managed landscapes.

Many studies have examined the role of landscape factors, including plant diversity, on pollinator abundance and colony growth[6–9], but the role of particular plant species in mediating bee-pathogen dynamics is largely unknown. For example, previous work has linked bumble bee pollen collection and colony growth to land-use patterns, and found that quantity, rather than quality, of pollen was most important for growth[8]. However, this work did not consider the role of pathogens. Conversely, a recent study that incorporated a range of landscape factors and pesticide use data found that use of the fungicide chlorothalonil was the best predictor of the pathogen *Nosema* in four declining bumble bee species[9], but this work did not consider the role of particular plant species or pollen quality. Although pathogens can be horizontally transferred among bees at shared flowers[10,11], and flower species can differ in their transmission probabilities[10,11], there is currently no published work suggesting that particular plant species may play significant roles in mediating bee-pathogen dynamics.

Pollen is the sole source of lipids and protein for bees, and varies widely in nutritional content[12], morphology, and chemistry[13]. Pollen nutritional quality, including protein, is important for individual bee size[14] and metrics of colony performance[15,16] and pollen macronutrient ratio shapes bumble bee foraging preferences[17]. Pollen quality also affects the expression of genes relating to host immune function[18], and pollen starvation increases the likelihood of bees dying when infected with a common gut pathogen[19]. Previous work has shown that nectar chemistry can mediate bee disease[20], and one study found that pollen from different plant species affects

honey bee tolerance of the pathogen *Nosema ceranae* and expression of immune genes[21]. Thus, interspecific variation in pollen composition may have a critical but largely unknown effect not only on bee performance, but also interactions with pathogens[21–23].

We conducted a series of laboratory experiments and a field survey to investigate the effect of pollen diet on bee disease and health using both the common eastern bumble bee, *Bombus impatiens* (Apidae), and the European honey bee, *Apis mellifera* (Apidae). Bumble bees can be infected with a diversity of pathogens, including *Crithidia bombi* (Trypanosomatidae), a protozoan gut pathogen contracted at flowers by fecal transmission[11]. *Crithidia* reduces learning and foraging efficiency in worker bees[24], slows colony growth rates (especially at the start of the season[25]), increases worker mortality, and reduces queen fitness under stressful conditions[19]. *Crithidia* infection is common, with a prevalence of over 80% in *B. impatiens* in some regions[26]. Honey bees can also be infected by a diversity of pathogens, including an obligate intracellular pathogen *Nosema ceranae* (Microsporidia), which has been implicated in colony losses. Field experiments suggest that *Nosema* infection can cause a rapid collapse of otherwise healthy colonies[27].

To ask whether pollen from different plant species could influence bee-pathogen dynamics, we first compared the effects of pollen from three different plant species on bumble bee infection intensities. Upon finding that sunflower pollen dramatically reduced infection intensity in multiple experiments, we assessed the effects of sunflower pollen on bumble bee microcolony performance in the presence and absence of infection. We then explored the generality of our findings with another bee-pathogen system, the European honey bee and the pathogen *Nosema*. Finally, we tested the hypothesis that increased sunflower crop area reduces *Crithidia* in wild bumble bees at the farm level, to assess whether our laboratory results could

extend to the field. Taken together, our findings suggest that sunflower pollen may serve as a novel tool to manage bee disease dynamics.

## Results

**Effects of pollen diet on *Crithidia* in bumble bees.** We first tested the hypothesis that pollen from different plant species varies in its effects on bumble bee infection intensities. We focused on three monofloral pollens commonly grown in large monocultures in agroecosystems and visited by bees: rape (*Brassica campestris*), sunflower (*Helianthus annuus*), and buckwheat (*Fagopyrum cymosum*), as well as a mixed diet composed of the three monofloral pollens. We experimentally inoculated bees with *Crithidia*[20], provided them with monofloral pollen diets of each species or the pollen diet mix, and measured subsequent infection intensity. Sunflower pollen significantly reduced *Crithidia* infection in bumble bees compared to all other pollen diets ($\chi^2_{(3)} = 111.2$, $P < 0.001$). Infection levels were 20- to 50-fold lower in bees fed sunflower pollen than either rape or buckwheat pollen, respectively (Fig. 1.1A). Moreover, two-thirds of the sunflower-fed bees had no detectable infection after one week of treatment. We found no effect of pollen diet on bee survival ($\chi^2_{(3)} = 4.04$, $P = 0.257$; Fig. 1.1B; Fig. S1.1; Appendix A: Supplementary Information: Text 1), suggesting minimal mortality costs. In a separate experiment, we allowed infection levels to build for one week before providing pollen treatments, and we found a 5- to 8-fold reduction of infection within bees fed sunflower pollen compared to a wildflower pollen mixture or buckwheat pollen, respectively ($\chi^2_{(2)} = 17.2$, $P < 0.001$; Fig 1.1C).

The medicinal effects of sunflower pollen were consistent across pathogens collected from two locations, as well as with two different sources of sunflower pollen. In addition to our

original results using *Crithidia* from Massachusetts, USA (Fig. 1.1A), sunflower pollen also reduced *Crithidia* infection intensity by 30-fold in a separate experiment where bees were infected with a pathogen lineage isolated in North Carolina, USA (Pollen treatment $\chi^2_{(1)} = 30.7$, $P < 0.001$; Fig. S1.2). We also compared domesticated sunflower pollen from two sources (**Methods**). Both showed medicinal effects compared to bees fed a wildflower pollen mixture (Pollen treatment: $\chi^2_{(2)} = 23.6$, $P < 0.001$; Fig. 1.1D), with sunflower pollen reducing disease at least 4-fold. There was no difference in pathogen reduction between the two sunflower sources ($Z = 0.601$, $P = 0.82$). Furthermore, the medicinal effects of sunflower pollen were not associated with any potential pesticide residues in the pollen diets (Appendix B: Table S1.1).

**Costs and benefits of sunflower pollen on bee health, reproduction and *Crithidia*.** To ask how sunflower pollen affects bee performance, we conducted a factorial experiment using microcolonies of queenless workers with infection (yes or no) crossed by pollen diet treatment (sunflower or buckwheat). We used buckwheat as a comparison to sunflower pollen because it supported high *Crithidia* levels (Fig. 1.1A) but has a similar protein content as sunflower[28], allowing us to compare pollens of relatively similar protein content but different effects on *Crithidia*. Over the course of the experiment, bees consumed more sunflower than buckwheat pollen ($\chi^2_{(1)} = 66.67$, $P < 0.001$; Fig. S1.3; Appendix A: Supplementary Information: Text 2), suggesting that the medicinal benefits of sunflower pollen were not due to lower pollen consumption, which can independently reduce *Crithidia* infection[29]. Consumption of sunflower pollen significantly increased nearly every measure of microcolony reproduction compared to buckwheat pollen, including number of eggs, larval number and mass, and probability of producing pupae ($P < 0.004$ in all cases; Fig. 1.2), but was marginally associated with increased

worker mortality ($\chi^2_{(1)} = 3.78$, P = 0.051; Fig. S1.4). Moreover, infection reduced egg production in bees fed buckwheat but not sunflower (Infection x Pollen interaction $\chi^2_{(1)} = 10.34$, P = 0.0013; Fig. 1.2D), indicating that for this performance metric, sunflower pollen consumption can alleviate the negative effects of infection.

**Effects of pollen diet on *Nosema* in honey bees.** Having shown strong, consistent reductions in infection in bumble bees fed sunflower pollen, we then tested the effect of sunflower pollen on the pathogen *Nosema* in European honey bees. We experimentally infected groups of honey bees with *Nosema* and then fed them either buckwheat pollen, sunflower pollen, or no pollen as a negative control. At both 10 d (Z = -4.72, P < 0.001) and 15 d (Z = -3.06, P = 0.006) post-infection, sunflower pollen reduced *Nosema* infection in honey bees relative to buckwheat pollen (Fig. 1.3A; Appendix A: Supplementary Information: Text 3). Averaged across both time periods, infection intensity was 29% lower in sunflower- than buckwheat-fed bees, although still more than twice as high as in bees denied pollen. Despite this reduction in infection, the consumption of sunflower pollen came at a cost of increased mortality relative to buckwheat-fed bees (hazard ratio = 3.8, Z = 5.175, P < 0.001) and was similar to mortality in bees given no pollen (Z = -0.75, P = 0.74; Fig. 1.3B and Fig. S1.5).

**Effect of sunflower plantings on *Crithidia* in bumble bees at the farm scale.** We sampled worker *B. impatiens* from 22 farms (approx. mean distance of 2.5 km between farms) in western Massachusetts, USA, between July 27 and September 18, 2015. Farms ranged in size between 0.3 ha and 62.9 ha, with an average size of 16.3 ± 3.9 ha (Mean ± SE). We found a significant, negative relationship between the area of sunflower planted on farms and *Crithidia* infection

intensity (linear mixed model $\beta = -0.26 \pm 0.010$ SE, likelihood ratio $\chi^2_{(1)} = 6.88$, $P = 0.009$; Fig. 1.4). This corresponds to a 23.2% decrease in infection intensity on the linear scale (95% CI: 6.25% to 37.0%) for every 10-fold increase in sunflower area, or a 50% decrease for every 425-fold increase in sunflower area. Sampling date and sunflower area were not confounded (Pearson's $r = 0.02$). However, there was a significant decrease in infection intensity over the course of the sampling period, which spanned 52 d from the beginning of August through late September ($\beta = -0.038 \pm 0.013$ SE, likelihood ratio $\chi^2_{(1)} = 14.46$, $P < 0.001$). We sampled farms with different management practices (organic and conventional) and different varieties of sunflower (see **Methods**), but farm did not explain significant variation in infection intensity ($\chi^2_{(1)} = 0.24$, $P = 0.62$).

## Discussion

Sunflower pollen dramatically and consistently reduced *Crithidia* in bumble bees across a series of laboratory experiments. Sunflower pollen also resulted in greater bumble bee microcolony reproduction than buckwheat pollen, which matched sunflower pollen in protein content but did not reduce *Crithidia* infection. Additionally, these results were reflected in a field survey of pathogen infection intensity, which was reduced on farms with greater sunflower area. Sunflower is a common native plant in much of the US and is widely planted for agriculture worldwide. Thus, the consequences of this bee-pathogen-sunflower interaction may be widespread; in the US, almost two million acres are planted with sunflower[30], and in Europe, about ten million acres are planted with sunflower annually[31]. Furthermore, we found a significant relationship between infection intensity and sunflower coverage without considering other factors that could also explain variation in parasite infection, such as farm management

practices, farm size, other flowering crops, or landscape context. Thus, while there is substantial unexplained variation in *Crithidia* infection intensity (Fig. 4), our results suggest that the relationship between infection and sunflower plantings is consistent across a wide range of contexts.

We also found that sunflower pollen reduced *Nosema* in honey bees, although the effect was less dramatic than it was for *Crithidia* in bumble bees. Our results are consistent with previous work that demonstrates *in vitro* antimicrobial effects of secondary metabolite extracts from sunflower pollen against bacteria and fungi[32] and the medicinal value of sunflower honey against *Nosema* in honey bees[33], but vastly expands the breadth and medicinal potential of sunflower pollen by demonstrating dramatic reductions in a distantly related pathogen of bumble bees. However, despite the reduction in *Nosema* infection, the consumption of sunflower pollen by honey bees came with a cost of greater mortality. Thus, any anti-parasitic benefits of sunflower pollen need to be viewed within the context of mortality costs for honey bees. Future work that examines the relative benefits of sunflower pollen consumption across a variety of doses for healthy vs. infected bees may indicate appropriate procedures for use as commercial dietary supplements.

Although our experiments were not designed to determine the mechanism(s) behind the medicinal effects of sunflower, the results allow us to rule out some potential mechanisms and suggest others for future research. The nutritional components of sunflower, buckwheat, rape, and many other species of pollen have been explored[12,15,28,34,35], and a low protein pollen diet may induce physiological costs[15,36] and reduce longevity of parasitized bees[21]. Although honey bee-collected sunflower pollen is notably low in total protein compared to pure pollen collected directly from flowers,[28,35] buckwheat is equally low[28] (both 14-15 g/100 g compared to 27 g/100

g in rape), suggesting that low protein is not the mechanism. Similarly, although sunflower pollen is low in some amino acids including methionine, glutamic acid and proline[28,35], all of these components are also relatively low in buckwheat pollen and much higher in rape pollen[28], which does not correspond with relative medicinal effects. By contrast, concentrations of some key fatty acids correspond with relative medicinal quality (i.e., highest in sunflower, intermediate in rape, and low in buckwheat), including linoleic, decanoic and lauric acids, which are antimicrobial in honey bee brood combs[37], and must be acquired in the diet[38]. Because fatty acids are ubiquitous components of pollen[37], identifying fatty acids that affect bee disease could have broad implications for discovering additional medicinal pollens, as well as breeding medicinal traits.

There are four additional, non-mutually exclusive hypotheses that could explain the medicinal properties of sunflower pollen. First, Asteraceae pollen is notable for its conspicuous spines on the outer coat[39]. Given that *Crithidia* is a gut parasite that attaches to the hindgut wall[40], sunflower pollen could reduce parasitism by scouring the hindgut of parasite cells. Second, if sunflower pollen has laxative properties, it may decrease gut passage time and flush *Crithidia* and *Nosema* from bumble and honey bees before the pathogens can adhere to the gut. In a similar vein, the nectar alkaloids nicotine and anabasine can reduce *Crithidia* infection[20], and these compounds also reduced gut passage time and sugar assimilation in the Palestine sunbird[41]. Although gut physiology is certainly different between sunbirds and bees, these results are consistent with the hypothesis that diet components could act as laxatives. Third, sunflower pollen could affect insect immune function. Recent work showed that sunflower pollen extract increased rather than suppressed *Crithidia* growth *in vitro*[42], suggesting that the effect of sunflower pollen on *Crithidia* may be mediated by the bee host environment. Insect immune

function occurs through a variety of processes, including melanization and encapsulation of foreign material, which is initiated by the activation of phenoloxidases[43]. It is possible that sunflower pollen may mediate *Crithidia* infection through changes in PO activity, encapsulation, or fat body production. Fourth, indirect pathogen resistance may also be mediated through changes in the host microbiome rather than the host itself. Gut microbiota can play a key role in *Bombus* resistance to *Crithidia*[44,45], and diet can alter bee microbiome communities[46]. Thus, sunflower pollen may increase bee resistance to *Crithidia* via changes in the gut microbiome. Further research is needed to address each potential mechanism.

In nature, pollen consumption by bees will be affected not only by availability, but also by bee preference. For example, bumble bees prefer to visit plant species that produce pollen with a high protein to lipid ratio[17]. However, bees can also alter foraging preferences when infected by *Crithidia*[47,48]. Interestingly, infection with *Nosema* increased honey bee attraction to sunflower honey, which also reduced infection[33], suggesting self-medication behavior. However, honey bees have also demonstrated a relatively low attraction to sunflowers, preferring to forage on other plants surrounding sunflower fields, including corn, clover and flowering trees[49,50]. Controlled experiments that assess bee preference for sunflower as a function of pathogen infection will yield important ecological insights. In addition to foraging preference, farm management practices can also shape bee disease dynamics. For example, a greater use of the fungicide cholorothalonil was positively related to *Nosema* prevalence in four declining North American bumble bee species[9]. Interestingly, we found that the negative relationship between infection and sunflower crop area was robust to farm management practices (organic vs. conventional). Nonetheless, understanding bee disease dynamics at the landscape level will

require knowledge of the combined effects of flower species identity, bee foraging preferences, and interactions with farm management practices.

For both food security and biodiversity conservation, there is a critical need to move beyond documentation of pollinator declines and identify solutions to reduce bee disease and improve bee health. Sunflower pollen reduced the severity of infection by pathogens in bee species that are important for pollination services in natural and agroecosystems. Many beekeepers already provide pollen supplements to their colonies, and all levels of government, as well as growers, nonprofits, and the general public, are investing in plantings to improve pollinator habitat[51]. As both a domesticated crop and native wild species, sunflower could be prioritized for inclusion in agroecosystems and regionally appropriate native habitat. Our discovery that sunflower pollen reduced infection of multiple bee pathogens suggests the potential for simple, easily implemented approaches that could be tested for their ability to reduce disease and increase bee health.

## Methods

**Effects of pollen diet on *Crithidia* in bumble bees.** *Crithidia* inoculum. Infected ('source') colonies were used to make *Crithidia* inoculum. The original *Crithidia* cells infecting colonies came from three wild *B. impatiens* workers collected from Stone Soup Farm (Hadley, MA, USA: 42.363911 N, -72.567747 W) unless otherwise noted. To make inoculum, bees were dissected from the source colony daily using an established protocol[20]. Bee digestive tracts (excluding the honey crop) were removed, placed into a 1.5 mL microcentrifuge tube with 300 µL of 25% strength Ringer's solution (Sigma-Aldrich, St. Louis, MO, USA), finely ground, and vortexed for 5 seconds. Each sample was allowed to rest at room temperature for 4-5 hours. *Crithidia* cells

were counted from a 0.02 µL sample per bee with a Neubauer hemacytometer[20]. We mixed 150 µL of the supernatant with 25% strength Ringer's solution to achieve a concentration of 1200 cells $\mu L^{-1}$. The inoculum was then mixed with an equal volume of 50% sucrose solution to yield inoculum with 600 cells $\mu L^{-1}$ and 25% sucrose. Experimentally infected bees were starved for 4-6 hours and then fed a 10 µL drop of inoculum with 6,000 *Crithidia* cells, which is within the range of concentrations bees are exposed to when foraging on flowers in the wild[52]. Only bees that consumed the entire droplet were used in experiments.

*Monofloral and mixed pollen.* Monofloral pollen diets (rape, sunflower or buckwheat - *Brassica campestris*, *Helianthus annuus* and *Fagopyrum cymosum*, respectively) were obtained by sorting honey bee collected pollen pellets (Changge Hauding Wax Industry, China) initially by color. We then verified microscopically that pollen pellets within treatment were morphologically consistent and as expected for that species. Pollen was provided to bees as a paste made by mixing ground pollen pellets with distilled water to achieve a uniform consistency, which required different amounts of water depending on pollen species (pollen: water ratio: sunflower & buckwheat: 5:1; rape: 1.67:1; pollen mix of equal weights of the three monofloral pollens: 3.33:1).

Newly emerged adult worker bees (callows) obtained from pupal clumps were removed from six uninfected *B. impatiens* colonies (n = 272 bees). All *B. impatiens* colonies were provided by BioBest LTD (Leamington, Ontario, Canada), and experimental colonies were confirmed to be pathogen-free bi-weekly by screening five workers (see *Crithidia* inoculum). We regularly supplied all colonies with pollen loaves made of 30% sucrose solution mixed with ground honeybee–collected wildflower pollen (Koppert Biological Systems; Howell, MI, USA). Each day, newly emerged callows were collected from pupal containers, weighed to the nearest

0.01 mg, and randomly assigned to one of the four pollen diets. Bees were randomly assigned to treatment within experimental colony and, when relevant, date of emergence, for all experiments here and below. Bees were housed individually in a growth chamber in darkness at 28°C and fed 500 µL of 30% sucrose solution and a small ball of their respective pollen treatment daily for 9 days. Bees were inoculated two days after emergence, so that bees consumed their respective pollen treatments both before and after infection.

*Crithidia* infection intensity was measured as *Crithidia* cells per 0.02 µL (hereafter "cell counts") one week after bees were infected (n = 234 bees due to mortality). After 7 d, *Crithidia* infection intensity reaches a sufficient level for measurement within the bee host[53]. Each experimental bee was dissected (see *Crithidia* inoculum). We removed the right forewing of each bee and mounted them on glass slides to measure radial cell length, a proxy for bee size[54].

*Consistency with a different pathogen strain. Crithidia* infection can be heavily influenced by genotypic variation in hosts and pathogens[55], which may yield genetically distinct strains with varying susceptibility to host immune defenses[56] and potentially responses to pollen diet. Thus, we repeated our experiment testing the effects of pollen diet on a different set of colonies infected with a strain obtained from wild *B. impatiens* collected in Raleigh, North Carolina, USA (J.C. Roulston Arboretum: 35.794056 N, -78.698186 W). Given the strong negative effects of sunflower pollen on *Crithidia* (see Fig. 1.1A), we used only sunflower pollen (*H. annuus*) and buckwheat pollen as our control (*F. cymosum*). In addition, adult workers (rather than newly emerged callow bees) were used in this experiment to ensure that results were consistent across bees of varying ages. Worker bees were used from three colonies, and bees were inoculated and *Crithidia* pathogen loads were measured (n = 149 bees).

*Effect of diet post-infection.* We asked whether sunflower pollen could reduce *Crithidia*

infection in bees that already reached their asymptotic infection levels. Individual *B. impatiens* adult workers from three colonies were inoculated with *Crithidia* (North Carolina, USA strain) and fed a wildflower pollen mixture (Koppert Biological Systems; Howell, MI, USA) and 30% sucrose solution for 7 days. Each bee was then randomly assigned to one of three pollen diets: sunflower, buckwheat, or the same wildflower mix for 7 more days. By including a wildflower mix pollen treatment, we were able to compare monofloral pollen treatments to a more natural and diverse mix of pollens. Bees were then sacrificed (n = 74) and *Crithidia* pathogen loads were measured.

*Consistency using two sources of sunflower pollen*. Domesticated sunflower is a major oil crop distributed worldwide[35]. Breeding practices have modified a wide array of economically important traits, including seed and oil production[57], resistance to plant diseases and pests[58], and resistance to drought[59]. We compared the medicinal effects of sunflower pollen from China versus sunflower pollen from the USA. Adult *B*. *impatiens* workers from three colonies were inoculated with *Crithidia* (n = 120 bees) and fed either sunflower pollen collected from an organic farm in Wisconsin, USA (44.731641 N, -91.948666 W, Cobalt II cultivar - NuSeed Inc.), sunflower pollen collected in China (Changge Hauding Wax Industry, China), or the wildflower pollen mixture. We measured pathogen loads (n = 110 bees) after 7 days.

*Statistical analyses.* All statistical analyses here and below were conducted using R version 3.1.2[60] (Supplementary Information: Methods 1). To test how pollen diets affected *Crithidia* infection intensity, generalized linear mixed models were used to analyze *Crithidia* cell counts using "glmmTMB"[61], with pollen diet as a fixed effect, bee size as a covariate, and experimental bee colony and inoculation date (if applicable) as random effects. Significance of terms was evaluated with a likelihood ratio chi-squared test, implemented via the "drop1()"

function. Tukey's HSD tests were used for post hoc pairwise comparisons. All bees that died before their scheduled dissection date were excluded from analyses. To test how pollen diets affected bee survival, mixed-model Cox proportional hazards tests were used[62], with pollen diet and bee size as fixed effects, and inoculation date (if applicable) and experimental bee colony as random effects. To assess the effects of pollen diet on mortality, log-likelihood of models were compared with and without pollen diet treatment as a predictor. Significance of terms was tested with a Wald chi-squared test, implemented via the Anova function in package "car"[63]. Plots (here and throughout) were produced with ggplot2[64], survminer[65] and cowplot[66].

**Costs and benefits of sunflower pollen for bee health, reproduction and *Crithidia*.** Using queenless *B*. *impatiens* microcolonies, we tested the impact of pollen diet and *Crithidia* infection on mortality, reproduction and *Crithidia* infection in a 2x2 factorial design manipulating pollen diet (sunflower or buckwheat) and *Crithidia* infection (uninfected or infected). When unmated workers are isolated from the queen, one will gain dominance and lay haploid (male) eggs. Microcolonies are an effective approach to estimate the effects of diet and pathogen infection on whole-colony reproduction[15,20,67]. We used 20 replicate microcolonies per treatment for a total of 80 microcolonies, carried out in two rounds (or blocks) of 40 microcolonies, with five workers per microcolony. The first 40 microcolonies were constructed using workers from two colonies, with 5 replicates per treatment per colony of origin. The second set of 40 microcolonies were constructed from two new colonies of origin.

Microcolonies were randomly assigned to infection and diet treatments within rounds and colonies of origin. Bees were inoculated with *Crithidia* as in '*Crithidia* inoculum' or given a sham control inoculum of 10 µL of sucrose solution without *Crithidia* cells. We maintained

microcolonies in a growth chamber at 28˚C in darkness and fed them 400 mg of pollen each and ad libitum 30% sucrose solution, replaced and replenished 5 d per week. Pollen diets were made as in 'Monofloral and mixed pollen'. We measured pollen and sucrose solution consumption (in g) 5 days per week, calculating the total mass consumed (or used) per bee per hour. Pollen consumption was corrected for evaporation by subtracting the average weight lost to evaporation over 24 hr for each pollen type. To determine the average weight lost to evaporation, 15 samples of each pollen type were placed into empty microcolony containers without bees and in the same growth chamber for 24 hr. Each pollen sample was weighed at 0 hr and at 24 hr to determine the net weight lost to evaporation.

For each microcolony, we recorded the date of first eggs laid, male emergence and weight (which occurred in 5 of the 80 microcolonies) and worker mortality. Microcolonies were terminated 35 days post-egg laying, or if 4 out of the 5 worker bees died. We then measured *Crithidia* infection in the remaining worker bees (see *Crithidia* inoculum) and bee size. For each microcolony, the number of eggs, larvae, and pupae produced was counted and weighed. Because bees within microcolonies can vary in size and social dominance, which can affect food consumption and microcolony reproduction, we calculated a metric of within-microcolony size dimorphism [(largest bee radial cell/smallest bee radial cell)-1][20,68,69] for use as a covariate.

To analyze pollen and nectar consumption, *Crithidia* infection intensity, and microcolony reproduction, generalized linear mixed effects models were fit with distributions specific to the type of data analyzed (Appendix A: Supplementary Information: Methods 2). Unless otherwise noted, all models included fixed effects of pollen diet (sunflower or buckwheat), infection treatment (infected or uninfected), and (when significant) their interaction. All statistical tests included block as a random effect, which corresponded to microcolonies inoculated on the same

day. The block effect accounted for variation due to colony of origin (because each inoculation day used a different colony of origin) and variation due to different inoculation dates.

**Effects of pollen diet on *Nosema* in honey bees.** Newly emerged worker honey bees from three colonies were mixed together and placed into cages in groups of 50 bees per cage[70] with 50% sucrose solution. We experimentally infected the bees in each cage using a *Nosema* spore sucrose solution with a concentration of approx. 333,333 spores per bee[71,72]. Cages were randomly assigned to a pollen diet treatment and given a single 20 g ball of sunflower or buckwheat pollen paste, or no pollen as a negative control for 15 days. Prior studies have shown that *Nosema*-infected honey bees that do not consume pollen have significantly lower *Nosema* infection intensity than bees provided with pollen[71]. There were 11-12 replicate cages per pollen diet treatment. On days 10 and 15, samples of five bees and 10 bees per cage, respectively, were sacrificed to quantify *Nosema* infection intensity[71,72]. Any bees that died during the experiment were counted and removed from their cages.

We used generalized linear mixed effects models (R package glmmTMB) to test whether pollen diet affected *Nosema* infection intensity (spores per mL) on days 10 and 15 (Appendix A: Supplementary Information: Methods 3). *Nosema* infection intensity was used as the response variable; pollen treatment, days since inoculation, and their interaction were used as fixed predictors; cage was included as a random effect to account for repeated measures on each cage. Differences in survival were tested using a Cox Proportional Hazards mixed-effects model fit using "coxme"[62], with pollen diet as a fixed effect and cage as a random effect.

**Effect of sunflower plantings on *Crithidia* infection in bumble bees at the farm scale.** Bees were collected directly from sunflowers if available, or else from a variety of flowering crops. Each farm was sampled on a single date. We quantified the area of sunflower grown at each farm in $m^2$. We sampled a total of 667 *B. impatiens* workers (range: 19 – 62 bees per farm); all bees were sacrificed and we measured *Crithidia* infection (as in *Crithidia* inoculum).

We tested for spatial autocorrelation using a Monte-Carlo Mantel test and a Moran's I test using the "ape" and "ade4" packages in R[73,74]. We found no indication of spatial autocorrelation ($P > 0.15$), and so considered farms to be independent sampling locations. We analyzed infection intensity (*Crithidia* cell counts) with a generalized linear mixed model with negative binomial error distribution using the "glmmTMB" package in R[61]. Sunflower area ($\log_{10}$ area ($m^2$)) and Julian date of sampling were used as fixed covariates; farm was included as a random effect to account for non-independence of bees within a farm. Sampling date and sunflower area were not confounded (Pearson's $r = 0.02$). Significance of predictors was tested by likelihood ratio chi-squared tests, implemented via the "drop1" function in R.

**Pesticide analysis.** To ensure that results were not associated with pesticide residues on pollen, the USA and Chinese sunflower, buckwheat, and the wildflower mix pollens were analyzed for 213 pesticides and other agrochemicals (Agricultural Marketing Services' National Science Laboratories, United States Department of Agriculture, Gastonia, NC USA) (Appendix B: Supplementary Information: Table 1).

**Data Availability.** All data generated from this project will be archived in the Dryad Digital Repository upon acceptance. Custom scripts used for statistical analysis and plotting will be available on Github upon acceptance.

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## Author contributions

JJG, JL, LSA, DRT, and REI designed research; JJG and JL performed research; JJG and EPY analyzed data; JJG, LSA, REI and JL wrote the paper with feedback from all authors.

## Additional information

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

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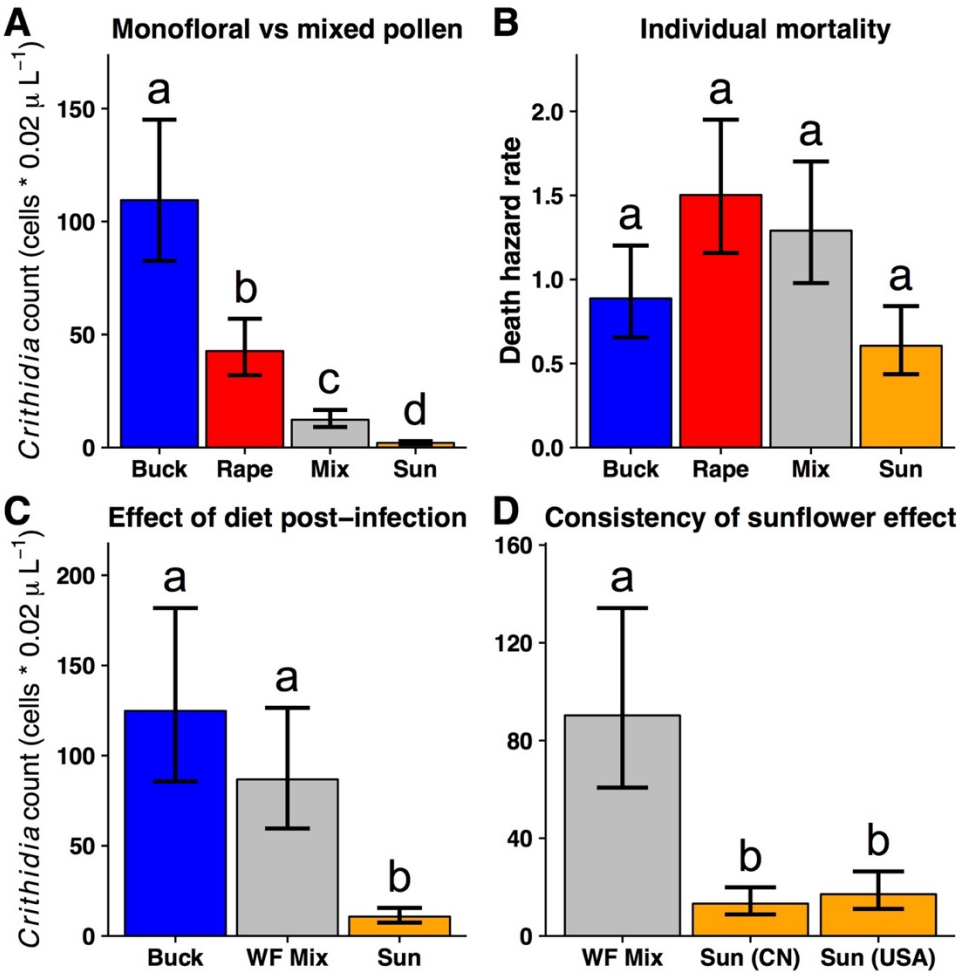
**Figure 1.1**. (A) Effects of pollen diets on *Crithidia* infection in individual *Bombus impatiens* workers. Bees were inoculated with *Crithidia* and fed a monofloral pollen diet commonly grown in large monocultures in agroecosystems: sunflower (*Helianthus annuus*; Sun), buckwheat (*Fagopyrum cymosum*; Buck), rapeseed (*Brassica campestris*; Rape), or a mixed diet composed of equal weights of the three monofloral pollens (Mix). (B) Pollen diets did not significantly affect rate of worker death over the 7 d experiment shown in (A). Y-axis shows exponentiated hazard rates ± 1 standard error. (C) *Crithidia* infection was allowed to build for one week post-inoculation before providing pollen treatments: sunflower (Sun), buckwheat (Buck), or a wildflower pollen mixture (WF Mix). (D) Inoculated bees were fed sunflower pollen from two sources, China (CN) or USA (USA), or a control wildflower pollen mixture (WF Mix). Bars and error bars indicate negative binomial model means ± 1 standard errors back-transformed (i.e., exponentiated) from the scale of the linear predictor. *Crithidia* counts represent raw counts of cells diluted in a gut homogenate. Error bars represent uncertainty in fixed effects portions of models only, and do not account for variability due to random effect. Different letters above each bar within panels indicate significant differences based on Tukey's HSD tests.


A
Monofloral vs mixed pollen
Crithidia count (cells * 0.02 µL⁻¹)
150
100
50
0
a
b
c
d
Buck
Rape
Mix
Sun
B
Individual mortality
Death hazard rate
2.0
1.5
1.0
0.5
0.0
a
a
a
a
Buck
Rape
Mix
Sun
C
Effect of diet post−infection
Crithidia count (cells * 0.02 µL⁻¹)
200
150
100
50
0
a
a
b
Buck
WF Mix
Sun
D
Consistency of sunflower effect
160
120
80
40
0
a
b
b
WF Mix
Sun (CN)
Sun (USA)

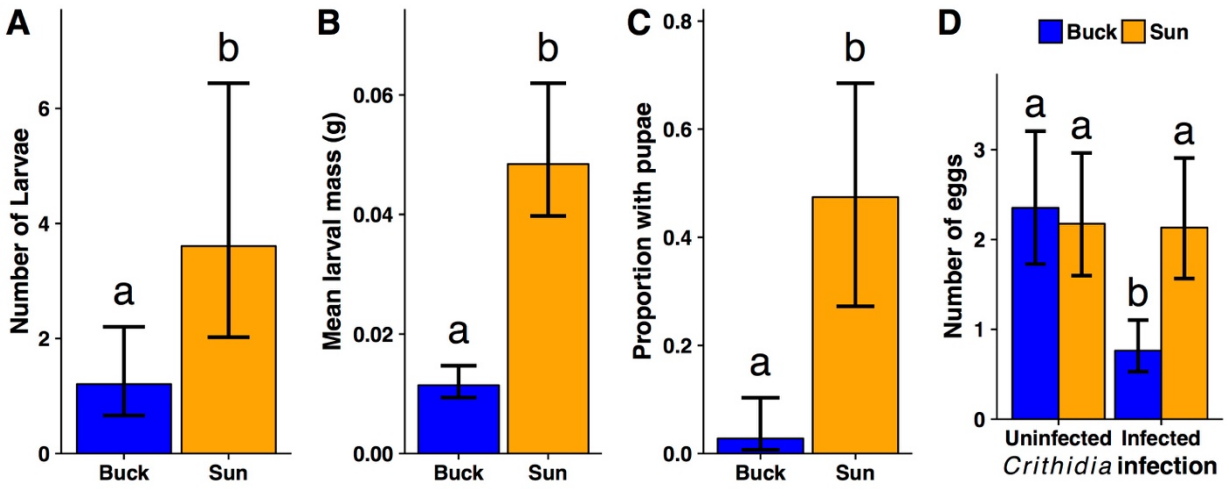



**Figure 1.2**. *Bombus impatiens* microcolony performance. Microcolonies were fed either buckwheat (Buck) or sunflower (Sun) pollen diets and either inoculated with *Crithidia* (Infected) or a *Crithidia*-free control solution (Uninfected). Infection did not significantly affect responses in A-C and so responses were averaged across infection treatments for these panels. (A) Mean number of larvae produced, (B) mean total larval mass, (C) proportion of microcolonies that produced pupae during the experiment, and (D) mean number of eggs produced. *Crithidia* infection reduced egg production in microcolonies fed buckwheat pollen, but not sunflower pollen. For all panels, error bars indicate ± 1 standard error. Error bars represent uncertainty in fixed effects portions of models only, and do not account for variability due to random effect.

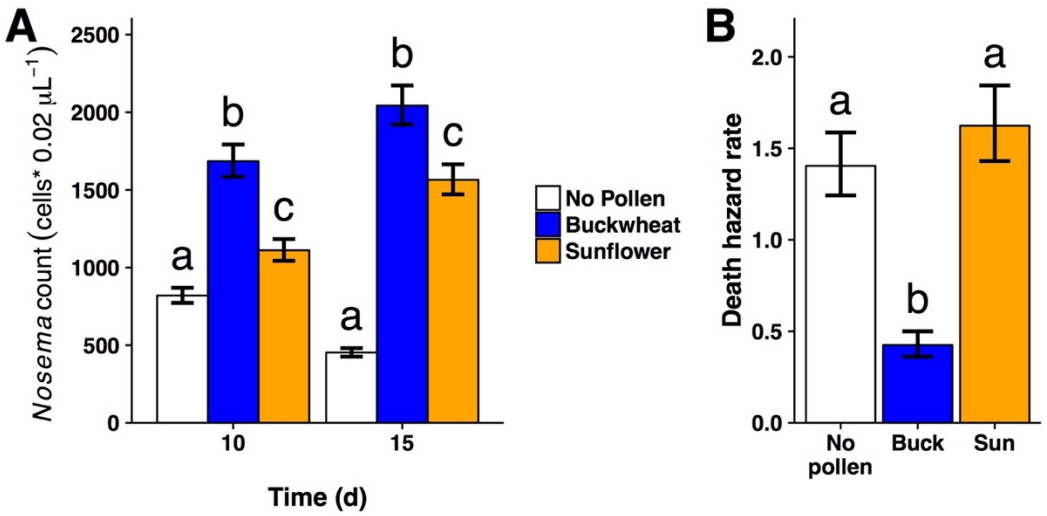



**Figure 1.3**. Effects of pollen diet on *Nosema* infection in honey bees (*Apis mellifera*). (A) Sunflower pollen reduced *Nosema* infection in honey bees by an average of 29% compared to buckwheat pollen across the two time periods. Bars and error bars indicate negative binomial model means and standard errors, back-transformed (i.e., exponentiated) from the scale of the linear predictor. Error bars represent uncertainty in fixed effects portions of models only, and do not account for variability due to random effect. (B) Exponentiated hazard rates ± 1 standard error for mortality on different pollen diets. Sunflower-fed bees died at nearly four times the rate of buckwheat-fed bees and had equivalent survival to bees with no pollen. Lower-case letters indicate significant differences based on post hoc pairwise comparisons; in (A), comparisons are made within each time point (10 d and 15 d).

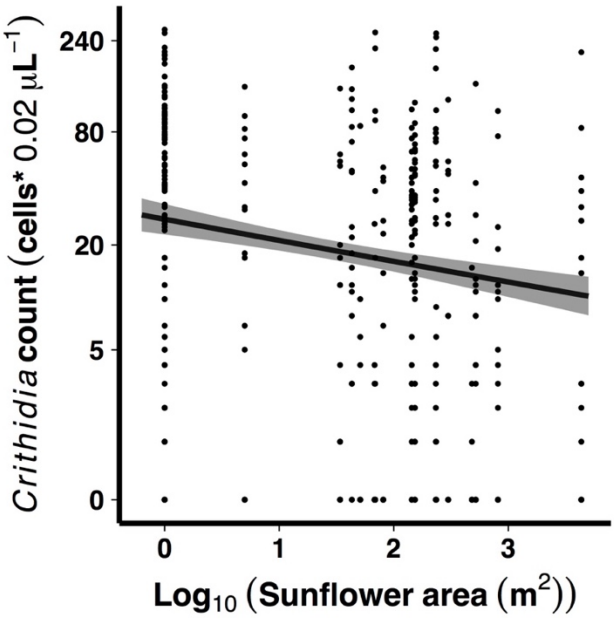



**Figure 1.4**. Negative relationship between the area of sunflower planted on farms and *Crithidia* infection intensity in *Bombus impatiens* workers. Line and shaded band indicate back-transformed mean *Crithidia* counts for area of sunflower planted $\pm$ 1 standard error; points show counts for individual bees.

## CHAPTER 2: The costs and benefits of sunflower pollen on bumble bee colony disease and health.

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### Abstract

Pathogen transmission between domesticated and wild host species has important implications for community ecology, agriculture and wildlife conservation. Bumble bees provide valuable pollination services that are vital for both wildflowers and agricultural production. Intense concerns about pathogen spillover from commercial bumble bees to wild bee populations, and the potential harmful effects of pathogen spillback to commercial bees, has stimulated a need for practical strategies that effectively manage bumble bee infectious diseases. Here, we assessed the costs and benefits of a medicinal sunflower pollen diet (*Helianthus annuus*) on whole-colony bumble bee disease and performance using commercial colonies of the common eastern bumble bee, *Bombus impatiens*, and its protozoan pathogen, *Crithidia bombi* (Trypanosomatida). We first found that a 1:1 mixture of sunflower combined with wildflower pollen reduced *C. bombi* infection prevalence and intensity within individual *B. impatiens* workers by nearly 4-fold and 12-fold, respectively, relative to wildflower pollen. At the colony level, a 1:1 mixture of sunflower and wildflower pollen reduced *C. bombi* infection prevalence by 11% averaged over a 10-week period and infection intensity by 30% relative to wildflower pollen. Colony performance was similar between pollen diets and infection treatments, including the number of workers and immatures produced, and size and weight of workers, drones and queens. Infection significantly reduced the probability of queen production in colonies fed a pure

wildflower pollen diet, but not colonies fed a mixed sunflower pollen diet, suggesting that the medicinal benefits of a mixed sunflower pollen diet can reverse the negative effects of infection on reproductive success. This study provides evidence that sunflower pollen as part of a mixed pollen diet can reduce infection in individual bees and whole colonies with no significant nutritional tradeoffs for colony worker production and most aspects of colony reproduction. A supplemental mixed sunflower pollen diet may provide a simple and effective solution to reduce disease and improve the health of economically and ecologically important pollinators.

**Key Words**

Tri-trophic interactions, disease ecology, parasitology, conservation, pollination biology, bee health, pollination services

## Introduction

Many pathogens can infect both domesticated and wild host species, creating the potential for pathogen transmission (Daszak et al. 2000, Dobson and Foufopoulos 2001, Power and Mitchell 2004), which can have important implications for community ecology, agricultural production, and species conservation (Lafferty and Gerber 2002, McCarthy et al. 2007, Pedersen et al. 2007). Consequently, the control of pathogens in domesticated species is paramount. For example, bumble bees are critically important for the conservation of plant biodiversity and provide pollination services vital to agricultural production (Klein et al. 2007, Ollerton et al. 2011, Potts et al. 2016). Since the late 1980's, domesticated bumble bee colonies have been used extensively for crop pollination services (Shipp et al. 1994, Whittington and Winston 2004, Velthuis and Van Doorn 2006). The increasing use of domesticated bumble bee species both

within and outside of their native ranges (Velthuis and Van Doorn 2006, Looney et al. 2019), along with high prevalence of infectious diseases (Arbetman et al. 2013, Graystock et al. 2013, Sachman-Ruiz et al. 2015), has generated concerns about pathogen spillover from domesticated to wild bee populations. (Colla et al. 2006, Otterstatter and Thomson 2008). *Furthermore, effective management of the pollination potential of commercial bumble bee colonies requires consideration of pathogen spillback. The transmission of pathogens from wild to domesticated bees could have deleterious effects on* worker bee abundance, life-span and foraging behavior (Cornman et al. 2012, Koch et al. 2017). *Thus,* strategies are needed that effectively manage bumble bee infectious diseases.

Diet may play a key role in mediating host-pathogen dynamics for bees. Pollen is a primary source of nutrition for bees and contains multiple nutritional components, including protein, lipids, amino acids, vitamins and carbohydrates, each of which vary widely in composition and concentration among plant taxa (Roulston and Cane 2000, Yang et al. 2013). Various aspects of pollen quality and nutrition have been linked to bumble bee colony growth and reproduction (Schmid-Hempel and Schmid-Hempel 1998, Vaudo et al. 2018), and increasing evidence points to pollen's role in bee-pathogen dynamics. For example, given that immune systems are energetically costly (Sheldon and Verhulst 1996), poor nutrition can weaken the bee immune system and increase symptoms of pathogen infection (Alaux et al. 2010, Di Pasquale et al. 2013, Roger et al. 2017). Conversely, poor host nutrition may affect the availability of resources for the pathogen, limiting pathogen growth and reproduction (reviewed in Pike et al. 2019). Pollen also contains a high diversity of plant secondary compounds, often orders of magnitude higher concentrations than found in nectar and vegetative tissues (Cook et al. 2013, Palmer-Young et al. 2018, 2019). Plant secondary compounds can mediate bee-pathogen

interactions by reducing parasitism, although this effect has mostly been studied within the context of nectar (Manson et al. 2010, Richardson et al. 2015, Koch et al. 2019).

Optimizing bumble bee health management requires the careful consideration of key plant species that play disproportionate roles in protecting pollinators against pathogens, as well as nutritional needs associated with colony growth and reproduction. The consumption of sunflower pollen (*Helianthus annuus*) greatly reduces the intensity of infection by the trypanosome *Crithidia bombi* in bumble bee, *Bombus impatiens*, workers (Giacomini et al. 2018, LoCascio et al. 2019, Adler et al. 2020). Giacomini et al. (2018) found that more than two-thirds of bees that consumed sunflower pollen had no detectable infection after one week, and the intensity of infection was reduced 20- to 50-fold compared to other pollen diets. Sunflower pollen also resulted in greater bumble bee microcolony reproduction (i.e., production of male drones) compared to buckwheat (*Fagopyrum cymosum*) pollen, which matched sunflower pollen in crude protein content but did not reduce *C. bombi* infection (Giacomini et al. 2018). These results suggest that crude protein content is not the mechanism behind the medicinal effect of sunflower pollen in bumble bees. However, sunflower pollen is traditionally considered a poor quality diet for bumble bees due multiple factors, including relatively low protein content (Yang et al. 2013), deficiency in three essential amino acids for bee development (Nicolson and Human 2013), and conspicuous spines on the outer pollen wall (Blackmore et al. 2007), which may act as a digestive barrier that prevents nutrient assimilation (Vanderplanck et al. 2018). For sunflower pollen to be an effective treatment for reducing pathogens in domesticated bumble bee colonies, the benefits of pathogen reduction need to outweigh the nutritional costs of sunflower pollen consumption for colony growth and reproduction.

The goal of this study was to assess the costs and benefits of sunflower pollen on whole-colony bumble bee disease and performance. We used commercial colonies of the common eastern bumble bee *B. impatiens* and its protozoan pathogen *C. bombi* (Trypanosomatida) to ask the following questions: (1) What is the minimum dose of sunflower pollen mixed with wildflower pollen that provides medicinal benefits? Given that sunflower pollen is low in protein content and lacks some essential amino acids for bee development (Nicolson and Human 2013, Yang et al. 2013), it is not advisable to feed bumble bee colonies a diet exclusively of sunflower pollen. Instead, we needed to determine the minimum proportion of sunflower pollen mixed with wildflower pollen that would provide medicinal benefits to infected bees relative to a pure sunflower diet. We predicted that the medicinal effect of sunflower pollen would be significant at concentrations less than 100% sunflower pollen, but lacking at very low sunflower pollen concentrations. (2) We then used the minimum proportion of sunflower pollen mixed with wildflower pollen that provided a medicinal effect in a whole-colony experiment to ask: What are the costs and benefits of sunflower pollen on colony-level infection and performance? We predicted that a diet of sunflower mixed with wildflower pollen would minimize colony growth and reproductive costs that are associated with a poor nutritional diet, while providing medicinal benefits by reducing pathogen infection. (3) Finding that a diet with sunflower pollen could not completely clear infection in bumble bee colonies after 10 weeks of continuous pollen diet treatment, we asked: Can *C. bombi* develop resistance to the medicinal effects of sunflower pollen? Given that rapid evolution of resistance to medicines is documented in a wide variety of pathogens (Cohen 1992, D'Costa et al. 2011), we predicted that *C. bombi* would develop resistance to sunflower pollen. Taken together, results from this study suggest that sunflower

pollen supplements in a mixed pollen diet could reduce pathogens in domesticated colonies of bumble bees, reducing the potential for pathogen transmission between domestic and wild bees.

## Methods

### *Study System*

*Bombus impatiens* is a native eusocial bee species in North America and ranges from Maine to Ontario to the eastern Rocky Mountains and south through Florida (Kearns and Thomson 2001). Colonies are annual, founded by single inseminated queens. The main bumble bee species domesticated in North America is *B. impatiens*. Standard commercial *B. impatiens* colonies come with a queen and 50 – 100 workers and reach a peak of several hundred individuals typically within 12 weeks (JJG, *personal observation*). Later in the colony lifecycle, colonies switch from rearing workers to the production of males and daughter queens. In wild colonies, the males and new queens disperse and mate, and the inseminated new queens overwinter (Goulson 2003).

*Crithidia bombi* (Zoomastigophora:Trypanosomatidae) is an infectious protozoan gut pathogen that can be contracted at flowers via fecal transmission and can also be horizontally transmitted within colonies (Schmid-Hempel and Durrer 1991, Durrer and Schmid-Hempel 1994). *C. bombi* infection reduces learning and foraging efficiency in worker bumble bees (Gegear et al. 2005, 2006), is correlated with slower colony growth rates, especially early in the colony life cycle (Shykoff and Schmid-Hempel 1991a), and is correlated with the reduced likelihood of successful reproduction in wild colonies (Goulson et al. 2018). *C. bombi* infection is common; for example, *C. bombi* infected over 60% of wild-caught *B. impatiens* in western

MA (Gillespie 2010), suggesting potential for pathogen transmission among wild and commercial bees.

Domesticated sunflower (*H. annuus*) is a major oilseed crop cultivated worldwide and a native US wildflower (Reagon and Snow 2006). Nearly two million acres of sunflowers are planted in the US (Holcomb 2015) and ten million acres are planted in Europe annually (Strange et al. 2016). The high abundance of cultivated sunflowers combined with large nectar and pollen yields make it an important resource for bees, despite being considered a poor quality diet (Nicolson and Human 2013). Many bee species, including bumble bees, are known to visit sunflowers (Aslan and Yavuksuz 2010, Riedinger et al. 2014, but see Tepedino and Parker 1982, Fell 1986), and we have identified *H. annuus* pollen from the corbiculae of wild-caught *B. impatiens* workers foraging on sunflower (*unpublished data*).

### *Experimental methods*

***Preparing C. bombi inoculum.*** Experiments used *C. bombi* originally harvested from three wild *B. impatiens* workers collected near Stone Soup Farm, Hadley, MA, USA (42.363911 N, -72.567747 W) and housed in commercial colonies of *B. impatiens*. The *Crithidia* species was identified in a previous study by our group (Figueroa et al. 2019). Briefly, the CB-SSU rRNA F2 and CB-SSU rRNA B4 primers and PCR conditions described in Schmid-Hempel and Tognazzo (2010) were used to Sanger sequence the 18S small subunit rRNA gene. BLAST searches were then conducted against the National Center for Biotechnology Information's nr/nt database. The sequences matched *C. bombi* and had the same level of divergence to *C. expokei* as reported in Schmid-Hempel and Tognazzo (2010), confirming the species identity as *C. bombi*. The commercial colonies were free of the pathogen prior to infection with the field-collected *C.*

*bombi*. Both the *C. bombi* source colonies and experimental colonies (described below) were from BioBest LTD (Leamington, Ontario, Canada). Colonies were fed with 30% sucrose solution and mixed wildflower pollen throughout their lifetimes and housed in a dark room at 21 – 24ºC and ~50% rh. We made *C. bombi* inoculum for each experiment using an established protocol (Manson et al. 2010, Richardson et al. 2015, Giacomini et al. 2018). Briefly, bee digestive tracts, excluding the honey crop, were removed with forceps, placed into a 1.5 mL microcentrifuge tube with 300 µL of distilled water, and ground with a pestle. We allowed each sample to rest at room temperature for 4-5 hours so that gut material settled and the *C. bombi* cells could ascend into the supernatant. Flagellate *C. bombi* cells were counted from a 0.02 µL sample of supernatant per bee with a Neubauer hemacytometer under a compound light microscope at 400X magnification. We then mixed 150 µL of the supernatant with distilled water to achieve a concentration of 2400 cells $\mu L^{-1}$. The sample was then mixed with an equal volume of 50% sucrose solution to yield inoculum with 1200 cells $\mu L^{-1}$ in 25% sucrose.

***Measuring C. bombi infection.*** Each experimental bee was dissected using similar techniques as *Preparing C. bombi inoculum* with the addition that all tools were washed with 70% ethanol and thoroughly dried between bees. We counted flagellate *C. bombi* cells from a 0.02 µL sample of supernatant per bee with a Neubauer hemacytometer (Manson et al. 2010, Richardson et al. 2015, Giacomini et al. 2018). The proportion of flagellate vs. aflagellate *C. bombi* cells changes as infections develop in pollen-fed bees (Logan et al. 2005). However, assessing infection levels based solely on flagellate *C. bombi* should not alter interpretation of the results since infection age was consistent across our different treatments. Prevalence was recorded as the presence (1 or more *C. bombi* cells) or the absence of *C. bombi* cells per 0.02 µL of each sample. *C. bombi* infection intensity was measured as the number of flagellate *C. bombi*

cells per 0.02 μL and only included bees with at least 1 or more *C. bombi* cells. We removed the right forewing of each bee and mounted them on glass slides to measure marginal cell length, a proxy for bee size (Nooten and Rehan 2020).

***What is the minimum dose of sunflower pollen mixed with wildflower pollen that provides medicinal benefits?***

Experimental adult worker bumble bees (n = 120 bees) were obtained from three commercial *B. impatiens* colonies (40 bees/colony) that were determined to be un-infected by screening five workers from each colony using the methods described in *Measuring C. bombi infection*. Workers were removed from their colonies of origin and placed into individual plastic containers (7.5cm x 10cm x 5cm) with mesh screen flooring. We starved the bees for 4-6 hours and then fed each a 10 μL drop of inoculum that contained 12,000 *C. bombi* cells, which is within the concentration range bees are exposed to when foraging on flowers in the wild (Schmid-Hempel and Schmid-Hempel 1993). Only bees that consumed the entire droplet were used in the experiment. Bees were then randomly assigned, within colony of origin, to one of four experimental pollen diets: (1) pure sunflower pollen diet (100% Sun), (2) 1:1 mixture of sunflower pollen to wildflower pollen (50% Sun), (3) 1:3 mixture of sunflower to wildflower pollen (25% Sun), or (4) pure wildflower pollen (100% Wild). We based ratios on the weight of honey bee-collected pollen pellets. Sunflower pollen was obtained from Changge Hauding Wax Industry (China). We sorted honey bee-collected sunflower pollen pellets by color to remove impurities and verified a pure batch of sunflower pollen by staining five samples with fuschin dye and visually identifying the lack of non-sunflower pollen with a compound light microscope at 400X magnification. Honey bee-collected mixed wildflower pollen pellets were purchased

from Koppert Biological Systems (Howell, MI, USA) and microscopically confirmed to contain < 5% Asteraceae pollen, identified by having spines on the exine (Blackmore et al. 2007). The chemical and nutritional quality of wildflower pollen can vary based on plant species composition. While we did not identify the pollen species that made up the wildflower mixture used in this study, several studies have used wildflower pollen mixtures of unknown species composition as a control, showing sunflower pollen reduces *C. bombi* infection relative to wildflower pollen (Giacomini et al. 2018, LoCascio et al. 2019). Pesticide residues in pollen used in this study were not measured. However, the same sunflower and wildflower pollen lots were used for all experiments throughout this study and are from the same suppliers as in Giacomini et al. (2018), which did measure pollen pesticide levels. More pesticide residues were found in wildflower compared to sunflower pollen, all but two of which were at trace levels. The two that were above trace levels were both miticides used to treat varroa mites in honey bee colonies. Sunflower pollen also contained a different miticide used to treat varroa in honey bees. Given that pesticide levels were low overall and higher in wildflower than sunflower pollen, it seems unlikely that pesticides mediated the results.

Experimental pollen diets were provided to bees as a paste produced by mixing ground pollen pellets, weighed to the appropriate ratios of sunflower:wildflower pollen, and adding distilled water to achieve a uniform consistency. Each day for a week we fed inoculated bees fresh pollen paste packed into an inverted lid of a 1.5 mL microcentrifuge tube, and 1 mL of 30% sucrose via a filled and inverted plastic 1.5 mL microcentrifuge tube plugged with cotton (Richmond Dental & Medicine, Charlotte, NC, USA). On day 7, we assessed *C. bombi* prevalence and infection intensity (see *Measuring C. bombi infection*). The 7-day period allowed

sufficient *C. bombi* growth to quantify infection intensity within host bumble bees (Schmid-Hempel and Schmid-Hempel 1993, Otterstatter and Thomson 2006).

***Statistical analyses.*** All statistical analyses here and below were conducted using R version 3.5.2 (R Core Team 2014). All figures were generated using the ggplot() function from the "ggplot2" package (Wickham 2016). We used generalized linear mixed models (GLMMs) to analyze how pollen diets affected *C. bombi* infection prevalence (presence/absence) and intensity (cells per 0.02 μL in infected bees only) using the package "glmmTMB" (Brooks et al. 2017). *C. bombi* prevalence models were fit with a binomial distribution and infection intensity models were fit with a negative binomial distribution. Models included pollen diet (100% sunflower, 50% sunflower, 25% sunflower or wildflower mixture) and bee size estimated as marginal cell length (covariate) as fixed effects and experimental bee colony as a random effect. Significance of fixed effect terms was evaluated with a likelihood ratio chi-squared test, implemented via the drop1() function in R. Tukey's Honest Significantly Difference tests were used for post hoc pairwise comparisons between pollen diets using the "emmeans" package (Lenth 2020). All bees that died before their scheduled dissection date were excluded from the *C. bombi* infection analysis. A total of 90 bees survived until dissect date ($N_{100\%} = 23$, $N_{50\%} = 21$, $N_{25\%} = 21$, $N_{0\%} = 25$). We tested how pollen diets affected bee survival using mixed-effects Cox proportional hazards models (Therneau 2015), with pollen diet and bee size as fixed effects, and experimental bee colony as a random effect. We used Analysis of Deviance type III tests from the "car" package to test the significance of terms.

***What are the costs and benefits of sunflower pollen on colony-level infection and colony performance?***

We conducted a two-way factorial laboratory experiment crossing *C. bombi* infection (yes/no) with pollen diet treatment. *Bombus impatiens* colonies were randomly assigned to a *C. bombi* infection treatment (*C. bombi* inoculum (infected) or a sham inoculum (un-infected)) and pollen diet treatment (50% sunflower (1:1 sunflower:wildflower pollen) or wildflower pollen) for 10 weeks, which is within the range of a natural life cycle for *B. impatiens*. A 1:1 ratio of sunflower to wildflower pollen (50% sunflower pollen) provided similar medicinal benefits as 100% sunflower pollen for individual bees (see *Results*) and so was used as the sunflower pollen diet in this whole-colony experiment (hereafter referred to as sunflower pollen diet for simplicity within the context of this experiment). We used 9-11 replicate colonies per treatment (N = 45 total colonies), split into four blocks of 9-11 colonies. Samples sizes per treatment were not identical because a small number of colonies were damaged upon receipt from the commercial supplier. Blocks corresponded to day of the week that we performed initial colony inoculations and weekly parasite screenings. On any given day of the week, there was at least one replicate of each treatment combination represented so that treatment was not confounded with block. All colonies were confirmed to be *C. bombi*-free by screening ten workers from each colony upon receipt.

***Infection treatment.*** A fresh *C. bombi* inoculum was made for each block of colonies on their assigned inoculation day (as in *Preparing C. bombi inoculum*). We made the sham inoculum following the same procedure used to make the *C. bombi* inoculum, but using un-infected worker bumble bees from a different *B. impatiens* colony that was confirmed to be *C. bombi*-free. From each experimental colony, we removed 15 adult worker bees and placed each

into 7-dram snap cap vials (Qorpak, Clinton, PA, USA). We starved the bees on the lab bench for 3 – 5 hours. We then hand-fed all bees 10 μL of *C. bombi* or sham inoculum as appropriate. After consuming the entire drop, all inoculated bees were returned to their colonies. To ensure infections established in the infected treatments, we gave each colony an additional 5 mL of either *C. bombi* or sham inoculum via an open-faced petri dish (35 mm) placed directly into the colony for 24 hours.

***Pollen treatment.*** The sunflower pollen diet was made by mixing an equal portion (1:1 ratio by weight) of sunflower and wildflower pollen. The wildflower pollen diet solely contained honey bee-collected wildflower pollen. We gave all colonies their assigned pollen treatment (sunflower or wildflower) one week after the infection treatment started, thus allowing *C. bombi* infection to spread and become established within the colonies. Colonies received fresh pollen diet (approx. 50 g balls of ground pellets mixed with distilled water to form a paste) and 30% sucrose solution (750 mL) weekly until the termination of the experiment at 10 weeks post-inoculation.

***Measuring C. bombi infection and colony performance.*** Starting one week post-inoculation, each week we removed 10% of the workers (up to 10) from each colony to measure *C. bombi* prevalence and infection intensity (see *Measuring C. bombi infection*). Weekly, each colony box, excluding the sucrose reservoir, was weighed to the nearest 0.1 g, and drones were recorded and removed. Upon termination of the experiment, colonies were weighed (final weight) and then immediately frozen and stored at -18 ºC. We dissected frozen colonies and counted and weighed eggs, larvae, pupae, workers, drones and new queens. We combined the number of eggs, larvae and pupae into a single variable that represented the number of immature stages. The right forewings of all drones and queens (n = 0 - 93 per colony) from each colony

were removed and mounted on glass slides to measure the length of the marginal cell to estimate bee size of each caste. The average weight of workers, drones, queens and immatures was calculated by dividing the total weight of each by the number counted for each colony. Post-dissection, we pressure washed each plastic colony box to remove organic material and weighed each box to the nearest 0.1 g to subtract the box weights from the colony weights.

***Statistical analyses.*** Generalized linear mixed effects models (GLMMs) using the package "glmmTMB" (Brooks et al. 2017) were used to analyze how pollen diets affected *C. bombi* infection prevalence within colonies (fit with a binomial distribution) and infection intensity (fit with a negative binomial distribution), only for colonies experimentally inoculated with *C. bombi* (n = 20). Throughout the experiment, we did not detect *C. bombi* within colonies given the sham inoculum. The models included pollen diet, week, and their interaction as fixed effects, bee size as a covariate and both block and experimental colony as random effects. AIC scores were used to evaluate model fits using the AICtab() function in the "bbmle" package (Bolker and R Development Core Team 2020) to select random effect terms that produced the best fit model (i.e., lowest AIC score by 2 units). We checked for autocorrelation between adjacent weekly intervals using the acf() function in the base R "stats" package (R Core Team 2014). Linear contrasts using Tukey's method for P-value adjustment were used for post hoc pairwise comparisons between pollen diets at each weekly interval, or between weekly intervals, using the "emmeans" package (Lenth 2020).

Differences between pollen diets and infection treatments in the number, size and weight of adult bees and immature stages were analyzed either with linear mixed-effects models using the "lmerTest" package (Kuznetsova et al. 2017) for response variables that met the assumptions of normality or GLMMs using the "glmmTMB" package for those that followed a negative

binomial. The probability of producing new queens and the probability of producing any drones were modeled using GLMMs fit with a binomial distribution. The models included pollen diet, infection treatment and their interaction as fixed effects, and block as a random effect. Colony weight gain over time was analyzed with a GLMM, which included pollen diet, infection treatment and sample week (time), and two- and three-way interactions as fixed effects, and block and colony as random effects. Using the "emmeans" package, we computed estimated marginal means and pairwise comparisons using Tukey's p-value adjustment for pollen diets, infection treatments and sample week, when applicable. We applied the Benjamini-Hochberg method to control false discovery rate and reduce the chance of type 1 errors from multiple testing of correlated dependent variables (Benjamini and Hochberg 1995).

***Can C. bombi develop resistance to the medicinal effects of sunflower pollen?***

We tested for the development of sunflower-pollen resistant *C. bombi* at the end of the 10-week whole-colony experiment. We first created a 'potentially resistant' (PR) lineage of *C. bombi* using bees that had been exposed to sunflower pollen for 10 weeks from the whole-colony experiment, and a 'non-resistant' (NR) lineage sourced from the original *C. bombi* used to create the infection treatment for the whole-colony experiment. We then conducted a 2x2 factorial experiment crossing inoculum type (PR or NR) with pollen diet (100% sunflower vs. wildflower) and measured subsequent *C. bombi* infection. We hypothesized that *C. bombi* previously exposed to sunflower pollen (PR) would have greater ability to infect and grow in new sunflower-fed hosts, compared to *C. bombi* with no prior exposure to sunflower pollen (NR). If evolved resistance to sunflower comes with a tradeoff and reduces the ability to infect hosts

consuming other diets, then the PR lineage may have reduced infection compared to NR in hosts fed wildflower pollen.

To create the PR inoculum, we randomly chose three colonies from the whole-colony experiment from the *C. bombi* infection/sunflower pollen diet treatment combination. We removed 15 workers from each colony and placed them as groups into plastic containers with screen floors to establish microcolonies (i.e., one 15-worker microcolony established from each parent colony). The microcolonies were established to house the PR infection until we were ready to run the experiment. Each microcolony was fed 30% sucrose solution and sunflower pollen daily for two weeks. We dissected 7 bees from each microcolony (see *Preparing C. bombi inoculum*) and counted *C. bombi* cells (see *Measuring C. bombi infection*). Mean *C. bombi* counts were similar across microcolonies ($F_{2,18} = 0.319$, $P = 0.731$), suggesting an even representation of *C. bombi* cells from each of the three colonies. Therefore, we combined the samples to produce the PR *C. bombi* inoculum that was continuously exposed to sunflower pollen. A non-resistant (NR) inoculum was created using infected bees sourced from the same ancestral colony that the PR lineage started from, but were never exposed to sunflower pollen. An alternative approach would have been to create NR inoculum from infected wildflower colonies from the whole-colony experiment; we did not do so because we kept the ancestral source colony (which was also fed wildflower pollen) and creating the NR inoculum from the ancestral colony allowed us to start the experiment more quickly. For the NR inoculum, the supernatant of 21 bees (same number used to make the PR inoculum) was mixed together using the same procedure as for the PR inoculum. The mean *C. bombi* cell counts of the combined supernatant prior to diluting with distilled water and sucrose solution for the two inocula (PR and NR) were similar (9950 and 10550 cells/μL, respectively).

To conduct the factorial experiment, we removed 40 *B. impatiens* workers each from three new uninfected parent colonies, placed them into 7 dram snap-cap vials and starved them for 4 – 6 hours. Each bee (N = 120 total) was then hand-fed 10 μL of either PR or NR inoculum. Inoculated bees were transferred to individual plastic containers as in Question 1 and then randomly assigned to a pollen diet treatment (100% sunflower vs. wildflower) to yield 10 bees per colony-treatment combination. Fresh pollen and sucrose were provided for 7 d. All bees were sacrificed on day 7, at which point we measured infection prevalence, intensity, and bee size (see *Measuring C. bombi infection*). A nearly even number of bees died per treatment before the scheduled dissection date (8–11 bees per treatment). A total of 19 bees from the NR-Sunflower treatment, 22 PR-Sunflower, 19 NR-Wildflower and 22 PR-Wildflower were in the final analysis.

***Statistical analysis.*** We used GLMMs to analyze how treatments affected *C. bombi* infection prevalence and intensity, as in Question 1. Pollen diet (sunflower or wildflower), inoculum type (PR or NR), and their interaction were included as fixed effects and bee size as a covariate. Experimental bee colony was included as a random effect. Significance of fixed effect terms and post-hoc pairwise comparisons were conducted as in Question 1. All bees that died before their scheduled dissection date were excluded from the *C. bombi* infection analysis. We tested how pollen diets affected bee survival using mixed-effects Cox proportional hazards models (Therneau 2015), with pollen diet, inoculum type, their interaction and bee size as fixed effects, and experimental bee colony as a random effect. We used Analysis of Deviance type III tests from the "car" package to test the significance of terms.

## Results

### *What is the minimum dose of sunflower pollen mixed with wildflower pollen that provides medicinal benefits?*

Pollen diet had a significant effect on *C. bombi* prevalence ($\chi^2_{(3)} = 38.426$, $P < 0.0001$) and infection intensity *(*$\chi^2_{(3)} = 18.675$, $P = 0.0003$). Prevalence of infection ranged from 13% to 92% of bees with a detectable infection, with the highest prevalence in a 100% wildflower pollen diet and the lowest in a 100% sunflower pollen diet (Figure 2.1a). Post-hoc pairwise comparisons revealed significant differences in prevalence of infection between 100% sunflower and both the 100% wildflower (0% sunflower) and 25% sunflower treatments ($Z = -4.51$, $P < 0.0001$; $Z = -2.93$, $P = 0.018$; respectively), but not between 100% sunflower and 50% sunflower treatments ($Z = -1.63$, $P = 0.36$). The 50% sunflower treatment reduced *C. bombi* prevalence by nearly 4-fold compared to 100% wildflower pollen ($Z = -3.61$, $P = 0.0018$). For *C. bombi* infection intensity, pairwise comparisons revealed a similar pattern (Figure 2.1b); 100% sunflower significantly reduced infection intensity compared to 100% wildflower and 25% sunflower treatments ($Z = -4.68$, $P < 0.0001$; $Z = -2.82$, $P < 0.025$; respectively), but not 50% sunflower ($Z = -0.83$, $P = 0.84$). The 50% sunflower treatment reduced *C. bombi* infection intensity by nearly 12-fold compared to 100% wildflower pollen ($Z = -3.28$, $P = 0.0057$). We found no effect of pollen diet on bee survival ($\chi^2_{(3)} = 4.889$, P = 0.180).

### *What are the costs and benefits of sunflower pollen on colony-level infection and colony performance?*

***C. bombi infection.*** We analyzed the effect of pollen diet and time on infection dynamics in infected colonies only. Pollen diet significantly affected colony-level *C. bombi* infection

(Figure 2.2). Models indicated a significant interaction between pollen diet and time on prevalence ($\chi^2_{(8)} = 19.049$, P = 0.015; Figure 2.2a). *C. bombi* infection prevalence was similar for sunflower and wildflower pollen diets at the first sampling period ($t_{(1393)} = 0.641$, P = 0.522), suggesting that inoculated colonies started with comparable infection levels prior to receiving pollen treatments. Infection prevalence within wildflower colonies then increased over the 10 weeks of sampling, culminating with 90 to 100% of bees sampled from inoculated wildflower colonies testing positive for infection. In comparison, prevalence ranged from 33% to 100% within sunflower colonies by week 10. Post-hoc linear contrasts revealed significant differences between pollen diets on *C. bombi* infection prevalence for weeks 5, 6, 7, 9 and 10 ($t_{(1393)} > 1.9$, P < 0.049 in all cases), ending with the largest difference between treatments at week 10. There was a spike in *C. bombi* prevalence in sunflower colonies at week 8, which muted the statistical difference between pollen diets in this week ($t_{(1393)} = -0.169$, P = 0.866). Moreover, variation in the prevalence of infection within colonies was much lower for wildflower colonies compared to sunflower colonies, revealed by a significant Fligner-Killeen test of homogeneity of variances ($\chi^2_{(1)} = 33.568$, P = 0.0059), and the variance decreased significantly throughout the 10-week experiment in wildflower ($\chi^2_{(8)} = 34.769$, P < 0.0001) but not sunflower ($\chi^2_{(8)} = 6.521$, P = 0.589) colonies.

Pollen diet and time also affected *C. bombi* infection intensity ($\chi^2_{(1)} = 5.665$, P = 0.017; $\chi^2_{(8)} = 36.044$, P < 0.0001; respectively; Figure 2.2b), but the interaction was not significant ($\chi^2_{(8)} = 11.134$, P = 0.194). Averaged across sampling weeks, *C. bombi* infection intensity for sunflower colonies was 30.3% lower than wildflower colonies (sunflower = 29.9 ± 3.75 cells per 0.02 μL; wildflower = 42.9 ± 4.80 cells per 0.02 μL; mean ± SE). Variation in *C. bombi* infection intensity was also lower for sunflower compared to wildflower colonies, revealed by a

Fligner-Killeen test of homogeneity of variances ($\chi^2_{(1)} = 37.387$, $P < 0.0001$). For the significant effect of time, post-hoc pairwise tests revealed that week 4 was significantly different from all other weeks ($P < 0.0215$ for all comparisons), with lower infection levels by at least 40%. The covariate bee size had a significant effect on both infection prevalence ($\chi^2_{(1)} = 3.969$, $P = 0.046$) and intensity ($\chi^2_{(1)} = 4.022$, $P = 0.045$), although in opposite directions. Larger bees were less likely to be infected ($\beta = -0.7659$), but had greater infection intensities if they were infected ($\beta = 0.2852$).

***Colony performance.*** There was a significant interaction between pollen diet and time on colony weight gain ($\chi^2_{(7)} = 14.098$, $P = 0.0495$; Figure 2.3), but not between infection treatment and time ($\chi^2_{(1)} = 7.580$, $P = 0.3711$) or between pollen diet and infection treatment ($\chi^2_{(1)} < 0.001$, $P = 0.983$). At the start of the experiment, the average colony weight was 629.2 ± 1.5 g (mean ± SE) and was not significantly different between pollen diets ($t_{(285)} = -0.073$, $P = 0.942$) or infection treatment ($t_{(285)} = -0.242$, $P = 0.809$). After 10 weeks, colonies gained an average of 151.2 g ± 7.06 g (mean ± SE). Post-hoc pairwise comparisons revealed that wildflower colonies surpassed sunflower colonies in terms of weight gain starting at week 7 and continuing through the end of the experiment ($t_{(285)} < -2.459$, $P < 0.015$, for weeks 7, 9 and 10), with the exception of week 8 ($t_{(285)} = -1.627$, $P = 0.105$). By the end of the experiment (week 10), wildflower colonies gained approximately 4% more weight than sunflower colonies. Infection treatment did not significantly affect colony weight gain ($\chi^2_{(1)} = 0.361$, $P = 0.548$).

By the end of the experiment, colonies contained on average 189.05 ± 17.68 workers (mean ± SE), but queen and drone production were highly variable (see Table 1 for means and statistical tests of colony performance metrics). Pollen diet and infection treatments did not have significant main effects on worker production or the average weight or size of workers, nor on

the number of immature stages counted at the end of the experiment (Table 2.1). However, there was a significant interaction between pollen diet and infection treatment on the average weight of combined immature stages, with un-infected wildflower colonies having nearly double the weight of all other treatments. There was also a significant interaction between pollen diet and infection treatment on the number of drones produced, with sunflower pollen rescuing the negative effects of *C. bombi* infection on drone production. None of the treatments had a significant effect on average drone weight or size. There was a significant interaction between pollen diet and infection treatment on the probability of producing new queens, with uninfected sunflower and infected wildflower colonies nearly half as likely to produce new queens compared to infected sunflower and uninfected wildflower colonies. There was no effect of pollen diet, infection treatment or their interaction on queen size or weight. Overall, these analyses show similar colony performance between pollen diets and infection treatments, with the addition that a mixed sunflower pollen diet rescued the negative effect of infection on queen and drone production.

***Can C. bombi develop resistance to the medicinal effects of sunflower pollen?***

Consistent with our first experiment, there was a significant effect of pollen diet on *C. bombi* prevalence ($\chi^2_{(1)} = 25.066$, $P < 0.0001$) and infection intensity ($\chi^2_{(1)} = 4.695$, $P = 0.0303$), with sunflower-fed bees having 69% lower *C. bombi* prevalence and nearly 3-fold lower infection intensity than wildflower-fed bees (Figure 2.4). However, there was no effect of inoculum type on prevalence ($\chi^2_{(1)} = 1.538$, $P = 0.2149$) or infection intensity ($\chi^2_{(1)} = 0.013$, $P = 0.9110$), nor were there significant interactions between pollen diet and inoculum type for prevalence ($\chi^2_{(1)} = 0.305$, $P= 0.5809$) or intensity of infection ($\chi^2_{(1)} = 0.0003$, $P = 0.9860$). We

also found no effect of pollen diet, inoculum type, or their interaction on bee survival ($\chi^2_{(1)} < 1.9$, $P < 0.2$ for all).

**Discussion**

The goal of this study was to assess the costs and benefits of sunflower pollen on bumble bee colony disease and performance. Given concerns of pathogen transmission between domesticated and wild bee populations (Power and Mitchell 2004, Colla et al. 2006, Sachman-Ruiz et al. 2015), and that *C. bombi* infection levels in *B. impatiens* can drive pathogen dynamics in other wild bee species (Figueroa et al. 2020), understanding how to control pathogens in managed populations of bees is critically important. This study provides evidence that a 1:1 mixture of sunflower combined with wildflower pollen reduces *C. bombi* infection prevalence and intensity in both individual *B. impatiens* workers and at the colony level, supporting our prediction that a mixed sunflower pollen diet could provide similar medicinal effects compared to a pure sunflower pollen diet. In a follow-up laboratory experiment, *C. bombi* was unable to rapidly evolve resistance to sunflower pollen over the course of the whole-colony experiment. A supplemental mixed sunflower pollen diet may provide a simple and effective solution to reduce disease and improve the health of economically and ecologically important pollinators.

Mixing sunflower with wildflower pollen can be an effective treatment for reducing *C. bombi* infection without sacrificing colony performance, which may reduce the risk of pathogen spillover from commercial colonies to wild bees, or spillback from wild bees to commercial colonies. Top-down negative effects of pathogens on pollination services can result from either reduced bee population sizes or deleterious effects on bee foraging behavior. Numerous studies have found that *C. bombi*-infected bumble bees are less efficient foragers, with reduced pollen

collection rates and weaker ability to learn floral reward associations and flower handling techniques (Shykoff and Schmid-Hempel 1991b, Gegear et al. 2005, 2006). While the influence of bee pathogens and diseases on pollination services is poorly understood, infection in bees can be negatively correlated with pollen movement between flowers, reducing plant reproduction (Gillespie and Adler 2013, Lach et al. 2015). In contrast, Theodorue et al. (2016) did not find an indirect effect of *C. bombi* infection on plant reproduction in urban ecosystems, which may have been the result of greater bee abundance in urban versus rural areas. In our whole-colony experiment, a mixed sunflower pollen diet reduced pathogen levels without the risk of a negative tradeoff in worker production, which is the major pollination workforce within a bumble bee colony. Whether sunflower pollen benefits pollination services by mediating host-pathogen interactions in bumble bees remains an open question.

By consuming pollen and nectar, pollinators can be considered herbivores that specialize on certain plant tissues. Generalist herbivores may benefit from a mixed diet when some components of the diet compensate for nutrient deficiencies or dilute toxic plant secondary compounds (Bernays et al. 1994). Similarly, generalist pollinator species may benefit from consuming mixed diets comprised of complementary pollen species that balance nutrient demands and dilute negative effects of nutritionally poor pollen, such as sunflower pollen (Eckhardt et al. 2013, Nicolson and Human 2013). For example, in a recent study using bumble bee microcolonies, bees that consumed a pure sunflower pollen diet had a significantly shorter lifespan than bees that consumed broad bean (*Vicia faba*, Fabaceae), rapeseed (*Brassica napus*, Brassicaceae) or Cucurbitaceae pollen, but the negative effects were eliminated when bees consumed a mixed pollen diet with 50% sunflower pollen (McAulay and Forrest 2019). Some pollen diets are inadequate for generalist bumble bee development (Génissel et al. 2002, Tasei

and Aupinel 2008), and many studies demonstrate that worker foraging preferences are largely based on nutritional quality of pollen (Ruedenauer et al. 2016, Vaudo et al. 2016, Kriesell et al. 2017). Pollen diets vary considerably in nutrient content and concentrations (Roulston and Cane 2000), as well as secondary metabolites (Palmer-Young et al. 2018) and digestibility (Vanderplanck et al. 2018), each of which can have profound impacts on colony growth. Our study provides further support that a 1:1 mixture of sunflower and wildflower pollen can compensate for nutrient deficiencies of monofloral sunflower pollen at the bumble bee colony level, without significantly sacrificing the medicinal benefits of sunflower pollen. Our experiment was not designed to test whether the medicinal benefits of sunflower pollen in mixed pollen diets show a linear or step-function relationship; visual inspection of the relationships suggests a linear relationship for prevalence but a step-function relationship for intensity (compare Figure 1a and 1b). An experiment with finer gradations in sunflower pollen proportions would be needed to test these patterns. Nonetheless, our results demonstrate that sunflower pollen as part of a mixed wildflower pollen diet can reduce disease and maintain production of colonies, and by extension, sunflowers planted as part of a diverse wildflower mixture may allow bees to naturally resist pathogen infection.

Compared to a wildflower diet, we found that the sunflower diet significantly reduced negative effects of infection on queen and drone production. Infection significantly reduced the probability of queen production in wildflower but not sunflower colonies, while infection significantly reduced the probability of drone production independent of pollen diet. However, infected sunflower colonies that did produce drones yielded on average four times as many as infected wildflower colonies. Similarly, in a previous study using *B. impatiens* microcolonies, sunflower pollen consumption alleviated the negative effects of *C. bombi* infection on egg

production compared to buckwheat pollen (Giacomini et al. 2018). Taken together, these results strongly suggest that the medicinal benefits of a mixed sunflower pollen diet can reverse the negative effects of *C. bombi* infection on reproductive success. While queen and drone production play an important role in bumble bee population dynamics, and provide a practical estimate of reproductive success (Muller and Schmid-Hempel 1992, Pelletier and McNeil 2003, Crone and Williams 2016), further investigation of the effects of a mixed sunflower pollen diet and *C. bombi* infection on mating success, queen overwintering and colony founding success is needed to better understand how a sunflower pollen diet ultimately affects bumble bee reproductive success.

In the whole-colony experiment, body size and mass of all adult castes (i.e., workers, drones, queens) was similar for pollen diets with and without sunflower, suggesting that a mixed sunflower pollen diet can minimize adverse effects on larval development associated with a pure Asteraceae diet (Tasei and Aupinel 2008, Vanderplanck et al. 2018). Maintenance of bee body size has important consequences for pollination services, as evidence suggests that intra-specific variation in body size can drive patterns in pollination efficacy, such that larger-bodied bees are more effective pollinators (Jauker et al. 2016). Body size and mass of workers are also important factors for determining bumble bee colony reproduction since larger workers are able to forage more effectively than smaller workers and can carry larger nectar and pollen loads per foraging trip (Goulson et al. 2002), although smaller workers are less vulnerable to starvation (Couvillon and Dornhaus 2010). Size and mass of queens also are important. Larger queens are more successful in nest usurpation contests (Richards 1978), better able to thermoregulate (Heinrich 1979), and have higher overwintering survival (Holm 1972).

Body size and mass in the whole-colony experiment were not affected by *C. bombi* infection. Energetic costs associated with the host's ability to defend against parasites can affect host body size and mass under stressful conditions (Van Heugten et al. 1996, Moret and Schmid-Hempel 2000, Bonneaud et al. 2003), such as a nutritionally deficient diet. Under favorable conditions, bumble bees can tolerate *C. bombi* infection without adverse effects (Brown et al. 2000, 2003). However, when infection is combined with nutritional stress, the risk of mortality increases and resource allocation patterns within the colony change, resulting in bees that dedicate more energy into their fat body and less into their reproductive system (Brown et al. 2000, 2003). The nutritional profile of the sunflower pollen treatment in the whole-colony experiment was likely adequate since it was a mixture of sunflower and wildflower pollen. In addition, all colonies in this study received consistent access to abundant nectar and pollen throughout the experiment, making it less likely that we would observe negative effects of infection on bee size or mass. Nonetheless, habitat loss and lack of floral resources play major roles in bee declines globally (Goulson et al. 2015), warranting further investigation of the interaction between floral resources, sunflower pollen and *C. bombi* infection to shed light on colony growth consequences under field conditions.

At the end of the whole-colony experiment, we discovered that colonies inoculated with *C. bombi* and fed mixed sunflower pollen had lower mean parasite loads than colonies fed wildflower pollen, but infection was not reduced as dramatically as in previous individual bee experiments (see Giacomini et al. 2018). Several non-mutually exclusive hypotheses may explain the inability of sunflower mixed with wildflower pollen to eliminate infection at the whole-colony level. One explanation could be rapid development of *C. bombi* resistance to the medicinal effects of sunflower pollen within a colony. Numerous studies have demonstrated the

rapid evolution of infectious microbes to repeated antibiotic treatments (Van den Bergh et al. 2016, Capela et al. 2019, Liu et al. 2020). In bumble bees, *C. bombi* lineage (defined loosely as a unique genotype) plays a major role in the ability to establish an infection within a colony. In one study, bumble bee colonies that were given a mixture of *C. bombi* lineages filtered out less infective lineages within just five serial passages between workers (i.e., transmission between hosts), or just 35 days (Yourth and Schmid-Hempel 2006). *C. bombi* also evolved resistance to inhibitory phytochemicals after only 6 weeks of exposure (Palmer-Young et al. 2017). Thus, it is plausible that a sunflower pollen diet could rapidly select for a novel *C. bombi* lineage resistant to the medicinal effects of sunflower pollen. However, we did not detect any differences in infection prevalence or intensity between *C. bombi* lineages (e.g., inoculum types) that were exposed to sunflower pollen or not, suggesting that *C. bombi* was unable to rapidly evolve resistance to sunflower pollen during the 10-week experiment.

Our finding that sunflower pollen did not completely clear infection within a colony could be because bees were confined to a box with increasing density of individuals over time. For fecal-orally transmitted parasites, such as *C. bombi*, the probability of susceptible hosts becoming infected should increase with increasing host density (Anderson and May 1979), due to increased density of social interactions between infected and susceptible hosts (Otterstatter and Thomson 2007). Domesticated bumble bees are typically confined to a single container for a significant period of their life cycle prior to being placed in greenhouses or outdoors for crop pollination. Confinement and greater density of individuals likely increases the deposition of contaminated bee feces and the likelihood of repeated exposure to susceptible individuals. Hygienic behaviors, such as localized deposition of feces or deposition away from the nest, are well known in eusocial insects and are thought to convey anti-parasite benefits (Michener and

Michener 1974, Weiss 2006). It is thus plausible that a multi-box system for commercially reared bumble bees, in combination with a mixed sunflower pollen diet, may separate contaminated feces from the brood and reduce *C. bombi* transmission between individuals and colonies. Such a hypothesis warrants further investigation, although the space requirements needed for a multi-box system in a commercial rearing facility may be cost prohibitive.

Recent work suggests that the effect of sunflower pollen on *C. bombi* may be mediated in part by the bee host rather than via a direct effect of pollen on the pathogen. For example, sunflower pollen extract increased rather than suppressed *C. bombi* growth *in vitro* (Palmer-Young 2017). While the mechanism underlying the medicinal effect of sunflower pollen is unknown (Adler et al. 2020) and outside the scope of this study, it is plausible that sunflower pollen reduces *C. bombi* infection via changes in host physiological functions, such as an immune response, or via direct interactions with the *C. bombi* cells within the host, such as nutrient limitation. Brunner et al. (2014) found that pollen-starved bumble bees showed reduced immune responses to infection, including the upregulation of energetically costly antimicrobial peptides and putative immune signaling molecules. A sunflower pollen diet with low protein content and missing essential amino acids would be expected to negatively affect host immunocompetence, and thus increase parasite growth. However, changes in host nutrition may affect the availability of resources for the pathogen and subsequently limit pathogen growth and reproduction. Logan et al. (2005) reported higher *C. bombi* infection levels in bumble bees fed pollen compared to pollen-starved bees. The bumble bee colonies in our study had access to a sufficient quantity of pollen, evident by each colony's inability to completely finish the weekly pollen balls that were supplied to them. Moreover, the sunflower pollen treatment consisted of a 1:1 mixture of sunflower:wildflower pollen to increase the nutritional profile of the sunflower

pollen treatment. Thus, it seems unlikely that poor nutrition was the sole cause of reduced *C. bombi* growth within sunflower pollen-fed bees. Sunflower pollen could also mediate infection through physiological changes in the host induced by the spiny morphology of the pollen grains, or, since *C. bombi* is a gut parasite that attaches to the hindgut wall (Gorbunov 1996), sunflower pollen could reduce infection by scouring the hindgut of parasite cells (Huffman and Caton 2001). Further work aimed at establishing how a sunflower pollen diet reduces *C. bombi* infection may open up new areas of inquiry into mechanisms mediating bee health, as well as identifying floral traits that could be incorporated into pollinator landscapes.

Despite the evidence that sunflower pollen reduces *C. bombi* infection in commercial *B. impatiens* colonies, several obstacles should be addressed prior to applying a medicinal sunflower pollen diet. First, since a mixed sunflower pollen diet reduces but does not eliminate infection, it is important to identify how such a reduction relates to the rate of pathogen spillover from commercially managed colonies to wild bees. Contact rate, rather than the duration of contact, may drive risk of *C. bombi* infection for bumble bees (Otterstatter and Thomson 2007, Sah et al. 2018), suggesting that reduced *C. bombi* prevalence and intensity within commercially managed bumble bee colonies will concordantly reduce pathogen spillover rates. Similarly, sunflower pollen supplements could increase resistance to pathogen spillback from wild bees to commercial colonies. Second, it is important to identify cost-effective sources of environmentally friendly sunflower and wildflower pollen that are not contaminated with pesticides or pathogens. Pesticides are commonly used on sunflower crops to suppress weeds, herbivorous insects and plant pathogens (Elbert et al. 2008) and can pose a substantial risk for bees (Whitehorn et al. 2012). Moreover, honey bee-collected pollen used for feeding commercially managed bumble bees comes with the risk of pathogen contamination (Graystock

et al. 2016, de Sousa Pereira et al. 2019). Sterilization of non-local pollen used to feed bumble bees should be encouraged to reduce the transmission of infectious bee diseases among managed and wild bees.

## Conclusion

Managing pollinator populations requires the careful consideration of key plant species that play disproportionate roles in protecting against pathogens, as well as nutritional needs associated with growth and reproduction. This study provides evidence that sunflower pollen as part of a mixed pollen diet can reduce infection in individual bees and at the whole-colony level and recover negative effects of infection on colony reproduction, with no significant nutritional tradeoffs for colony worker production. The reduction of pathogens within bumble bee colonies is a significant concern for commercial producers of domesticated bumble bees, growers that use bumble bee colonies for pollination, and conservation biologists worldwide. We conclude that a mixed sunflower pollen diet could be an effective strategy for reducing bumble bee disease.

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## Data Availability

Data and R scripts for the statistical analyses are publicly available at

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**Table 2.1**. Mean numbers, sizes and weights of adult bees and immature stages in *Bombus impatiens* colonies either inoculated (Inoc.) with the protozoan gut parasite *Crithidia bombi* (Inf.) or a sham control (Uninf.) and fed either sunflower pollen (1:1 ratio of sunflower to wildflower pollen) or wildflower pollen mix, along with statistical test results. Differences between treatments were analyzed using linear mixed effects models, with the exceptions of worker size, colony weight, and drone and queen production, which were analyzed using generalized linear models. For linear mixed effects models, *F*-scores (*St.*) and denominator degrees of freedom are listed in parentheses under *St.* ; the numerator degrees of freedom was always one. For generalized linear models, Type II Wald chi-square values are listed (*St.*). Statistically significant factors ($P < 0.05$) are in bold and marked with an asterisk. Values under treatments represent Mean (SE).

| | | | | | | | | | Pollen | | Inoc. | | Pollen x Inoc. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **Inf. Sun** | | **Inf. Wild** | | **Uninf. Sun** | | **Uninf. Wild** | | ***St.*** | ***P*** | ***St.*** | ***P*** | ***St.*** | ***P*** |
| **Workers** (No.) | 126 | (39.51) | 227 | (36.34) | 202 | (37.46) | 190 | (37.46) | 1.72 (35) | 0.20 | 0.34 (33) | 0.56 | 2.86 (33) | 0.10 |
| **Worker wt.** (mg) | 138 | (12.88) | 145 | (11.61) | 130 | (12.05) | 152 | (12.05) | 1.33 (36) | 0.26 | 0.00 (36) | 0.98 | 0.39 (36) | 0.53 |
| **Drones** (No.) | 35 | (22.07) | 8.09 | (4.68) | 15.2 | (9.14) | 47.7 | (28.5) | 0.06 (1) | 0.80 | 0.78 (1) | 0.38 | 4.69 (1) | **0.03*** |
| **Prob. of Drones** | 0.56 | (0.17) | 0.45 | (0.15) | 0.8 | (0.13) | 0.9 | (0.09) | 0.00 (1) | 0.98 | 4.51 (1) | **0.03*** | 0.58 (1) | 0.45 |
| **Drone wt.** (mg) | 66.4 | (27) | 107 | (28.64) | 108 | (24.9) | 94.7 | (20.04) | 0.33 (15) | 0.57 | 0.40 (1) | 0.54 | 1.32 (14) | 0.27 |
| **Queens** (No.) | 26.1 | (13.17) | 18.3 | (8.92) | 7.23 | (3.69) | 25.1 | (12.34) | 1.13 (1) | 0.29 | 1.29 (1) | 0.26 | 3.63 (1) | 0.06 |
| **Prob. of Queens** | 0.8 | (0.15) | 0.46 | (0.19) | 0.38 | (0.19) | 0.92 | (0.09) | 0.10 (1) | 0.76 | 0.01 (1) | 0.90 | 6.42 (1) | **0.01*** |
| **Queen wt.** (mg) | 366 | (71.07) | 513 | (67.44) | 600 | (79.29) | 496 | (66.42) | 0.09 (32) | 0.76 | 2.44 (30) | 0.13 | 3.24 (29) | 0.08 |

**Table 2.1** (continued)**.**

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Immatures** (No.) | 152 | (53.65) | 217 | (50.79) | 197 | (51.81) | 153 | (51.81) | 0.08 (34) | 0.78 | 0.07 (33) | 0.80 | 2.12 (33) | 0.16 |
| **Immature wt.** (mg) | 189 | (47.91) | 164 | (38.82) | 136 | (30.86) | 308 | (69.8) | 2.40 (28) | 0.13 | 0.51 (27) | 0.48 | 4.97 (27) | **0.03*** |
| **Worker size** (mm) | 2.53 | (0.02) | 2.54 | (0.02) | 2.5 | (0.02) | 2.53 | (0.02) | 1.39 (1) | 0.24 | 0.72 (1) | 0.40 | 0.47 (1) | 0.49 |
| **Queen size** (mm) | 3.7 | (0.11) | 3.63 | (0.13) | 3.66 | (0.14) | 3.82 | (0.12) | 0.11 (14) | 0.74 | 0.33 (14) | 0.58 | 0.84 (14) | 0.38 |
| **Drone size** (mm) | 2.79 | (0.08) | 2.81 | (0.09) | 2.76 | (0.1) | 2.71 | (0.06) | 0.02 (18) | 0.89 | 0.62 (18) | 0.44 | 0.16 (18) | 0.69 |
| **Colony wt.** (g) | 718 | (5.72) | 733 | (5.72) | 714 | (5.72) | 729 | (5.72) | 5.22 (1) | **0.02*** | 0.36 (1) | 0.55 | 0.00 (1) | 0.98 |
| **Colonies** (No.) | (N = 9) | | (N = 11) | | (N = 10) | | (10 = N) | | | | | | | |

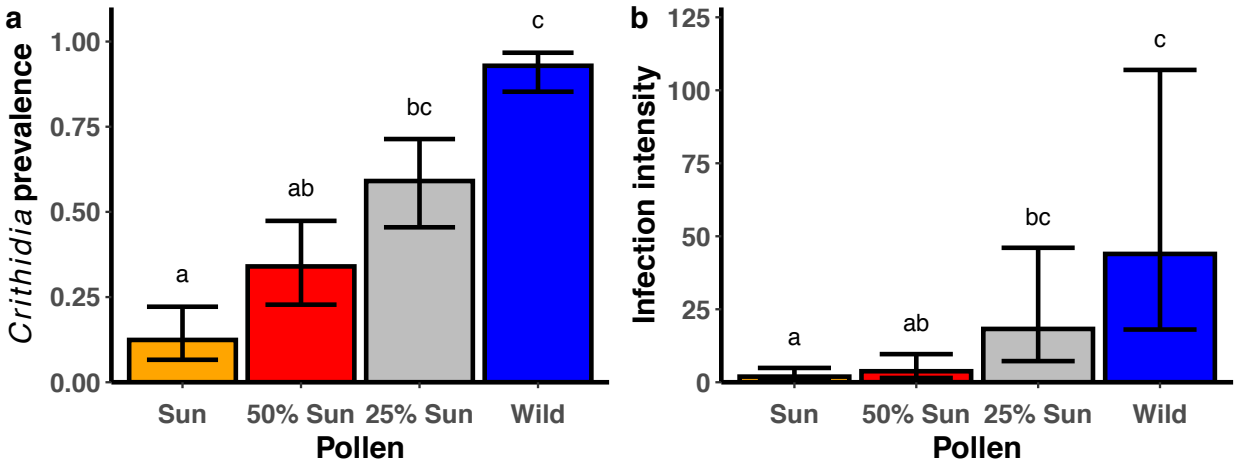


**Figure 2.1**. *Crithidia bombi* infection prevalence and intensity for *Bombus impatiens* workers fed varying ratios of sunflower to wildflower pollen mix. (**a**) Average infection prevalence (the proportion of parasitized bees) and (**b**) average infection intensity of infected bees only (cells per 0.02 μL) were significantly lower in bees fed 100% sunflower (Sun) and a 50% sunflower:wildflower pollen mixture compared to wildflower pollen. Different letters above bars indicate statistically significant differences based on pairwise comparisons using Tukey's HSD tests. Bars and error bars indicate binomial (**a**) and negative binomial (**b**) model means and standard errors, back-transformed from the scale of the linear predictor.

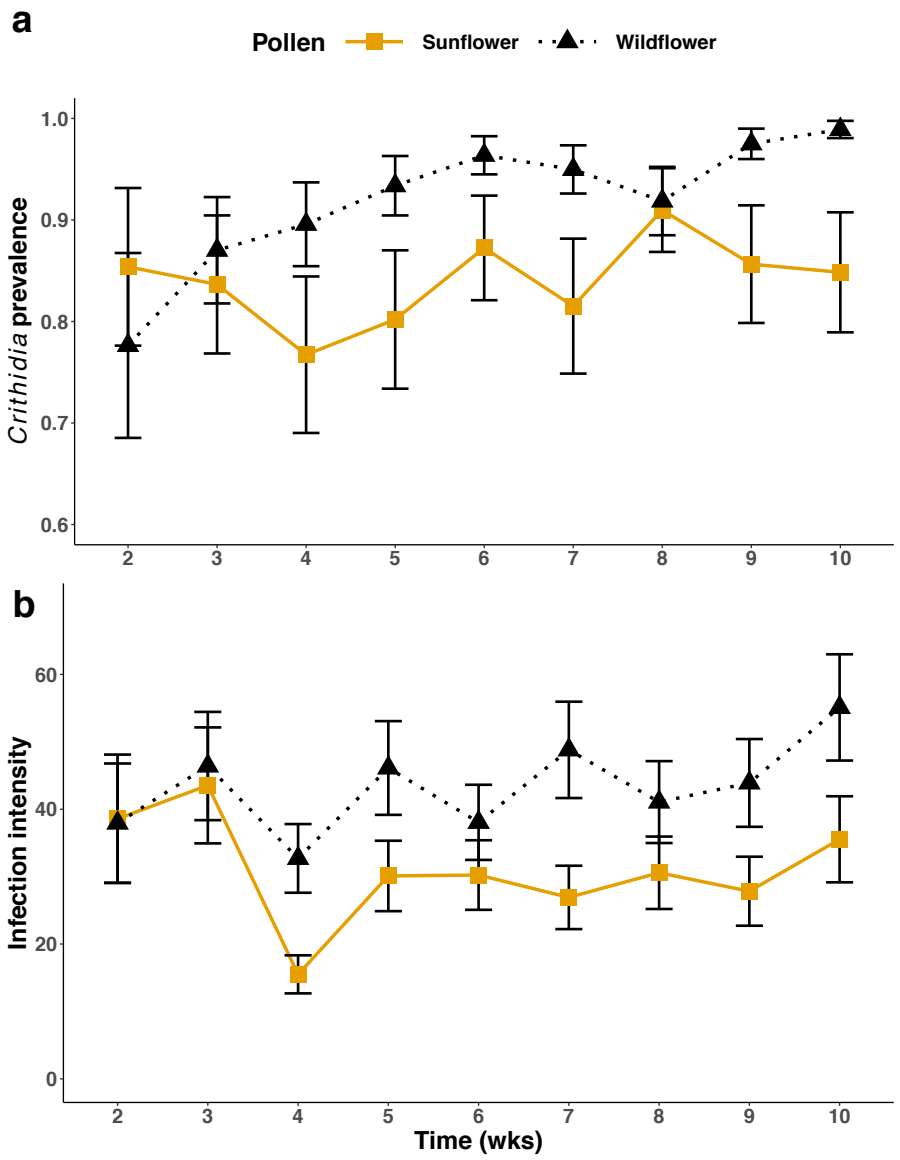


**Figure 2.2**. *Crithidia bombi* infection prevalence for *Bombus impatiens* (**a**), measured as the proportion of bees sampled from an infected colony, and average infection intensity of infected bees only (cells per 0.02 µL) (**b**) over 10 weeks for experimentally infected bumble bee colonies fed either a sunflower pollen diet (1:1 ratio of sunflower to wildflower pollen; yellow squares) or wildflower pollen diet (black triangles). Points and error bars indicate binomial (**a**) and negative binomial (**b**) model means and standard errors, back-transformed from the scale of the linear predictor.

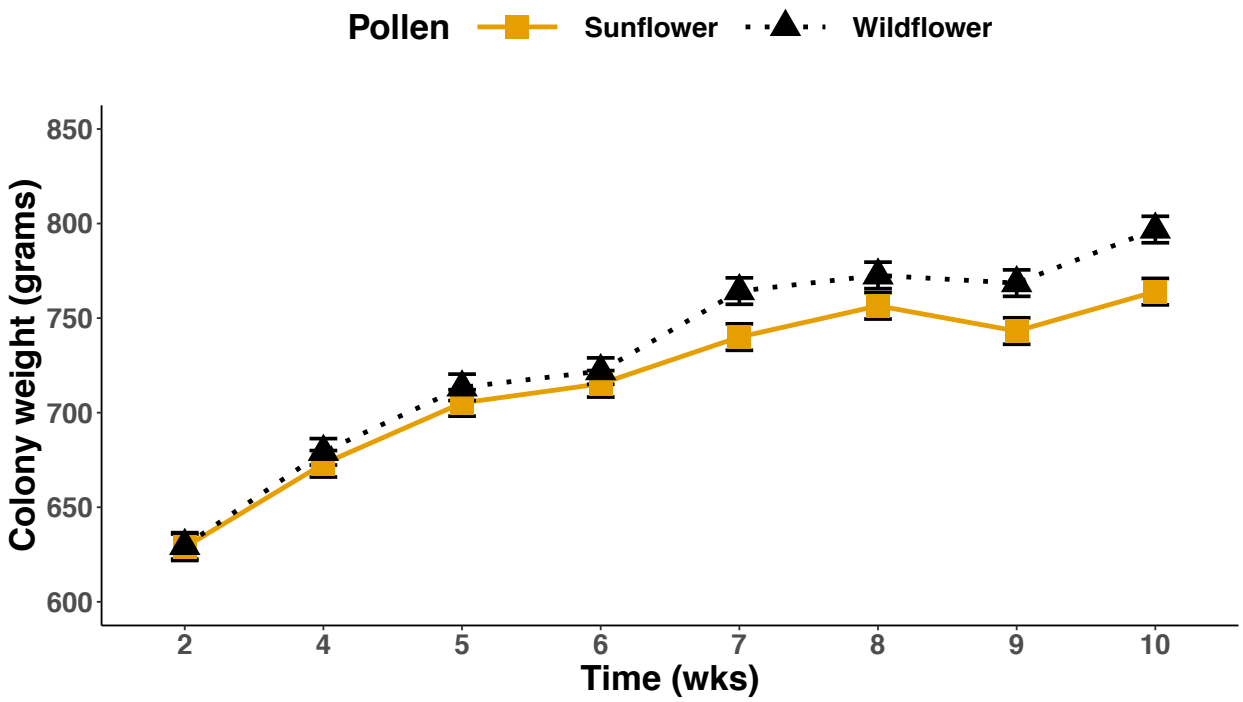


**Figure 2.3**. Change in weight (g) of *Bombus impatiens* colonies fed either sunflower pollen diet (1:1 ratio of sunflower to wildflower pollen; S; yellow squares) or wildflower pollen diet (W; black triangles). Points and error bars indicate model means and standard errors, back-transformed from the scale of the linear predictor.

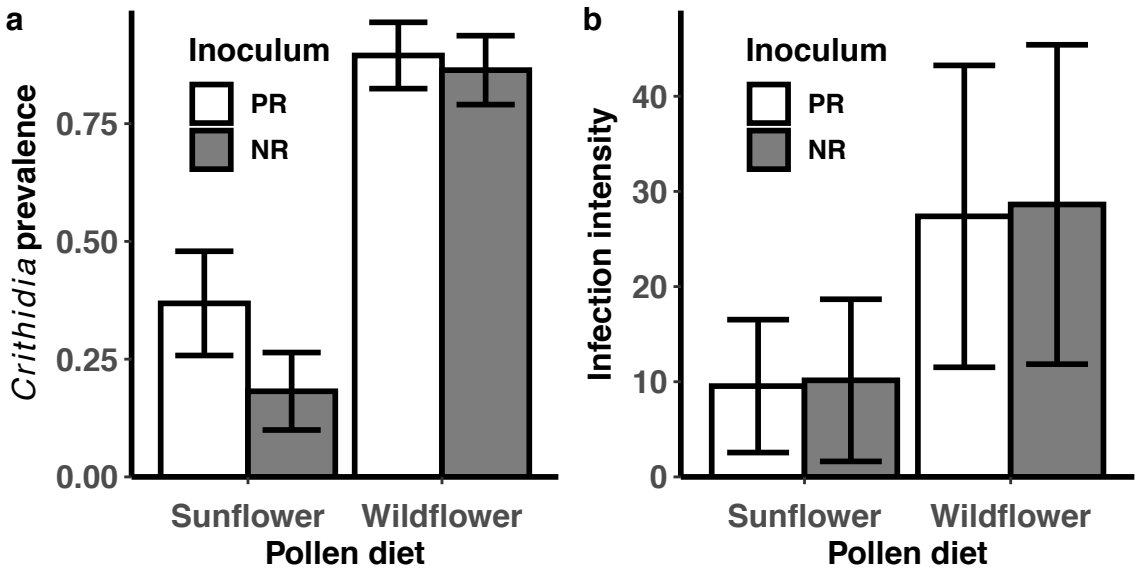


**Figure 2.4**. *Crithidia bombi* infection prevalence and intensity for *Bombus impatiens* workers fed either 100% sunflower pollen or a wildflower pollen mix and inoculated with either a potentially resistant inoculum of *C. bombi* exposed to sunflower pollen (PR) or a non-resistant inoculum (NR) that shared the same lineage as the PR, but was not exposed to sunflower pollen. (**a**) Prevalence of infection (the proportion of parasitized bees) and (**b**) average infection intensity of infected bees only (cells per 0.02 μL) was similar for inoculum types, but significantly lower for bees fed sunflower pollen. Bars and error bars indicate model means and standard errors, back-transformed from the scale of the linear predictor.

# CHAPTER 3: Sunflower pollen induces rapid excretion in bumble bees: implications for host-pathogen interactions

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## Abstract

Host diet can have a profound effect on host-pathogen interactions, including indirect effects on pathogens mediated through host physiology. In bumble bees (*Bombus impatiens*), the consumption of sunflower (*Helianthus annuus*) pollen dramatically reduces infection by the gut protozoan pathogen *Crithidia bombi.* One hypothesis for the medicinal effect of sunflower pollen is that consumption changes host gut physiological function, causing rapid excretion that flushes *C. bombi* from the system. We tested the effect of pollen diet and *C. bombi* infection on gut transit properties using a 2x2 factorial experiment in which bees were infected with *C. bombi* or not and fed sunflower or wildflower pollen diet. We measured several non-mutually exclusive physiological processes that underlie the insect excretory system, including gut transit time, bi-hourly excretion rate, the total number of excretion events and the total volume of excrement.

Sunflower pollen significantly reduced gut transit time in uninfected bees, and increased the total number of excretion events and volume of excrement by 66 % and 68 %, respectively, in both infected and uninfected bees. Bees with more intense *C. bombi* infections had fewer excretion events, but only when fed sunflower pollen. Here we show that a sunflower pollen diet can affect host physiology gut function, causing more rapid and greater excretion. These results provide important insight into a mechanism that could underlie the medicinal effect of sunflower pollen for bumble bees.

**Keywords:** host-pathogen interactions, rapid excretion, insect excretory system, protozoan pathogens, host physiology

## Introduction

Host diet can have a profound effect on host-pathogen interactions (Price et al. 1980, Agrawal 2000, de Roode et al. 2013). In addition to direct toxic effects of host diet on pathogens (Milan et al. 2012, Palmer-Young et al. 2016, Koch et al. 2019), diet can also have indirect effects on pathogens mediated through host physiology (Huffman and Caton 2001, Brunner et al. 2014, Pike et al. 2019). In the case of intestinal pathogens, host diet could influence pathogen growth and reproduction via changes in host immune system (Schlüns et al. 2010, Cotter et al. 2011, Brunner et al. 2014), host microbiome (David et al. 2014, Knutie 2020) and excretory functions (Hoste 2001, Huffman and Caton 2001), which may result in the cleansing of the host intestinal tract. Despite the commonality of intestinal pathogens in insects and vertebrate animals (Poulin 2011), surprisingly few studies have identified the mechanism(s) behind host diets that reduce pathogen infection. Here we focused on how pollen diet affects bumble bee gut

physiology and the implications for intestinal pathogen infection by the protozoan *Crithidia bombi*.

Bees provide pollination services for a wide variety of crops and wildflowers worldwide (Klein et al. 2006, Ollerton et al. 2011a, Garibaldi et al. 2013, Potts et al. 2016), but are threatened by a number of interacting stressors including pathogens (reviewed in Goulson et al. 2015a). Diet plays a key role in mediating host-pathogen dynamics for bees. Pollen in particular serves as the primary source of nutrition for bees, and varies tremendously among plant taxa in both composition and concentration of macronutrients (Roulston and Cane 2000, Yang et al. 2013) and plant secondary chemistry (Cook et al. 2013, Palmer-Young et al. 2018a, 2019). Poor nutrition can weaken an energetically costly immune system and increase symptoms of pathogen infection (Alaux et al. 2010, Di Pasquale et al. 2013, Roger et al. 2017), or conversely may limit resources for pathogens, reducing pathogen growth and reproduction (reviewed in Pike et al. 2019). In addition, a variety of plant secondary compounds found in nectar can reduce pathogen infection in bees (Manson et al. 2010, Baracchi et al. 2015, Richardson et al. 2015, Koch et al. 2019). Despite accumulating evidence that nectar and pollen diets play a key role in mediating pathogen infection in host bees, few studies have identified the mechanisms involved (but see Koch et al. 2019). Understanding how diet mediates bee host-pathogen interactions may lead to practical and efficient methods for improving pollinator health.

Prior research has shown that a diet with sunflower pollen dramatically reduces and can even clear *C. bombi* infection in *Bombus impatiens* worker and queen bumble bees (Giacomini et al. 2018, LoCascio et al. 2019, Adler et al. 2020, Fowler et al. 2020), but the mechanism(s) by which this effect occurs is unknown. Bees become infected with *C. bombi* by consuming contaminated excrement. *Crithidia bombi* cells pass through the esophagus, honey crop and

midgut, and then attach to and replicate along the hindgut walls (Gorbunov 1996, Otterstatter and Thomson 2006, Koch et al. 2019). One hypothesis for the medicinal effect of sunflower pollen is that diuretic or laxative qualities decrease gut passage time, which causes *Crithidia* to be excreted from the host hindgut before attaching or replicating. This is plausible since rapid metabolism and excretion is a common adaptation that many folivorous herbivores use to cope with ingestion of potentially toxic secondary plant metabolites (Ivie et al. 1983, Iyengar et al. 1987, Hasspieler et al. 1988, Kumar et al. 2014, Saremba et al. 2018). For example, Tadmor-Melamed et al. (2004) demonstrated that plant secondary metabolites in nectar stimulate rapid excretion in nectivorous Palestine sunbirds. Recent studies have reported a number of plant secondary metabolites in sunflower pollen, including hydroxycinnamic acid amides and flavanols (Kyselka et al. 2018, Palmer-Young et al. 2018a), phenolic acids (Kostić et al. 2019), as well as a variety of saturated fatty acids (Lin and Mullin 1999, Kostić et al. 2017). In bees, hydroxycinnamic acids upregulate detoxification genes (Mao et al. 2013) that are homologous to those that stimulate rapid excretion in other insects (Chahine and O′Donnell 2011), suggesting that phytotoxins in sunflower pollen may trigger rapid metabolism and subsequent excretion in bees. In addition, sunflower pollen grains are notable for their conspicuous spines that protrude from the outer exine surface (Blackmore et al. 2007). The consumption of rough foods can induce laxative effects, which flush gut pathogens from the digestive tract (Huffman and Caton 2001). Thus, the medicinal effect of sunflower pollen may result from chemical or physical properties of the pollen that stimulate rapid excretion in host bumble bees.

Excretion can be defined as the process by which noxious or useless chemical substances are removed from the metabolic pool of an organism (Cochran 1975). In insects, the Malpighian tubule-rectum complex is the principal excretory system and functions to remove excess ions,

water, nitrogen-containing compounds and other potentially harmful substances (O′Donnell 2008). The primary function of Malpighian tubules is urine production, which can be regarded as the mechanism of removing soluble substances with low molecular weight (e.g., K+, NA+, H2O) from the blood or hemolymph. The hindgut and rectum function to reabsorb useful molecules from the excreta and thus play a critical role in modification of the primary excretory fluid produced by the Malpighian tubules and the formation of dry fecal material. The rate at which these substances are removed or reabsorbed is largely believed to be a function of the nutritional state of the insect and the need to conserve water or ions (Madrell 1971). However, insects often encounter substances in their diet that are not nutritional, or may even be toxic (i.e., xenobiotics or plant secondary metabolites). These substances can be eliminated from the body either by passing through the gut unabsorbed or into the haemocoel, the latter of which likely involves metabolism or detoxification, followed by excretion (Cochran 1975, Esther et al. 2017). Rapid excretion in particular may be an important mechanism for coping with ingestion of xenobiotics to clear from the body before they interact with target sites, or may simply be a necessary step to remove accumulated substances immediately following metabolism by detoxification enzymes.

Here, we tested the hypothesis that sunflower pollen diet leads to rapid excretion in bumble bees infected with *C. bombi*, a physiological response that could flush *C. bombi* cells from the infected host. Workers of the common eastern bumble bee, *Bombus impatiens*, were infected with *C. bombi* or not and fed sunflower or wildflower pollen, and we measured subsequent gut physiological functions including gut transit time, bi-hourly excretion rate, the total number of excretion events, and the total volume of excrement. Results shed light on a mechanism potentially underlying the medicinal effect of sunflower pollen in bees, and more

broadly on how host diet can affect physiological processes that mediate host-pathogen interactions and infection dynamics.

## Methods

### *Study system*

*Bombus impatiens* is a native eusocial bumble bee species in North America that ranges from Maine to Ontario to the eastern Rocky Mountains and south through Florida (Kearns and Thomson 2001). Colonies are commercially reared in North America for crop pollination services (Klein et al. 2007, Ollerton et al. 2011b, Potts et al. 2016), which has greatly expanded its range throughout western North America (Looney et al. 2019). Colonies are annual, founded by single inseminated queens, and can be maintained and manipulated in the lab and field. *Bombus impatiens* is considered broadly polylectic (Cane and Sipes 2006), such that they collect and use pollen from a wide spectrum of plant families. For example, 80 colonies of commercially reared *B. impatiens* in southern Québec, Canada collected pollen from 128 plant species (or morphospecies) in 41 families (Gervais et al. 2020).

Bumble bees, including *B. impatiens*, are commonly infected by the protozoan gut pathogen *Crithidia bombi* (Zoomastigophora:Trypanosomatidae). For example, Gillespie (2010) found that *C. bombi* infected up to 80% of wild-caught *B. impatiens* in western MA, USA. Infection can be contracted at flowers via the consumption of contaminated excrement or horizontally transmitted within colonies or fecal-orally transmitted vertically from queens to workers (Schmid-Hempel and Durrer 1991, Durrer and Schmid-Hempel 1994). Studies have reported a range of effects of *C. bombi* infection on host bumble bees, including reduced learning and foraging efficiency in workers (Gegear et al. 2005, 2006), slower colony growth rates,

especially early in the colony life cycle (Shykoff and Schmid-Hempel 1991), and a reduced likelihood of successful reproduction in wild colonies (Goulson et al. 2018). Nutritional stress increases the mortality of infected workers (Brown et al. 2000) and reduces infected queen fitness (Brown et al. 2003b), indicating synergistic interactions between host diet and infection on bee performance.

Domesticated sunflower (*Helianthus annuus*) is a major oilseed crop that is cultivated worldwide and is a native US wildflower (Reagon and Snow 2006). Many bee species, including *B. impatiens*, are known to visit sunflowers for pollen (Aslan and Yavuksuz 2010, Riedinger et al. 2014, but see Tepedino and Parker 1982, Fell 1986), and we have identified *H. annuus* pollen from the corbiculae of wild-caught and commercially-reared *B. impatiens* workers foraging on sunflower (*unpublished data)*. Sunflower pollen is widely considered to be a relatively poor protein source for some bee species, including *B. impatiens*, due to low crude protein content (Yang et al. 2013) and missing essential amino acids (Tasei and Aupinel 2008, Nicolson and Human 2013, McAulay and Forrest 2018, Treanore et al. 2019).

**Experimental Methods**

We conducted a 2x2 factorial experiment in which bees were infected with *C. bombi* or not and fed a sunflower or wildflower pollen diet. We used worker bees from four experimental *B. impatiens* colonies from Koppert Biological Systems (Howell, MI, USA). Colonies and experiments were housed in a dark room at 21 – 24ºC and ~50% rh. All colonies were confirmed to be pathogen-free by screening ten workers upon receipt using methods described below in *Infection Treatment*. The *C. bombi* used in this study was harvested from three wild *B. impatiens* workers collected near Stone Soup Farm, Hadley, MA, USA (42.363911 N, -72.567747 W) in

2014 and housed in separate commercial colonies of *B. impatiens* (hereafter referred to as source colonies). The *Crithidia* species was identified in a previous study and confirmed to be *C. bombi* (Figueroa et al. 2019).

***Infection Treatment***. We used queenless microcolonies (i.e., small groups of workers) to efficiently establish infection in experimental bees and allow infection to build before transferring bees to individual containers for diet manipulations. Microcolonies were created by transferring seven adult worker bumble bees from uninfected *B. impatiens* colonies into plastic containers (7.5cm x 10cm x 5cm) with mesh screen flooring. A total of 30 microcolonies were constructed throughout the experiment, with 6 – 12 microcolonies per experimental colony. All microcolonies were regularly supplied with honeybee–collected wildflower pollen paste (collected locally in Raleigh, NC, USA) and 30% sucrose solution. After one week, enough time for each microcolony to establish and build honey pots, microcolonies were assigned to either a *C. bombi* or sham-control inoculation treatment.

To make *C. bombi* inoculum, sixteen adult worker bumble bees were dissected from a source colony using an established protocol (Manson et al. 2010, Richardson et al. 2015, Giacomini et al. 2018). Bee digestive tracts (excluding the honey crop) were removed, placed into a 1.5 mL microcentrifuge tube with 300 µL of distilled water, finely ground, and vortexed for five seconds. Each sample was allowed to rest at room temperature for 4-5 hours. *C. bombi* cells were then counted from a 0.02 µL sample of supernatant per bee with a Neubauer hemacytometer and a compound light microscope at 400X magnification. We mixed 200 µL of supernatant from ten samples (total volume = 2 mL) with distilled water to achieve a concentration of 2000 cells $\mu L^{-1}$. The inoculum was then mixed with an equal volume of 50%

sucrose solution to yield a *C. bombi* inoculum with 1000 cells $\mu L^{-1}$ and 25% sucrose. The sham-control inoculum was created by mixing distilled water and 50% sucrose solution to yield inoculum with no *C. bombi* cells and 25% sucrose. Each microcolony received 1 mL of either *C. bombi* inoculum (~142,857 *C. bombi* cells per bee) or the sham-control inoculum pipetted directly into empty honey pots and the brood dish. The dose of *C. bombi* inoculum roughly corresponds to a 20 μL drop of contaminated bee excrement with a concentration of 7,000 *C. bombi* cells $\mu L^{-1}$, which is within the natural range of concentrations bumble bees are exposed to when foraging on flowers in the wild (Schmid-Hempel and Schmid-Hempel 1993). Microcolonies continued to feed on fresh wildflower pollen and 30% sucrose daily for seven days, which is enough time to establish a *C. bombi* infection within a host bumble bee (Otterstatter and Thomson 2006).

***Pollen Diet Treatment***. We administered pollen treatments in five blocks of 36 bees (nine bees per treatment per block), for a total of 45 bees per treatment. For each block, we randomly selected three microcolonies per inoculation treatment, transferred workers to individual containers and randomly assigned each worker to either the sunflower or wildflower pollen treatment for 24 hours. Honeybee-collected monofloral sunflower pollen was purchased (Changge Huading Wax Industry, China), sorted to remove impurities and verified microscopically that pollen grains were morphologically consistent. The wildflower pollen diet was obtained from honeybee–collected wildflower pollen collected locally in Raleigh, NC, USA, and verified microscopically to contain less than 1% Asteraceae pollen. We identified Asteraceae pollen as grains with conspicuous spines that make up the outer exine wall. Pollen was provided to bees as a paste, made by mixing ground pollen pellets with distilled water to achieve a

uniform consistency, packed into the lid of a 1.5 mL microcentrifuge tube. Each bee also received 1 mL of 30% sucrose via a filled and inverted plastic 1.5 mL microcentrifuge tube plugged with cotton (Richmond Dental & Medicine, Charlotte, NC, USA). The weight of each pollen lid and nectar feeder was recorded before and after the 24-hour feeding period to estimate consumption.

***Measuring Gut Transit Properties***. The bumble bee digestive system does not divide waste into solids and liquids. Thus, hereafter we refer to bumble bee excretion as the combination of both urine and fecal material.

For each block, we randomly selected 24 bees (six per treatment combination) that had consumed a net positive amount of pollen and transferred each bee to an individual 7-dram snap cap vial. The remaining bees were discarded, while selected bees were starved of nectar and pollen for 2 hours and then hand-fed 10 uL of 30% sucrose solution mixed with red food coloring (McCormick & Company, Inc., Hunt Valley, MD, USA). After feeding on the red dye, each bee was gently placed, under red light only to reduce stress, onto a filter paper disc (Whatman, Grade 1) with an inverted plastic cup directly over it (hereafter referred to as a video chamber), all of which rested on a glass table. A video camera (Canon VIXIA HF R800) was positioned directly below the glass table such that all 24 paper discs fit within the camera's field of view. As soon as all 24 bees were placed in their video chambers, we began recording for 13 hours under ambient outdoor and fluorescent white light. In pilot tests, the red dye was visible in excrement deposited on filter paper, but gut transit time varied widely, necessitating the use of video for long-term observation. After the video session, all bees were removed from their video chambers and dissected. *Crithidia bombi* infection intensity (number of flagellate cells per 0.02

uL) was measured using a standard protocol (Manson et al. 2010, Richardson et al. 2015, Giacomini et al. 2018). We removed the right forewing of each bee to measure marginal cell length, a proxy for bee size (Nooten and Rehan 2020). Evidence suggests that substantial metabolic costs are associated with ovary production in worker bumble bees which could affect host gut physiology (De Loof 2011, Blacher et al. 2017). Developed ovaries can be readily observed during the dissection process used to measure *C. bombi* infection intensity. We thus recorded ovary development (presence or absence) for each bee, and later removed bees with developed ovaries from the analysis.

***Estimating gut transit time, total number, excretion rate and total volume.*** We measured four estimates of gut physiology: gut transit time, total number of excretion events per 13-hour period, bi-hourly excretion rate, and total volume of excrement (estimated as area of excrement on filter paper). Gut transit time in this study refers to the amount of time in which substances in the diet pass through the entire digestive system. We also estimated the total number of excretion events, the bi-hourly excretion rate and total volume, which were all strongly correlated (Appendix D; Table S3.1). Despite such a correlation, we believe it is important to analyze each in order to shed light on non-mutually exclusive physiological processes that underlie the insect excretory system. To estimate gut transit time, we observed the videos and recorded the time to observation of each of the first five red-dyed excretions for each bee. We only observed the first five red-dyed excretions since most bees (> 80 %) failed to deposit a sixth red-dyed excretion by the end of the 13-hour period. We also used the video to record the cumulative number of excretion events every two hours and calculated a bi-hourly excretion rate for each bee by

extracting the slope from bee-specific linear models of cumulative number of excretion events regressed against time (two-hour intervals).

Each paper disc was photographed using a Canon EOS Rebel DSLR camera under UV light and against a black background. Bee excrement naturally fluoresces under UV light (JJG, pers. obs.), which allowed us to illuminate bee excrement that would be invisible under white light. For each bee, we used imageJ software (Schindelin et al. 2012) to measure the area of excrement deposited onto each paper disc as an estimate of total volume of excrement and to measure the total number of excretion events as the number of non-overlapping spots with an area larger than 5 $mm^2$ (to ignore splatter). Spots that overlapped but were obviously separate events were manually counted and added to the total. While the number of excretion events estimated using the still images and the video were strongly correlated ($t = 4.561$, $df = 108$, $p < 0.0001$, $R = 0.402$), un-dyed excretion events often were not observable in the video but were in the fluorescent still images. Thus, using the still images to estimate the total number of excretion events should be more accurate than the videos.

***Statistical Analysis.*** All statistical analyses were conducted using R version 4.0.2 (R Core Team 2020). Figures were produced with "ggplot2" (Wickham 2016) and "survminer" (Kassambara et al. 2016). In total, we were able to track the gut transit properties of 110 bees; 27 infected-sunflower, 26 uninfected-sunflower, 30 infected-wildflower and 27 uninfected-wildflower. Ten bees were excluded from the analyses due to mortality (n = 6) or the presence of well-developed ovaries (n = 4). Bee size was strongly associated with nectar consumption ($F_{1, 112} = 26.534$, $p < 0.0001$) and was thus excluded from analyses.

*Pollen and nectar consumption.* We used linear mixed effect models fit with the "lme4" package (Bates et al. 2015) to analyze the effect of pollen diet and infection treatment on the amount of pollen (mg) and nectar (mg) consumed by bees prior to measuring gut transit properties. Pollen diet, infection treatment and their interaction were included as fixed effects, with block as a random effect. Pollen and nectar consumption were not statistically correlated based on Pearson's product-moment correlation ($R = -0.118$, $t = -1.259$, $df = 112$, $p = 0.211$). We also analyzed the relationship between *C. bombi* infection intensity (infected bees only) and pollen diet and their interaction (all fixed effects) on the amount of pollen and nectar consumed in separate models, with block as a random effect. We used Analysis of Deviance Type II tests to assess significance of fixed effect terms and t-tests (simple slopes analysis) using the "interactions" package (Long 2019) to test if slopes significantly differed from zero for each pollen diet in the *C. bombi* infection intensity models.

*Gut transit time.* We used separate Cox mixed-effect models (i.e., time-to-event models) using the "coxme" package (Therneau 2015) to analyze the timing of each of the first five red-dyed excretions during the thirteen hour video period. Time-to-event models are useful when the outcome of interest is not only if the event occurred, but also when the event occurred. To analyze the effect of pollen diet and infection treatment on gut transit time, we included pollen diet, infection treatment and their interaction as fixed effects, continuous covariates of pollen consumption and nectar consumption, and block as a random effect. We also modeled the relationship between *C. bombi* infection intensity and gut transit time for bees inoculated with *C. bombi,* with pollen diet, *C. bombi* infection intensity and their interaction as fixed effects, the continuous covariates of pollen consumption and nectar consumption and block as a random

effect. We used Analysis of Deviance Type II tests to assess significance of fixed effect terms. For time-to-event models in which fixed effect parameters were significant, we assessed *post hoc* comparisons between treatments by refitting a cox proportional hazards (coxph) model with a single treatment term that represented the combination of pollen diet and infection treatment. Significance of post-hoc pairwise comparisons between treatment combinations was assessed using log-rank tests using the pairwise_survdiff function in the "survminer" package (Kassambara et al. 2020). For all gut transit time models, we set wildflower pollen diet and the sham control inoculation (uninfected bees) as the reference levels for pollen diet and infection treatment, respectfully. For each model, we report the hazard ratio (HR) for each significant treatment with respect to the reference treatment. For categorical factors, the hazard ratio indicates the change in the expected number of red-dyed excretions per one unit of time (1 hour) compared to the reference level. For the continuous variable *C. bombi* infection intensity, the hazard ratio indicates the change in the expected number of red-dyed excretions per one unit of time if intensity rises by one unit.

*Total number and volume of excretion events and bi-hourly excretion rate.* We used separate generalized linear models (GLMs) to analyze the effect of pollen diet and infection treatment on total number of excretion events (negative binomial distribution with a log-link function), the total volume of excrement deposited (gamma distribution with a log-link function), and the bi-hourly excretion rate (gamma distribution with an inverse-link function). Mixed-effect models with a negative binomial distribution were fit using the "glmmTMB" package (Magnusson et al. 2017) and those with gamma distributions were fit using the "lme4" package (Bates et al. 2015). Pollen diet, infection treatment and their interaction were included as fixed effect parameters

along with pollen consumption and nectar consumption as continuous covariates. To analyze the relationship between the gut transit parameters (total number of excretion events, total volume and bi-hourly excretion rate) and *C. bombi* infection intensity for bees that were inoculated with *C. bombi*, we used GLMs with the fixed effects pollen diet, *C. bombi* infection intensity and their interaction. For all GLMs, we assessed the significance of potential random effects of microcolony nested within block and microcolony nested within source experimental colony using AIC and log-likelihood ratios to compare full and reduced models with and without random effect terms in a stepwise fashion. The GLM with block as the random effect term provided the best fit in every case, with the exception of the bi-hourly excretion rate model where including a random effect term resulted in singularity (i.e., random-effect variance estimates of zero). We used Analysis of Deviance Type II tests from the "car" package (Fox and Weisburg 2011) to assess significance of fixed effect terms in each GLM and the "emmeans" package (Lenth 2020) to extract estimated marginal means.

**Results**

***Pollen and nectar consumption.*** On average, bees consumed approximately 3.3 times more wildflower pollen than sunflower pollen, based on weight (pollen diet: $\chi^2 = 491.830$, $p < 0.0001$; Fig. 1a), but pollen diet did not affect nectar consumption ($\chi^2 = 0.025$ $p = 0.875$, Fig. 1b). There was no effect of infection treatment or pollen diet by infection interaction on pollen or nectar consumption ($\chi^2 < 1.5$, $p > 0.2$ for all cases). Among infected bees only, there was a significant interaction between pollen diet and *C. bombi* infection intensity on the amount of pollen consumed ($\chi^2 = 11.521$, $p = 0.0007$, Fig. 1c). The simple slopes analysis indicated a significant positive relationship between infection intensity and pollen consumption for wildflower-fed bees

(t = 2.273, p = 0.026) and a marginally significant negative relationship for sunflower-fed bees (t = -1.904, p = 0.062). There was no effect of infection intensity or the interaction of pollen diet and infection intensity on the amount of nectar consumed ($\chi^2 < 1.4$, $p > 0.2$ for both cases).

***Gut transit time.*** We found a significant interaction between pollen diet and infection treatment on timing of the first ($\chi^2 = 9.347$, $p = 0.002$; Fig. 2) and second ($\chi^2 = 4.141$, $p = 0.042$) excretions. Post-hoc pairwise comparisons revealed that among uninfected bees, sunflower pollen significantly reduced gut transit time (i.e., bees excreted faster) relative to wildflower pollen (HR = 2.5, p = 0.003; HR = 2.2, p = 0.016, for first and second excretions respectively). There were no main effects or interactions between pollen diet and infection treatment on the timing of the third, fourth and fifth excretions ($\chi^2 < 1.9$, $p > 0.17$ in all cases). There was a moderately significant negative relationship between *C. bombi* infection intensity and the timing of the fifth excretion (HR = 0.9893, $\chi^2 = 4.239$, p = 0.0395), corresponding to a 1.07% decrease in the expected hazard for observing the fifth excretion relative to a one unit increase in *C. bombi* infection intensity. There was no relationship between *C. bombi* infection intensity and the timing of the first four excretions ($\chi^2 < 0.219$, $p > 0.224$ in all cases). The covariates of nectar and pollen consumption did not have a significant effect on gut transit time ($\chi^2 < 0.119$, $p > 0.324$ in all cases).

***Total number and volume of excretion events.*** Bees that consumed sunflower pollen had approximately 66% more excretion events ( $\chi^2 = 5.787$, $p = 0.016$; Fig. 3a) that covered approximately 68% more area ($\chi^2 = 4.699$, $p = 0.030$; Fig. 3b) of the paper discs compared to bees that consumed wildflower pollen. There was no effect of infection treatment or the pollen

diet by infection interaction on total excretion events or volume ($\chi^2 < 0.9$, $p > 0.2$ for all cases). There was a significant positive relationship between volume and both nectar ($\beta = 1.132$, $\chi^2 = 7.999$, $p = 0.0047$) and pollen consumption ($\beta = 13.060$, $\chi^2 = 5.560$, $p = 0.0184$). Back-transforming the model coefficients to the scale of the response, this corresponded to an approximate 31% increase in volume per every 100 mg of nectar consumed and a 10-fold increase per mg of pollen consumed. The amount of pollen or nectar consumed did not explain significant variation in the number of excretion events ($\chi^2 < 1.988$, $p > 0.178$ for both).

In infected bees only, there was a significant interaction between pollen diet and *C. bombi* infection intensity on the total number of excretion events ($\chi^2 = 5.165$, $p = 0.023$). For bees that consumed sunflower pollen, there was a negative relationship between *C. bombi* infection intensity and the number of excretion events, whereas there was a positive relationship between *C. bombi* infection intensity and the number of excretion events for bees that consumed wildflower pollen (Fig. 4a). There was no significant interaction between pollen diet and *C. bombi* infection intensity for the total volume of excretion ($\chi^2 = 0.011$, $p = 0.917$), but there was a significant negative relationship between *C. bombi* infection intensity and the total volume of excrement, regardless of pollen diet ($\beta = -0.003$, $\chi^2 = 5.035$, $p = 0.025$; Fig. 4b). Back-transforming the model coefficient to the scale of the response, this corresponded to a nearly equal decrease in excrement volume (measured as area, in $mm^2$) per one-unit increase in *C. bombi* cell count.

***Bi-hourly excretion rate.*** Pollen diet did not significantly affect bi-hourly excretion rate ($\chi^2 = 0.024$, $p = 0.876$), but infection nearly doubled the rate ($\chi^2 = 9.717$, $p = 0.002$; Fig. 5). The pollen by infection interaction, and nectar and pollen consumption, did not significantly affect bi-hourly

excretion rate ($\chi^2 < 0.75$, $p > 0.35$ for all cases). For infected bees, the interaction between pollen diet and infection intensity did not affect bi-hourly excretion rate ($\chi^2 = 0.011$, $p = 0.917$), but there was a significant negative relationship between *C. bombi* infection intensity and bi-hourly excretion rate, regardless of pollen diet ($\beta = 0.016$, $\chi^2 = 6.736$, $p = 0.009$; Fig. 4c). Back-transforming the model coefficient to the scale of the response, this corresponded to an approximately 1.42% decrease in bi-hourly excretion rate per each unit increase in *C. bombi* infection intensity.

**Discussion**

Host diet can have important implications for their physiology, with potential ramifications for host-pathogen interactions. Here we found that consuming sunflower pollen resulted in greater and more rapid excretion in bumble bees compared to wildflower pollen. Specifically, gut transit time was significantly shorter in uninfected bees fed sunflower pollen, indicating a laxative or diuretic effect of sunflower pollen. Sunflower-fed bees also had a greater number of excretion events and excreted a much greater volume despite having consumed less pollen and an equal amount of nectar compared to wildflower-fed bees. These results provide important insight into a potential physiological mechanism underlying the medicinal effect of sunflower pollen on bumble bees.

The mechanism of action underlying the gut physiological responses to sunflower pollen consumption could be chemical, mechanical, or both. Herbivorous insects can cope with ingestion of potentially toxic compounds by removing them through a variety of non-mutually exclusive processes, including metabolizing toxins to less harmful derivatives, converting lipophilic compounds into hydrophilic metabolites that can be more easily excreted, and rapid

excretion. For example, in the specialist herbivore *Manduca sexta*, unmetabolized nicotine is rapidly excreted before it is modified (Kumar et al. 2014), but the larvae of generalist *Trichoplusia ni* rapidly metabolize nicotine to hydrophilic cotinine, cotinine-N-oxide and nicotine-N-oxide, which is followed by rapid excretion (Saremba et al. 2018). The metabolism of phytotoxins and the subsequent passage of non-toxic plant material through the digestive system of mutualist bee pollinators is also well-documented. In honey bees (*Apis sp.)* and bumble bees (*Bombus sp*.), a variety of detoxification genes are found in the genome, although to a lesser extent than in other phytophagous insects (Claudianos et al. 2006). Several studies have found that such genes play a major role in bee metabolism of phytotoxins and other xenobiotics found in honey and pollen (Mao et al. 2009, 2011, 2013, Johnson et al. 2012). It is thus a plausible hypothesis that sunflower pollen consumption causes the upregulation of detoxification enzymes in bumble bees, which in turn leads to rapid excretion.

The mechanism of action underlying the gut physiological response to sunflower pollen consumption could also be mechanical. Sunflower pollen grains are notable for their conspicuous spines that protrude from the outer exine surface (Blackmore et al. 2007). This echinate morphological trait is common throughout much of the Asteraceae family, including several relatives of *H. annuus* that also produced the same medicinal effect in bumble bees (LoCascio et al. 2019). To the best of our knowledge, a link between physical stimulation and rapid excretion in insects has not been well described in the literature. However, a recent study found that consumption of crushed *Taraxacum* pollen, which is also a member of the Asteraceae family, caused significant damage to the bumble bee gut lining (Vanderplanck et al. 2020). The authors speculate that crushing the pollen may have increased the density of sharp fragments that physically damaged gut epithelial cells. Alternatively, phytosterols found in *Taraxacum* pollen

equally damage the gut lining, suggesting that crushed pollen may have simply increased the bioavailability of phytosterols that are otherwise trapped within undigestible pollen grains. Indeed, sunflower pollen is particularly notable for poor digestibility in honey bees that inefficiently extract the chemical contents of sunflower pollen compared to pollen from other plant families (Nicolson et al. 2018). To the best of our knowledge, it is currently unknown why sunflower pollen is difficult to digest, but likely is a function of pollen grain morphology, namely a thick outer exine, small pores and echinate surface. It is thus plausible that echinate sunflower pollen irritates the gut lining of bees, which increases excretion rates. If mechanical irritation by spiky pollen causes rapid excretion in bumble bees, then pollen diets that contain a high proportion of pollen species with a spiky outer exine, regardless of plant family, or contain a proportion of empty (i.e., methanol-extracted) pollen grains with spikes, should induce rapid excretion. Alternatively, if removing the internal contents of sunflower pollen substantially slows the excretion processes, this would suggest that chemical properties of sunflower pollen are driving the effect.

The total number of excretion events was negatively associated with *C. bombi* infection intensity for bees fed sunflower pollen, but not wildflower pollen, suggesting that ingestion of sunflower pollen stimulates rapid and voluminous excretion which may reduce *C. bombi* cells in the host gut. To the best of our knowledge, evidence of diet-induced rapid excretion providing anti-parasitic benefits in insects is lacking. However, in the Hemipteran *Rhodnius prolixus,* consumption of the plant secondary metabolite burchellin reduces urine production via the release of antidiuretic hormones, which also reduced infection load of the gut protozoan pathogen *Trypanosoma cruzi* (Garcia and Azambuja 2004), indicating a link between excretion rates and infection of protozoan gut parasites. Thus, it is plausible that chemical constituents of

sunflower pollen may induce changes in host bumble bee excretion that subsequently reduce *C. bombi* cells from the digestive tract. Contrary to that hypothesis, Adler et al. (2020) recently demonstrated that several phytochemicals notable in sunflower pollen, including the secondary compounds triscoumaroyl spermidine and rutin, as well as several fatty acids, did not reduce *C. bombi* infection *in vivo* when added to other non-medicinal pollen diets. However, despite such a contradiction, it is important to point out that further research is needed to fully explore the phytochemical profiles of sunflower pollen before interactions between host bumble bees and phytotoxins can be ruled out as the mechanism. For example, while there is consistent evidence of relatively high concentrations of chlorogenic acid in sunflower (*H. annuus*) leaves, flower petals (Gai et al. 2020) and seeds (Weisz et al. 2009), there is mixed evidence of chlorogenic acid in pollen or nectar (Palmer-Young et al. 2018b, Kostić et al. 2019). Consumption of chlorogenic acid upregulates putative detoxification genes in insects (Li et al. 2004) and reduced *Crithidia* infection in bumble bees (Egan et al. 2018), suggesting the potential for a plant secondary compound to influence both host digestive processes and resistance to gut pathogens. Establishing a causal link between sunflower-mediated rapid excretion and reduced *C. bombi* infection should ideally be confirmed with further studies that assess a wide variety of chemical components in sunflower pollen, including synergistic effects between combinations of chemicals.

Alternatively, morphological properties of sunflower pollen may mechanically stimulate rapid excretion in host bumble bees and flush *C. bombi* from the digestive tract. Since *C. bombi* requires attachment to the walls of the hindgut for growth and replication (Gorbunov 1996, Otterstatter and Thomson 2006, Koch et al. 2019), one hypothesis is that damage of the host gut lining stimulates rapid excretion and disrupts attachment by *C. bombi*. In mammals, Huffman

and Canton (2001) discovered that physical irritation resulting from the consumption of spiky vegetation increased excretion rates in wild chimpanzees and removed pathogenic nematodes from the gut. Similarly, a spiky pollen diet may have effects on host gut physiology akin to those caused by high-fiber diets in many organisms. For example, a high fiber diet appears to play a major role in mucin regulation and often significantly increases daily mucin output in the excreta (reviewed in Montagné et al. 2004). Mucins are gel-forming glycoproteins with high carbohydrate content that are a major component of the insect intestinal mucus layer, and are known to protect against microbial infection and chemical damage by binding to microorganisms and particles that are removed by the mucociliary system (Derrien et al. 2010). Moreover, many protozoan pathogens express mucin-degrading enzymes, which can turn mucins into an important source of energy for pathogens or act as adhesion ligands for pathogen reproduction (Pellegrin et al. 1991, Hicks et al. 2000, Hoffman et al. 2020). While it is unknown if *C. bombi* utilizes mucin-degrading enzymes to extract nutrients from the host, *Crithidia* species are known to exist in either a non-motile form attached to the lining of the insect gut or as an elongated form that swims freely in the gut lumen (Brooker 1971, Gorbunov 1996), and thus may rely on host mucins for reproduction. Thus, a spiky sunflower pollen diet may influence the production of host mucins, preventing the establishment of *C. bombi* or facilitating expulsion.

In addition to diet, pathogens can also induce physiological changes that influence host excretion rates. We found that *C. bombi* infection nearly doubled the bi-hourly excretion rate in bumble bees. Pathogen-induced increase of host excretion rates may be adaptative for fecal-orally transmitted pathogens, since increased excretion rates may increase transmission rates. One study found that bumble bees excreted onto flowers significantly more when infected with *Crithidia* (Figueroa et al. 2019), which may increase the probability of a susceptible bee

encountering contaminated excrement while foraging among shared flowers. However, among infected bees, greater *C. bombi* infection intensity was associated with a lesser volume and a slower excretion rate. This suggests a limit on the ability of *C. bombi* to increase host bumble bee excretion rates and perhaps an opposing physiological response by host bumble bees as infection intensity increases. We also found that sunflower pollen consumption decreased gut transit time only in uninfected bees, indicating that *C. bombi* infection increases gut transit time, or slows down digestive functions in host bumble bees. Additionally, bees with greater *C. bombi* infection intensity that were fed sunflower pollen diet had fewer excretion events, whereas the reverse was found in wildflower-fed bees.

The rate at which urine and excreta production occurs may be a function of the nutritional state of the insect (Madrell 1971). If *C. bombi* competes with the host for nutrition, it is plausible that host excretion parameters could slow down to conserve water, ions or other nutrients. Indeed, recent work with honey bees infected with the trypanosomatid gut pathogen *Lotmaria passim*, found that a number of genes associated with starvation were upregulated in the hindgut at the late stage of infection, suggesting that gut epithelial cells are in poor nutritional status (Liu et al. 2020). In response, hosts could counter competition for nutrients by increasing food consumption, or by decreasing host activity to offset energy requirements. We did not detect compensation in food consumption by infected bumble bees, suggesting that energetic demands are low in infected worker bumble bees housed in ideal laboratory conditions. Measurable compensation is more likely in reproductive individuals (i.e., gynes or queens) in stressful environmental conditions. Experiments are needed that experimentally vary *C. bombi* infection intensities in worker bumble bees in variable conditions to determine if pathogen-induced physiological changes interact with nutrient competition and influence host excretion rates.

Our data indicate that sunflower pollen ingestion causes rapid excretion, but it is not clear if this effect is driven by a diuretic or laxative effect. The insect excretory system is primarily comprised of Malpighian tubules (MTs) and the hindgut. MTs largely function to transport solutes into the lumen, producing urine that contains various ions, amino acids, and waste materials, including toxins (O′Donnell 2008). The hindgut selectively resorbs water, ions, and metabolites, which results in a strongly hyperosmotic or hypoosmotic excreta containing fecal material and urine. Sunflower pollen may have diuretic properties if urine production by MTs increases, or may have laxative properties if gut transit time of fecal material decreases. The bee digestive system, however, does not divide waste into solids and liquids, but instead individual bee excretions contain both urine and fecal material. This makes it is difficult to differentiate between laxative and diuretic effects in bees. Further research that measures the urinary concentrations of ions (e.g., Na+ and K+) in bees may help indicate whether sunflower pollen causes a laxative or diuretic effect.

This study sheds light on the effects of floral rewards on the physiology of mutualist pollinators with potential implications for host-pathogen interactions. Research that aims to reveal the molecular mechanisms that underlie the effect of sunflower pollen consumption on host gut physiology and subsequent excretion patterns could improve our understanding of bee-pathogen dynamics and may lead to practical and efficient methods for improving pollinator health. This is particularly important since declines of many pollinator species are linked to pathogens (Potts et al. 2010, Goulson et al. 2015b) and such declines are of critical concern to food security and conservation of native ecosystems at global scales.

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**Author contributions**

JJG, LSA, and REI designed research; JJG performed research and analyzed data; JJG and REI wrote the paper with feedback from all authors.

**Data Availability**

All data generated from this project and custom scripts used for statistical analysis and plotting will be made publicly available upon publication.

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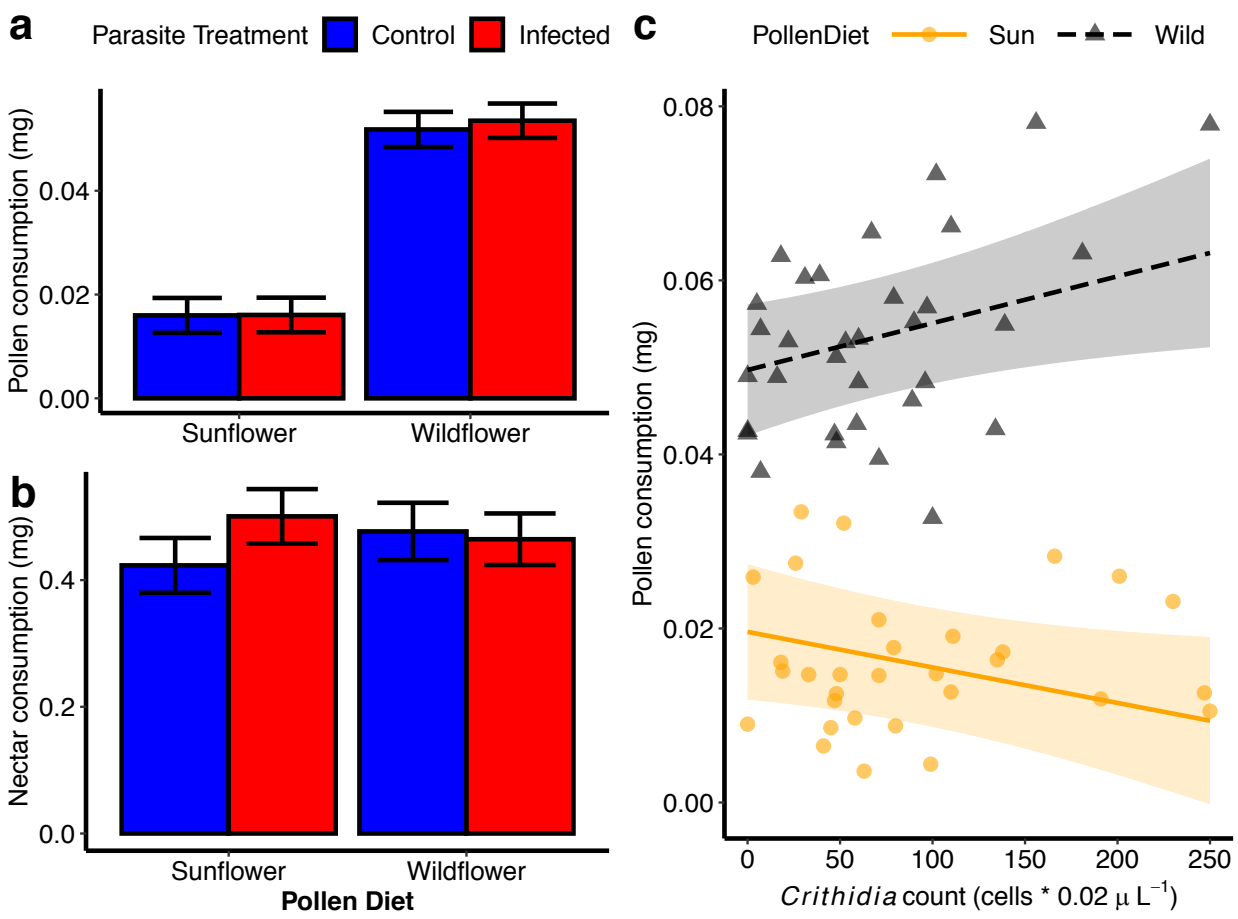


**Figure 3.1**. **(a)** Average amount of pollen and **(b)** average amount of nectar consumed by *Bombus impatiens* workers fed sunflower or wildflower pollen and inoculated with a sham control (control; blue bars) or *Crithidia bombi* (Infected; red bars). Bars and error bars indicate linear model means ± 1 standard error. **(c)** Relationship between *C. bombi* infection intensity, among infected bees only, and the average amount of pollen consumed for bees fed sunflower pollen (Sun; orange circles and solid line) or wildflower pollen (Wild; black triangles and dashed line). Lines and shaded band indicate model-adjusted mean pollen consumption ± 95% confidence intervals; points show raw data for individual bees.

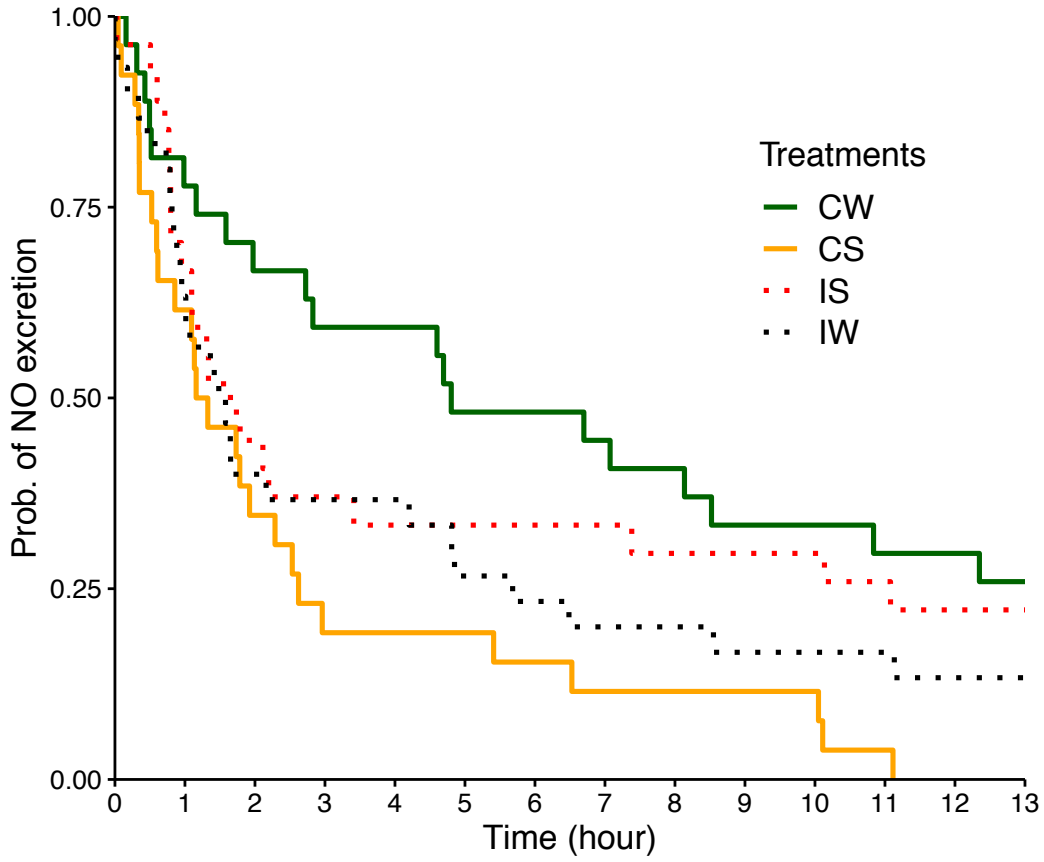


**Figure 3.2.** The probability of not observing the first red-dyed excretion (y-axis) over time (hour) of *Bombus impatiens* workers fed either sunflower pollen (S; solid orange or red dotted lines) or a wildflower pollen mixture (W; solid green or black dotted lines), and either infected with *Crithidia bombi* (I; dotted lines) or un-infected (C; solid lines). Estimates of probability were derived from a cox proportional hazards model fit to a survival curve based on a tabulation of the number at risk and number of observations at each unique time.

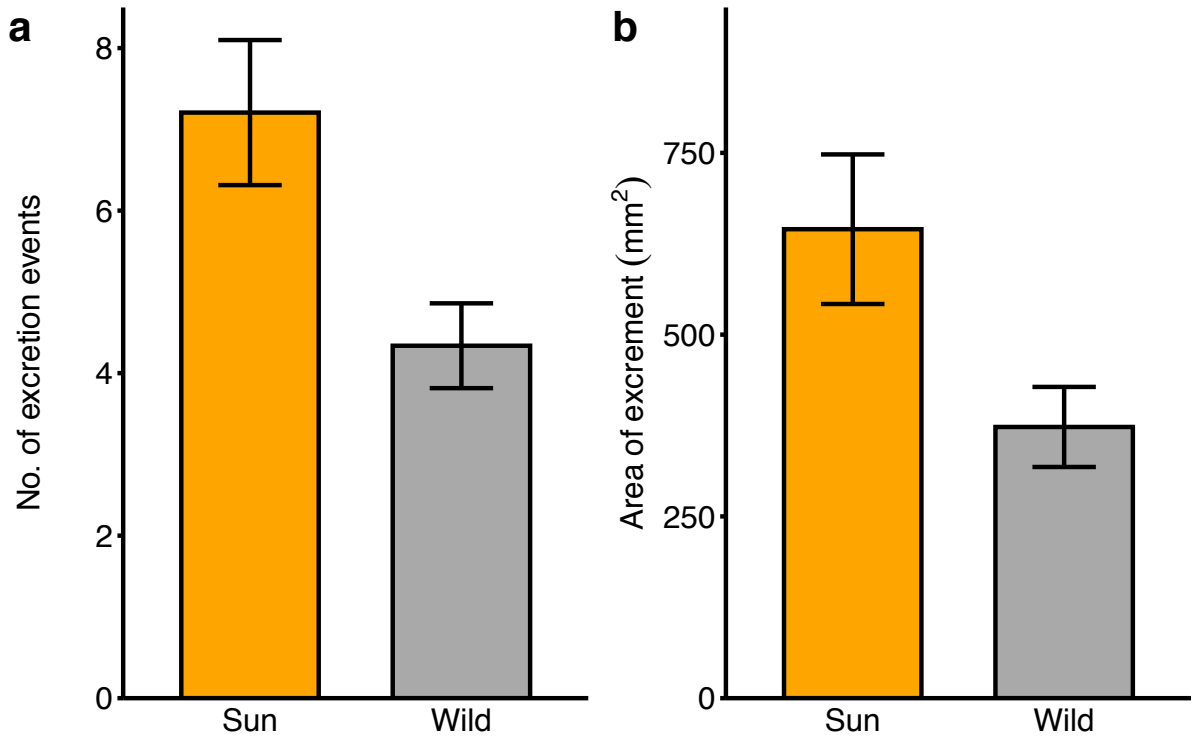


**Figure 3.3. (a)** Average number of excretion events and **(b)** average area of excrement deposited (an estimate of volume) after 13 hours by *Bombus impatiens* workers fed either sunflower (Sun; orange bars) or wildflower pollen (Wild; gray bars). Bars and error bars indicate generalized linear model means ± 1 standard error back-transformed from the scale of the linear predictor.

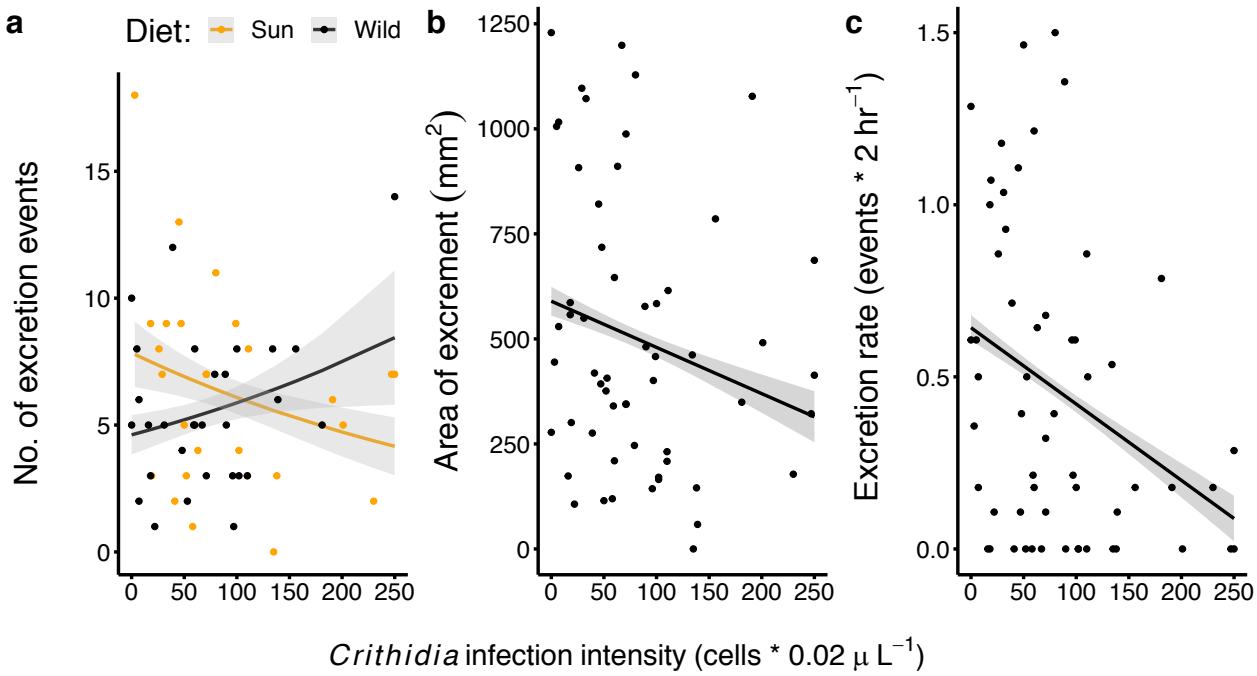


**Figure 3.4.** Relationship between *Crithidia bombi* infection intensity (infected bees only) and (**a**) the number of excretion events, (**b**) the area of total excretion (an estimate of volume), and (**c**) the bi-hourly excretion rate by host *Bombus impatiens* workers. The relationship between the number of excretion events and *C. bombi* infection intensity differed between bees fed either sunflower (Sun; orange lines) or wildflower pollen (Wild; black). Lines and shaded bands indicate back-transformed estimated model means ± 1 standard error; points show raw data for individual bees.

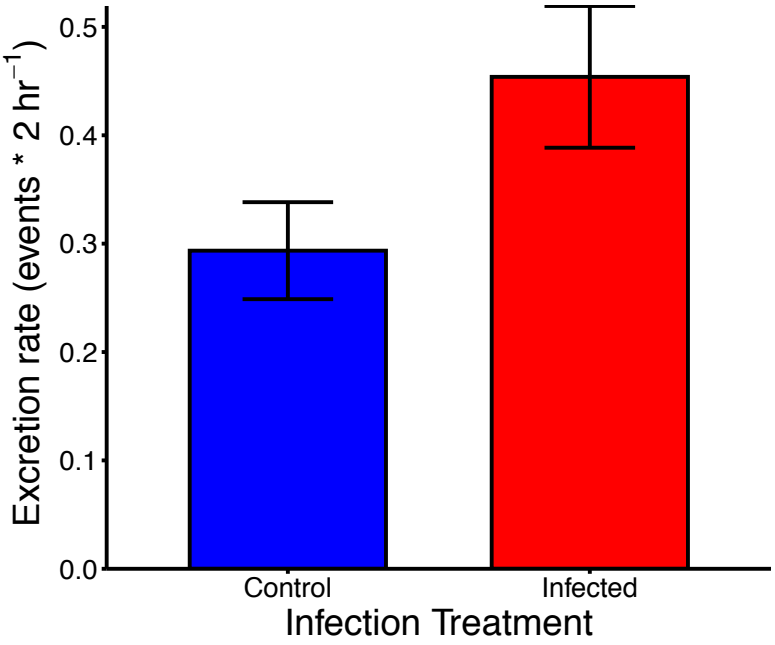


**Figure 3.5** Average bi-hourly excretion rate by host *Bombus impatiens* workers inoculated with a sham control (Control; blue bar) or *Crithidia bombi* (Infected; red bar). Bars and error bars indicate generalized linear model means ± 1 standard error back-transformed from the scale of the linear predictor.

# CHAPTER 4: Differential bumble bee gene expression associated with pathogen infection and pollen diet

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## Abstract

Diet and parasitism can have powerful effects on host gene expression. However, how specific dietary components affect host gene expression that could feed back to affect parasitism is relatively unexplored in many wild species. Recently, it was discovered that the consumption of sunflower pollen (*Helianthus annuus*) reduced severity of the gut protozoan pathogen *Crithidia bombi* in *Bombus impatiens* worker bumble bees. Despite the dramatic and consistent medicinal effect of sunflower pollen, very little is known about the mechanism(s) underlying this effect. However, sunflower pollen extract increases rather than suppresses *Crithidia* growth *in vitro*, suggesting that sunflower pollen reduces *Crithidia* infection indirectly via changes in the host. Here, we analyzed whole transcriptomes of *B. impatiens* workers to characterize the physiological response to sunflower pollen consumption and *C. bombi* infection to isolate the

mechanisms underlying the medicinal effect. *B. impatiens* workers were inoculated with either *Crithidia bombi* cells (infected) or a sham control (un-infected), and fed either sunflower or wildflower pollen *ad libitum*. Whole abdominal gene expression profiles were then sequenced with Illumina NextSeq 500 technology. Among infected bees, sunflower pollen upregulated immune genes, including the anti-microbial peptide hymenoptaecin, Toll receptors and serine proteases. In both infected and un-infected bees, sunflower pollen upregulated detoxification genes and genes associated with the repair and maintenance of gut epithelial cells. Among wildflower-fed bees, infected bees downregulated immune genes associated with phagocytosis and the phenoloxidase cascade. Taken together, these results indicate dissimilar immune responses between sunflower- and wildflower-fed bumble bees infected with *C. bombi*, a response to physical damage to gut epithelial cells caused by sunflower pollen, and a strong detoxification response to sunflower pollen consumption. The data generated in this study provides a strong foundation to further explore the chemical and structural properties of sunflower pollen that drive the medicinal effect in bumble bees.

## Introduction

Organisms are frequently exposed to a wide range of environmental challenges, such as fluctuations in nutrient availability, ingestion of toxins or invasion of pathogens. As a result, organisms modulate gene expression patterns at the transcriptional level in order to cope with such environmental challenges. Interactions between diet and pathogen infection can create feedbacks in gene expression that impact an organisms health. For example, in phytophagous insects, nutrient availability (Cotter et al. 2011, Di Pasquale et al. 2013, Brunner et al. 2014, Roger et al. 2017) or phytotoxins (Smilanich et al. 2009, Tan et al. 2019) in the diet can reduce

host immune gene expression, thus making a consumer more vulnerable to infection, or may enhance immune gene expression (Laurentz et al. 2012, Smilanich et al. 2018), thus conveying health benefits to the consumer. Despite the large body of literature on multitrophic interactions (McCann et al. 1998, Agrawal 2000, Abdala-Roberts et al. 2019), studies on whole genome transcriptomic responses to different diets and the interplay between diet and parasite infection remain rare. Here we focus on the relationship between pollen diet, bumble bees and a protozoan pathogen of bumble bees to shed light on how specific dietary components affect host gene expression that could feed back to affect pathogen infection.

Recently, it was discovered that consumption of sunflower pollen (*Helianthus annuus*) reduces severity of the gut protozoan pathogen *Crithidia bombi* in *Bombus impatiens* worker and queen bumble bees by at least 80% relative to control pollen (Giacomini et al. 2018, LoCascio et al. 2019a, Fowler et al. 2020). Despite the dramatic and consistent medicinal effect of sunflower pollen on *C. bombi* infection in *B. impatiens*, we have yet to identify the mechanism(s) underlying this effect. However, since sunflower pollen extract increased rather than suppressed *C. bombi* growth *in vitro* (Palmer-Young 2017), it is likely that sunflower pollen reduces *C. bombi* infection via changes in host physiological functions, such as immune and detoxification systems, or physical changes in the gut environment that prevent parasite growth and reproduction.

Chemical or physical properties of sunflower pollen may have an indirect negative effect in *C. bombi* mediated through changes in host bumble bee physiology. Sunflower pollen has relatively low protein content and lacks the essential amino acids methionine and tryptophan (Nicolson and Human 2012). Many microbial gut parasites rely on their host for nutrition (Coop and Holmes 1996), and thus poor host nutrition can limit parasite growth and reproduction.

However, the consumption of buckwheat pollen (*Fagopyrum escueluetum*), which matched sunflower pollen in crude protein and amino acid content, did not reduce *C. bombi* infection (Giacomini et al. 2018), and the consumption of a presumably nutritionally balanced sunflower pollen diet diluted with a diverse wildflower pollen blend (1:1 ratio by weight) did significantly reduce *C. bombi* infection in bumble bees (Giacomini et al. 2021), effectively ruling out poor host nutrition as the mechanism. Sunflower pollen also contains plant defensive compounds, including neochlorogenic acid, quercetin glycosides (Kostić et al. 2019), flavonoids (Kyselka et al. 2018, Palmer-Young et al. 2018), as well as a variety of saturated fatty acids and sterols that may have antimicrobial properties (Lin and Mullin 1999, Kostić et al. 2017). Adler et al. (2020) recently demonstrated that triscoumaroyl spermidine and rutin (a proxy for quercetin glycosides), as well as nine fatty acids found in sunflower pollen, failed to reduce *C. bombi* infection *in vivo* when spiked into non-medicinal control pollen diets. However, consumption of chlorogenic acid, which is similar in structure to neochlorogenic acid found in sunflower pollen (Kostić et al. 2019), reduced *Crithidia sp.* infection in bumble bees (Egan et al. 2018). Similar to what was found for sunflower pollen extracts, Palmer-Young et al. (2016) demonstrated that chlorogenic acid did not have a direct toxic effect on *Crithidia sp.* cells, thus indicating an indirect effect on *Crithidia sp.* infection mediated through changes in host bumble bee physiology. In addition, a recent study demonstrated that the consumption of phytosterols in *Taraxacum sp.* pollen, as well as crushed *Taraxacum sp.* pollen, caused substantial damage to the lining of the *B. terrestris* bumble bee digestive tract (Vanderplanck et al. 2020). Since *Crithidia sp.* require tight adhesion to the gut lining in bumble bees in order to establish infection (Koch et al. 2019), it is plausible that phytosterols in sunflower pollen or echinate spines, which are a particularly notable trait of

sunflower pollen morphology (Blackmore et al. 2007), cause damage to the gut lining, which in turn prevents adhesion and reduces proliferation of *C. bombi*.

Analyzing changes in bumble bee transcriptome in response to different diets and infection may shed light on the molecular pathways involved in the medicinal effect of sunflower pollen consumption in bumble bees infected with *C. bombi.* If sunflower pollen reduces *C. bombi* infection via changes in the host bumble bee immune system, then we would expect genes associated with canonical immune signaling pathways, including the Melanization & Encapsulation, Toll, Jak/STAT, IMD/JNK, or RNAi pathways described in bumble bees (Barribeau et al. 2015), to be differentially expressed in sunflower pollen-fed bees compared to bees fed the wildflower pollen control diet. In addition, a variety of detoxification genes are found in the genome of bumble bees (*Bombus sp*.), although to a lesser extent than other phytophagous insects (Claudianos et al. 2006). Several studies have found that such genes play a major role in bee metabolism of phytotoxins and xenobiotics found in honey and pollen (Mao et al. 2009, 2011, 2013, Johnson et al. 2012) and may also elicit an immune response in bees (Schmehl et al. 2014). Congruent expression of putative immune and detoxification genes may indicate that plant defensive chemicals play an important role in the medicinal effect. Alternatively, if echinate sunflower pollen decreases digestibility, or causes physical damage to the bee gut, then we may expect changes in gene expression associated with plasma membrane repair that mediate active resealing of membrane disruptions to maintain homeostasis.

There is an increasing interest in dietary ingredients that are appropriate to support insect pollinator health, including digestive and immune functions. Insect pollinators support numerous plant communities and are thus vitally important members of a diverse set of ecosystems. Bees are arguably some of the most important insect pollinators since many wildflowers and crops,

such as fruits, nuts, and vegetables in our diets, benefit enormously from bee pollination (Winfree et al. 2007, Garibaldi et al. 2013, Rogers et al. 2014). Recent estimates attribute an economic value of more than 15 billion dollars annually to the United States economy (Calderone 2012), of which bumble bees contribute substantially (Velthuis and Van Doorn 2006). Unfortunately, population declines have been observed for a number of bumble bee species worldwide (Goulson et al. 2005, Colla and Packer 2008) and the loss of native bumble bee species is likely to have far-reaching cascading effects on plant communities, ultimately threatening conservation of wildflowers, the ecosystems they support and sustainable crop management. Multiple stressors have been implicated in bumble bee declines, including poor nutrition, habitat loss and pathogens (Goulson et al. 2008, Cameron et al. 2011). Thus, identifying mechanisms that underly the medicinal effect of sunflower pollen in infected bumble bees may broaden our understanding of pollinator disease ecology and may provide opportunities for effective management of bee pathogens.

The objective of this study was to use a RNAseq-based whole transcriptome approach to identify key molecular pathways involved in the medicinal effect of sunflower pollen consumption in bumble bees infected with *C. bombi*. We analyzed differences in gene expression profiles of adult *B. impatiens* workers inoculated with live *C. bombi* cells or a sham control, and then either fed a medicinal sunflower pollen diet or a wildflower pollen control diet. Using a combination of traditional frequentist statistics and machine learning techniques, we found that consumption of sunflower pollen enhances bumble bee immune response to *C. bombi*, stimulates detoxification processes and upregulates genes associated with physical damage to gut epithelial cells.

## Methods

***Study System.*** *Bombus impatiens* is a native eusocial bee species in North America, ranging from Maine to Ontario to the eastern Rocky Mountains and south through Florida (Kearns and Thomson 2001). They are generalists that visit a range of agricultural and native plants. *B. impatiens* have been extensively domesticated for crop pollination services throughout much of North America (Shipp et al. 1994, Whittington and Winston 2004, Velthuis and Van Doorn 2006), subsequently making them a widely utilized study species. An annotated reference genome for *B. impatiens* (Sadd et al. 2015) is available from the National Center for Biotechnology Information (NCBI). At the time of this study NCBI BIMP_2.2 (GenBank assembly accession: GCA_000188095.4) contained 13,161 genes that code for 24,471 proteins.

*Crithidia bombi* (Zoomastigophora:Trypanosomatidae) is an infectious protozoan gut pathogen that can be contracted at flowers via fecal transmission and can also be horizontally transmitted within colonies (Schmid-Hempel and Durrer 1991, Durrer and Schmid-Hempel 1994). *Crithidia sp.* reduce learning and foraging efficiency in worker bumble bees (Gegear et al. 2005, 2006), slows colony growth rates, especially early in the colony life cycle (Shykoff and Schmid-Hempel 1991), reduces the likelihood of successful reproduction in wild colonies (Goulson et al. 2018), and reduces infected queen fitness (Brown et al. 2003). *Crithidia sp*. infection is common; for example, *Crithidia sp.* infected over 60% of wild-caught *B. impatiens* in western MA (Gillespie 2010) and commercial colonies can have high levels of infection (Gegear et al. 2005).

Sunflowers (*Helianthus* sp.) belong to a large and diverse family (Asteraceae) with over 32,000 described species (Funk 2009). Domesticated sunflower (*H. annuus*) is a major oilseed crop cultivated worldwide and a native US wildflower (Reagon and Snow 2006). With nearly

two million acres of sunflowers planted in the US (Holcomb 2015) and ten million acres planted in Europe annually (Strange et al. 2016), the high abundance of cultivated sunflowers combined with large nectar and pollen yields make it an important resource for bees.

***Preparing inoculation treatments.*** Live *Crithidia bombi* cells were harvested from three wild *B. impatiens* workers collected near Stone Soup Farm, Hadley, MA, USA (42.363911 N, -72.567747 W) and housed in commercial colonies of *B. impatiens*. The *Crithidia* species was identified in a previous study and confirmed to be *C. bombi* (Figueroa et al. 2019). Both the *C. bombi* source colony and experimental colony used in this experiment were purchased from Koppert Biological Systems (Howell, MI, USA). Colonies were fed with 30% sucrose solution and mixed wildflower pollen throughout their lifetimes and housed in a dark room at 21 – 24ºC and ~50% rh. We made *C. bombi* inoculum using an established protocol (Manson et al. 2010, Richardson et al. 2015, Giacomini et al. 2018). Briefly, bee digestive tracts of 15 workers, excluding the honey crop, were removed with forceps, placed into 1.5 mL microcentrifuge tubes with 300 µL of distilled water, and ground with a pestle. We allowed each sample to rest at room temperature for 4-5 hours so that gut material settled and the *C. bombi* cells could ascend into the supernatant. *Crithidia bombi* cells were counted from a 0.02 µL sample of supernatant per bee with a Neubauer hemacytometer under a compound light microscope at 400X magnification. We then mixed 150 µL of the supernatant with distilled water to achieve a concentration of 2400 cells $\mu L^{-1}$. The sample was then mixed with an equal volume of 50% sucrose solution to yield inoculum with 1200 cells $\mu L^{-1}$ in 25% sucrose. We made the sham inoculum following the same procedure as the *C. bombi* inoculum, but instead used the digestive tracts of five bees sourced from the un-infected experimental colony.

***Preparing pollen diets.*** We prepared two pollen diet treatments – sunflower and wildflower. Honey bee-collected sunflower pollen pellets were obtained from Changge Hauding Wax Industry (China) and sorted by color to remove impurities. We verified a pure batch of sunflower pollen by staining five samples with basic fuschin dye (Kearns and Inouye 1983) and visually confirming only sunflower pollen was present with a compound microscope at 400X magnification. Honey bee-collected mixed wildflower pollen pellets were obtained from Koppert Biological Systems (Howell, MI, USA) and microscopically confirmed to contain < 5% Asteraceae pollen, identified by having spines on the exine (Blackmore et al. 2007). Experimental pollen diets were provided to bees as a paste produced by mixing ground pollen pellets with distilled water to achieve a uniform consistency.

***Inoculation treatment.*** Experimental adult worker bumble bees were obtained from a single commercial *B. impatiens* colony that was determined to be un-infected by screening five workers using the methods described in ***Preparing inoculation treatments***. Workers were removed from their colonies of origin and placed into individual plastic containers (7.5 cm x 10 cm x 5 cm) with mesh screen flooring. We starved the bees for 3-5 hours and then fed each a 10 μL drop of either the *C. bombi* inoculum or sham control; bees were assigned at random to inoculation treatment. The dose of *C. bombi* inoculum contained 12,000 *C. bombi* cells, which is within the concentration range bees are exposed to when foraging on flowers in the wild (Schmid-Hempel and Schmid-Hempel 1993). Only bees that consumed the entire droplet (n = 120) were used in the experiment. In total we inoculated 60 workers with the *C. bombi* inoculum (hereafter referred to as infected bees) and 60 workers with the sham inoculum (hereafter referred to as un-infected bees).

Inoculated bees were then randomly assigned to either the sunflower (n = 60) or wildflower pollen diet (n = 60). Each day we fed inoculated bees fresh pollen paste packed, of their assigned treatment, into an inverted lid of a 1.5 mL microcentrifuge tube and 1 mL of 30% sucrose via a filled and inverted plastic 1.5 mL microcentrifuge tube plugged with cotton (Richmond Dental & Medicine, Charlotte, NC, USA). We harvested tissue samples for RNA extraction 72 hours post-inoculation. We selected this time period because *C. bombi* counts start to diverge between bees fed sunflower vs. wildflower pollen between 72 and 96 hours post-inoculation, with sunflower pollen starting to have lower counts than wildflower pollen (*Appendix E: Supplementary Text: Timing of sunflower pollen effect; Figure S4.1, Appendix F*). Moreover, a recent study demonstrated that consuming sunflower pollen for approximately the first 72 hours or all 7 days after inoculation reduced *C. bombi* intensity in bumble bees compared with control pollen (LoCascio et al. 2019b). We harvested tissue samples for RNA extraction from five workers per treatment that had the greatest average daily rate of pollen consumption (see ***Pollen consumption*** below). In total, we sequenced 20 samples: 5 replicates for each inoculation treatment and pollen diet.

***Pollen consumption.*** Previous work shows evidence of a dose-dependent medicinal effect of sunflower pollen in bumble bees, such that higher concentrations of sunflower pollen consumed produced a stronger medicinal effect (Giacomini et al. 2021). Because it was not feasible to control how much pollen an individual bee consumes, having an estimate of pollen consumption is thus important to effectively model the relationship between pollen diet and gene expression. To estimate consumption of pollen over the 72-hour period, we recorded the weight of each pollen feeder each day before placing it into the container with the bee and 24 hours later. We

accounted for feeder weight change caused by evaporation by placing an additional 30 pollen and nectar feeders (15 per pollen type) into containers that lacked a bee. Each day bees were provided fresh sucrose and pollen, yielding three days (post inoculation) of pollen consumption and evaporation measurements. We were not able to estimate nectar consumption because nectar feeders would often leak solution.

All statistical analyses using linear models were conducted using R version 4.0.2 (R Core Team 2020). To estimate pollen consumption, we calculated evaporation-adjusted net consumption based on change in weight of the pollen feeder for each bee per day. Using the evaporation controls, we fit separate linear regressions for each day and pollen type, with initial weight regressed against weight 24-hr later. We then used the *predict* function in R to calculate an evaporation-adjusted feeder weight, yielding a net consumption estimate for each bee each day. Consumption variables (day 1, day 2, day 3, average daily rate (mg/day) and total) were strongly correlated based on Pearson's product moment correlations ($t > 4.538$, $df = 34$, $p < 0.001$ for all combinations). We thus focused solely on average daily pollen consumption rate for all gene expression analyses (see ***Differential gene expression analysis***), as this was the metric used to select bees for RNA sequencing. We used ANOVA to test for differences in average daily pollen consumption rate between pollen diets and inoculation treatments for RNAseq bees. Model estimated means and Tukey-adjusted pairwise comparisons were obtained using the "emmeans" package (Lenth 2020).

***Efficacy of inoculation.*** To verify that bees inoculated with *C. bombi* were infected and that sunflower pollen reduced *C. bombi* infection relative to wildflower pollen, we measured the *C. bombi* prevalence and infection intensity of a random subset of the remaining bees that were not

selected for RNA extraction, but also consistently consumed their pollen treatments over the first 72 hours [n(sunflower pollen) = 15 bees, n(wildflower pollen) = 18 bees]. Each bee was dissected as in ***Preparing inoculation treatments***, with the addition that all tools were washed with 70% ethanol and thoroughly dried between bees to prevent cross-contamination. *Crithidia bombi* cells from a 0.02 μL sample of supernatant per bee were counted with a Neubauer hemacytometer at 400X magnification with a compound light microscope (Manson et al. 2010, Richardson et al. 2015, Giacomini et al. 2018). We measured prevalence as the presence (1 or more *C. bombi* cells) or the absence of *C. bombi* cells per 0.02 μL of each sample, and *C. bombi* infection intensity as the number of flagellate *C. bombi* cells per 0.02 μL. We also removed the right forewing of each bee to measure marginal cell length, a proxy for bee size (Nooten and Rehan 2020).

We used generalized linear models to analyze how pollen diets affected *C. bombi* infection prevalence and intensity. *Crithidia bombi* prevalence models were fit with a binomial distribution and infection intensity models were fit with a negative binomial distribution using the "MASS" package (Venables and Ripley 2002).

***RNA extractions and sequencing.*** For bees selected for RNA sequencing, at 72 hours post-inoculation, the bees were anesthetized in a container of dry ice for 2 min. Using flame-sterilized forceps, we removed the abdomen of each anesthetized bee and placed it into a sterile 2 mL microcentrifuge tube with 2 mL of RNA-stabilizing reagent (RNAlater; ThermoFisher, Waltham, MA, USA; cat. No. AM7021). Each abdomen was slightly torn open with forceps for the RNA-stabilizing reagent to fully saturate the tissue sample and stored at 4°C for 24 hours. All samples were then kept in a -80°C freezer until RNA extraction. *Crithidia bombi* is a gut

pathogen in bumble bees, and since our interests were in the effects diet, we chose to focus the sequencing on abdominal tissues where the two interact. We did not to use whole-bees to avoid potential tissue specific gene expression patterns (e.g., differences between brain and gut gene expression), which has been shown in other insects (Pulpitel et al. 2015) and may make it difficult to disentangle any gut specific responses.

Total RNA samples were submitted to the NCSU Genomic Sciences Laboratory for Illumina RNA library construction and sequencing. Purification of messenger RNA (mRNA) was performed using oligo-dT beads in the NEBNExt Poly(A) mRNA Magnetic Isolation Module. Complementary DNA (cDNA) libraries for Illumina sequencing were constructed using the NEBNext Ultra Directional RNA Library Prep Kit (NEB) and NEBNext Mulitplex Oligos for Illumina (NEB) using the manufacturer-specified protocol. Double-stranded cDNA was purified, end repaired, and "a-tailed" for adaptor ligation. Following ligation, samples were processed for a final fragment size (adapters included) of 400-550 bp using sequential AMPure XP bead isolation (Beckman Coulter, USA). Prior to library construction, RNA integrity, purity, and concentration was assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Chip. Library enrichment was performed and specific indexes for each sample were added during the protocol-specified PCR amplification. The amplified library fragments were purified and checked for quality and final concentration using an Agilent 2100 Bioanalyzer with a High Sensitivity DNA chip. The final quantified libraries were pooled in equimolar amounts for clustering and sequencing on an Illumina NextSeq 500 DNA sequencer, using a 75 bp x 2 single end sequencing reagent kit. The software package Real Time Analysis was used to generate raw bcl (base call files), which were then de-multiplexed by sample into fastq files. Low-quality bases and adapter sequences were removed from raw sequence data for each sample using the

Trimmomatic software package. Clean reads were mapped to the *B. impatiens* genome (BIMP 2.2) with HiSat2 version 2.1.0 (Kim et al. 2015) using default parameters. Gene expression was quantified using StringTie version 2.0 (Liao et al. 2014) to determine the number of reads uniquely mapping to exons and summed at the gene level using gene features annotated in the NCBI *B. impatiens* annotation file (BIMP 2.2; GCA_000188095.4).

***Differential gene expression analysis.*** We used a negative binomial generalized linear model using DESeq2 version 1.28.1 (Love et al. 2014) in R to test for differences in gene expression between treatments. We tested for effects of treatment (pollen diet x inoculation treatment) on gene expression and included pollen consumption rate as a continuous covariate to control for variation caused by differences in average daily pollen consumption among bees. We used the Wald test to assess the significance of differentially expressed genes (DEGs) and corrected for multiple testing using the Benjamini-Hochberg method with a cutoff of FDR < 0.05. Shrunken log2 fold changes for normalized gene counts were obtained using lfcShrink function in DESeq2 with a shrinkage estimator based on a normal prior (Love et al. 2014).

***Machine Learning analysis.*** Transcriptomic data often suffers from the 'curse of dimensionality' due to having many more features than samples (Clarke et al. 2008). Small sample sizes and the rapid loss of degrees of freedom thus make it a poor fit for traditional linear statistics, like regression and ANOVA (Bzdok et al. 2018). Standard analyses typically use multiple testing corrections to control false discovery rate. This method fails to consider the highly interactive system of the transcriptome and often fails to detect small changes in gene

expression. Machine learning, or artificial intelligence tools, can be used to address these challenges by building models from the data rather than fitting the data to rigid models.

We applied support vector machines (SVM) for classification using Weka 3.8.4 (Eibe et al. 2016) to the gene expression profiles of each pairwise treatment comparison. Classification in machine learning is the task of learning to distinguish data points that belong to two or more categories in a dataset. Feature selection techniques can then be used to select a reduced number of variables that can maintain accurate classification. For example, comparing infected bumble bees fed either sunflower or wildflower pollen, support vector machines can be used to determine how well pollen diet can be classified from gene expression profiles. Feature selection can then be used to determine a reduced set a of genes that accurately classify pollen diet, and are thus important. In order to optimize data dimensionality for feature selection, we first selected a subset of genes from each pairwise treatment from the DESeq2 models that were differentially expressed based on an un-corrected p-value $< 0.05$. Genes were then ranked using the InfoGain attribute evaluator and Ranker search method. This process evaluates the worth of an attribute by measuring the information gain with respect to the treatment. Specifically, InfoGain measures the difference in the Shannon's entropy of the system H(S) before a new attribute X is introduced, and H(S|X) is the entropy of the system after the attribute X has been introduced. We then created a series of ranked data sets, each included a subset of the top-ranked genes in a serial manner.

Preliminary classifier runs demonstrated that a support vector machine SMO, using the 10-fold (stratified hold-out) cross-validation method, correctly classified an average of 82.90% of genes (SD = 27.68 %), and thus was used in our analyses. For comparison, both of the two decision trees, J.48 and Random Forest, were only able to correctly classify 50% of the

instances. SMO Implements John Platt's sequential minimal optimization algorithm for training a support vector classifier by globally replacing all missing values and transforming nominal attributes, in our case genes, into binary attributes (Platt 1998). The machine-learning algorithm SMO has been successfully used to analyze gene expression profiles (Chen et al. 2016). We used the ranked data sets to train the SMO algorithm using both the 10-fold (stratified hold-out) and 66% split cross-validation method. For each data set, we repeated model training 100 times and used the classification performance metrics percent correct classification (%CC) and kappa statistic (k) to evaluate model performance.

We tested the efficacy of the optimized SMO model using a negative control method. We first created 10 randomized data sets using the DESeq2 normalized counts with treatment randomly assigned. We then re-ran the SMO model training using the number of attributes that provided the best classification. Since there are always two class types, the predicted correct classification rate from random assignment should be approximately 50%, based on the Law of Probability, and the kappa statistic should be close to zero for a randomized negative control to demonstrate that true learning occurred in the optimized SMO models.

***Gene Ontology enrichment analysis.*** Protein descriptions and gene ontology (GO) annotations for gene sequences were obtained using OmicsBox version 1.4.11 software (https://www.biobam.com/omicsbox/). First, a BED formatted file of gene coordinates was parsed from the NCBI BIMP 2.2 Annotation release. We then used bedtools version 2.29.2 (Quinlan and Hall 2010) to extract nucleotide sequences based on the BED file coordinates. A BLASTX search was then performed with an E-value of $10^{-25}$ against all arthropod sequences in the NCBI non-redundant database, with the number of hits restricted to 20, followed by GO

mapping and annotation for the resulting hits. We then ran InterProScan annotation for the sequences using the default settings and merged InterProScan GO annotations with BLASTX annotations. GO enrichment analysis was performed for all treatment comparisons to find significantly (FDR < 0.05) enriched GO biological process and molecular function terms in the test set of DEGs with respect to the reference set. We used the publicly available databases GeneCards (Stelzer et al. 2016) and UniProtKB/Swiss-Prot (Consortium 2019) as additional primary sources of information about DEGs.

***IPA canonical pathway analysis.*** We used Qiagen Ingenuity Pathway Analysis (IPA) software to further interpret the differential expression of genes in each treatment pairwise comparison. IPA Knowledge Base maintains a large set of databases that consist of curated metabolic and signaling pathways. Genes were manually mapped to human, mouse or rat ortholog gene IDs or other species' UniProtKB accession numbers for use in IPA. We also performed an additional blastx for all genes using the same methods described in ***Gene Ontology enrichment analysis***, but restricted to the *Homo sapiens*, *Mus* and *Rattus* taxonomies. Using these two different methods allowed us to double-check ambiguous orthologous gene symbols. We then performed a Core Analysis in IPA to determine enrichment of relevant canonical metabolic and signaling pathways that based on gene expression patterns. We repeated Core Analysis for each pairwise treatment comparison based on the list of important genes identified using machine learning. The significance of the association between each gene set and a Canonical Pathway was determined from a p-value of overlap calculated using a right-tailed Fisher's Exact Test. In addition, IPA calculates a z-score based on the gene expression fold change values of each gene to estimate the state of activation or inhibition of each pathway.

## Results

***Inoculation efficacy and pollen consumption.*** All but one of the non-RNAseq bees (1 of 18 bees) fed wildflower pollen were infected with *Crithidia*, suggesting successful inoculation for the RNAseq bees. Infected wildflower-fed bees had an average *Crithidia* intensity of 33.44 +/- 12.3 cells/0.02μL (mean +/- SE), which is an approximately 42 fold change increase compared to the initial inoculation. Sunflower pollen significantly reduced the prevalence of *Crithidia* infection by 35.07 % ($\chi^2 = 10.678$, $p = 0.001$) and the intensity of infection by 85.77 % ($\chi^2 = 5.866$, $p = 0.015$) relative to wildflower pollen. The average daily rate of pollen consumption did not affect *Crithidia* infection intensity or prevalence ($\chi^2 < 0.388$, $p > 0.533$, for both). Of the RNAseq bees, there was a significant effect of pollen diet on the average daily rate of pollen consumption ($F_{1,16} = 5.374$, $p = 0.034$), such that sunflower-fed bees had an approximately 24% lower average rate of pollen consumption compared to wildflower-fed bees (Figure S4.2, *Appendix F*).

***Assembly and BLAST.*** In total, between 13,702,311 and 60,011,808 cleaned reads were obtained after sequencing and trimming (Table S4.1). The average mapping rate of clean reads to the *Bombus impatiens* genome was 91.38 +/- 1.27 %, resulting in 22,726 unique transcripts. Reannotating the transcripts using OmicsBox blastx using the nr database against all arthropods yielded a total of 17,077 hits. The greatest number of top BLAST hits were found in *B. impatiens*, with the top five being *Bombus* (Figure S4.3, *Appendix F*), thus giving us confidence in our read quality.

***Differential gene expression - Infected bees: sunflower vs. wildflower***. Among infected bees, 40 genes were differentially expressed between sunflower- and wildflower-fed bees based on the DESeq2 model (FDR < 0.05; Table S4.2). Notably, four genes associated with the innate immune system were significantly upregulated in infected sunflower-fed bees, including the anti-microbial peptide *hymenoptaecin,* a serine protease inhibitor (dipetalogastin), *alkaline phosphatase 4* and *digestive cysteine proteinase 1* (Figure 4.1). Four genes associated with detoxification and oxidative stress were upregulated in infected sunflower-fed bees, including *glucose dehydrogenase [FAD, quinone]-like*, *cytochrome P450 9e2-like* and *oxidation resistance protein 1 isoform X6* and *beta−1,4−glucuronyltransferase 1* (Figure 4.2). Two genes associated with gut morphology were upregulated in sunflower-fed bees, including *endochitinase* and *nicastrin* (Figure 4.3).

Based on machine learning, we were able to correctly classify sunflower-fed from wildflower-fed infected bumble bees in 100% (SD = 0%) of the instances when using the top 160 through 105 ranked genes (Figure S4.4, *Appendix F*). The negative control method using a randomized set of the top 160 ranked genes demonstrated that true learning occurred in the optimized SMO model. Re-training the SMO model with the randomized data sets, the overall mean percent correct classification was 47.31 % (SD = 24.09 %), the average kappa statistic was -0.04 (SD = 0.46) and the AUROC was 0.48 (SD = 0.24), indicating that true learning occurred in the optimized SMO model with 160 top ranked genes. The IDs, gene functions and expression levels of the top 160 ranked genes are presented in Table S4.3 (*Appendix F*).

Notably, machine learning identified importance of a number of genes associated with the innate immune system that were upregulated in infected sunflower-fed bees (Figure 4.1), including Toll pathway receptor *spaetzle*, several serine proteases (*transmembrane protease*

*serine 9-like, serine protease inhibitor dipetalogastin and probable serine/threonine-protein kinase samkC*), and trypsins (*chymotrypsin-1, trypsin-3 and trypsin-1*), as well as *digestive cysteine proteinase 1*, *alkaline phosphatase 4*. Machine learning also identified several genes associated with detoxification and oxidative stress that were upregulated in infected sunflower-fed bees, including *UDP-glucuronosyltransferase 2B17-like, cytochrome P450 9e2-like, probable cytochrome P450 305a1, oxidation resistance protein 1, thioredoxin reductase 1 and beta−1,4−glucuronyltransferase 1* and *E3 ubiquitin-protein ligase MARCH5*, which plays an important role in ubiquitin-mediated protein degradation (Figure 4.2). Six genes associated with gut morphology, epithelium repair and maintenance were also identified by machine learning and upregulated in infected sunflower-fed bees, including *chitinase-3-like protein 1*, *actin 5c*, *catenin alpha*, *Partitioning defective 3 homolog*, and *lysosomal aspartic protease* (Figure 4.3).

***Functional enrichment - Infected bees: sunflower vs. wildflower***. We did not find significant enrichment of any GO biological process or molecular function terms for DEGs from the DESeq2 model. However, GO enrichment analysis based on the combination of the top 160 ranked genes identified by machine learning indicated significant enrichment of proteolysis, glucosidase, hydrolase, carboxypeptidase, exopeptidase and peptidase activities, as well as several carbohydrate metabolic processes (Figure S4.5, *Appendix F*), suggesting a metabolic response to xenobiotics and pollen nutrients (i.e., proteins, lipids and starches).

*IPA: Canonical Pathways*

A total of 104 out of the 160 top ranked optimal genes identified by machine learning were successfully mapped into IPA; 45 genes were uncharacterized and we were unable to find

human, rat or mouse orthologs for 11 genes. The top enriched canonical pathway was NRF2-mediated oxidative stress response (Table S4.4; *Appendix F*), which was predicted to be activated (z-score = 2.00, p-value <0.0001). This pathway elicits a cellular defense response to oxidative stress, including induction of detoxifying enzymes and antioxidant enzymes. Several other canonical pathways that overlap with the NRF2-mediated oxidative stress response pathway, and also play a role in response to oxidative stress were also enriched, including the thioredoxin pathway, acetone degradation, nicotine degradation II & III pathways, several melatonin degradation pathways, the epithelial adherens junction signaling pathway, and the aryl hydrocarbon receptor signaling pathway. The epithelial adherens junction signaling pathway also plays an important role in the maintenance of epithelial cell layers.

***Differential gene expression - Un-infected bees: sunflower vs. wildflower.*** Among un-infected bees, 11 genes were differentially expressed between sunflower- and wildflower-fed bees based on the DESeq2 model (FDR < 0.05; Table S4.5, *Appendix F*). The detoxification gene *dehydrogenase [FAD, quinone]-like* was upregulated in un-infected sunflower bees (Figure 4.2), along with *jerky protein homolog-like* (Figure 4.3), which is associated with the WNT signaling pathway and wound healing, and the *putative odorant receptor 92a*, which relays signals along the gut-brain axis. Only two of the ten significant genes overlapped with the DEG list from the comparison of infected sunflower- and wildflower-fed bees, one of which (*RNA-directed DNA polymerase from mobile element jockey-like)* was up-regulated in both lists, while the other (uncharacterized gene) was up-regulated in un-infected sunflower-fed bees, but was down-regulated in infected sunflower-fed bees compared to wildflower-fed bees, respectively.

Based on machine learning, we were able to correctly classify un-infected sunflower-fed from wildflower-fed bumble bees in 100% of the instances with the top 141 through 114 ranked

genes (Figure S4.6, *Appendix F*). The negative control method using a randomized set of the top 141 ranked genes demonstrated that true learning occurred in the optimized SMO model. Re-training the SMO model with the randomized data sets, the overall mean percent correct classification was 51.28 % (SD = 21.54 %), the average kappa statistic was 0.03 (SD = 0.44) and the AUROC was 0.51 (SD = 0.22), indicating that true learning occurred in the optimized SMO model with 141 top ranked genes. The IDs, gene functions and expression levels of the top 141 ranked genes are presented in Table S4.6 (*Appendix F*).

Similar to gene expression patterns by infected sunflower-fed bees compared to infected wildflower-fed bees, machine learning identified upregulation of detoxification enzymes (Figure 2) and genes associated gut morphology (Figure 3). Machine learning also identified two genes associated with the immune system that were upregulated in un-infected sunflower-fed bees, including the pro-inflammatory regulator *tyrosine-protein phosphatase* and *major royal jelly protein 1*, which has been shown to have antimicrobial effects in bees (Fratini et al. 2016) and upregulated in response to *Crithidia* infection (Riddell et al. 2014)(Figure 4.1). In addition, machine learning identified that un-infected sunflower-fed bees upregulated retrovirus-associated transposable elements (*gag-pol polyprotein, Transposon Tf2-9 polyprotein*), olfactory signaling in the gut (*odorant receptor 46a-like, Tachykinin-like peptides receptor 99D*), energy metabolism (*GDP-D-glucose phosphorylase 1-like* and *lysoplasmalogenase-like protein TMEM86A isoform X2*), enzymes involved in protein modification and glycosylation (*ectonucleoside triphosphate diphosphohydrolase 5 isoform X2, N-acetylgalactosaminyltransferase 6-like, Thyrotropin-releasing hormone-degrading ectoenzyme, methionine aminopeptidase 1*), and several factors associated with positive regulator of

transcription (*transcription initiation factor TFIID subunit 6-like, transcription factor IIIB 90 kDa subunit isoform X1, Gem-associated protein 2*).

***Functional enrichment - Un-infected bees: sunflower vs. wildflower.*** We did not find significant enrichment of any GO biological process or molecular function terms for DEGs identified in the DESeq2 model. However, GO enrichment analysis based on the combination of the top 141 top ranked genes identified by machine learning indicated that a number of biological processes involving pigmentation and oxidation-reduction (redox) reactions were significantly enriched (Figure S4.7, *Appendix F*), indicating a detoxification response in un-infected sunflower-fed bees.

*IPA: Canonical Pathways*

A total of 68 out of the 141 top ranked optimal genes identified by machine learning were successfully mapped into IPA; 58 genes were uncharacterized and we were unable to identify human, rat or mouse orthologs for 15 genes. Consistent with the IPA analysis for infected bees, a number of significant canonical pathways associated with xenobiotic metabolism, and gastrointestinal physiology were enriched in uninfected bees fed sunflower pollen. The top enriched canonical pathway was epithelial adherens junction signaling pathway (Table S4.4, *Appendix F*), followed by sorbitol degradation, stearate biosynthesis, calcium signaling, and protein kinase A signaling. The NRF2-mediated oxidative stress response and the LPS/IL-1 Mediated Inhibition of RXR pathways were also enriched, both of which were enriched and predicted to be activated in infected sunflower-fed bees compared to infected wildflower-fed bees. IPA was unable to predict activation of any of the enriched canonical pathways in un-

infected bees, likely based on the number of genes in the data set. However, NAD(P)H quinone dehydrogenase 1 (*Nqo1*), which is known to play an important role in the response to xenobiotics similar to cytochrome p450 enzymes, was up-regulated in un-infected sunflower-fed bees, suggesting activation of the NRF2-mediated oxidative stress response pathway. In addition, GDP-D-glucose phosphorylase 1 (*Gdpgp1*) was up-regulated in un-infected sunflower bees, which is associated with two enriched glycogen degradation pathways. Glycogen is a readily mobilized storage form of glucose that can be broken down by two enzymes working in tandem (glycogen phosphorylase and alpha-glucosidase) when energy is needed, suggesting a significant energy demand caused by sunflower pollen consumption. However, two myosin light chain genes (*Myl1* and *Myl2*) were down-regulated in sunflower-fed bees. These genes play a role in the structural constituent of muscle cells and a number of enriched canonical signaling pathways, including Ga12/13, RhoA, PAK, Apellin Cardiomyocyte, Calcium and Protein Kinase, suggesting that the energy demand was not the result of muscle contraction. Sorbitol dehydrogenase, which plays a major role in the Sorbitol Degradation pathway and catalyzes the conversion of the sugar alcohol sorbitol to fructose, was also downregulated in sunflower-fed bees.

***Differential gene expression - Sunflower-fed bees: infected vs. un-infected.*** Among sunflower-fed bees, 12 genes were differentially expressed between infected and un-infected bees based on the DESeq2 model (FDR < 0.05; Table S4.7, *Appendix F*). Of immune genes, the antimicrobial peptide *hymenoptaecin* and *alkaline phosphatase 4* were upregulated in infected bees, whereas the proteolytic enzyme *Trypsin alpha-3-like* was downregulated (Figure 4.1). Only one detoxification gene (*glucose dehydrogenase [FAD, quinone]-like*) was differentially expressed

between sunflower-fed bees, which was downregulated in infected bees (Figure 4.2). Similarly, only a single gut morphology gene *(endochitinas*e) was significantly upregulated in infected sunflower-fed bees (Figure 4.3).

Machine learning poorly predicted infection treatment among sunflower-fed bees (Figure S4.8, *Appendix F*), in most instances only reaching 80% correct classification with considerably large standard deviation (> 30%). The best machine learning classification was obtained from the top 80 through 78 ranked genes (% CC: 90.00 + /- 30.02; mean +/- SD), the IDs and gene functions for which are presented in Table S4.6 (*Appendix F*). Re-training the SMO model with the randomized data sets, the overall mean percent correct classification was 47.65 % (SD = 20.06 %), the average kappa statistic was -0.04 (SD = 0.36) and the AUROC was 0.48 (SD = 0.20). Substantial overlap between variation (SD) around the average percent correct classification between the negative control and optimized model, indicating that true learning failed in the SMO model with 80 top ranked genes.

***Functional enrichment - Sunflower-fed bees: infected vs. un-infected.*** We did not find significant enrichment of any GO biological process or molecular function terms for DEGs identified in either the DESeq2 model or the top 80 ranked genes identified by machine learning. Since machine learning poorly classified infection status among sunflower-fed bees, and so few genes were differentially expressed in the DESeq2 model, we did not perform IPA analysis to avoid misleading results.

***Differential gene expression - Wildflower-fed bees: infected vs. un-infected.*** Among wildflower-fed bees, 17 genes were differentially expressed between infected and un-infected

bees based on the DESEq2 model (FDR < 0.05; Table S4.9, *Appendix F*). One gene associated with the immune system (*digestive cysteine proteinase 1*) (Figure 4.1) and the detoxification gene *glucose dehydrogenase [FAD, quinone]-like* were downregulated in infected bees. No genes associated with gut morphology were differentially expressed between infected and un-infected wildflower-fed bees (Figure 4.3).

The best machine learning classification was obtained from the top 98 through 41 ranked genes (% CC: 100.00 + /- 00.00; mean +/- SD; Figure S4.9, *Appendix F*); the IDs, gene functions and expression levels are presented in Table S4.10 (*Appendix F*). The negative control method demonstrated that true learning occurred in the optimized SMO model. Re-training the SMO model with the randomized data sets, the overall mean percent correct classification was 47.70 % (SD = 20.13 %), the average kappa statistic was -0.03 (SD = 0.41) and the AUROC was 0.48 (SD = 0.22), indicating that true learning occurred in the optimized SMO model with 98 top ranked genes. Machine learning identified downregulation in infected wildflower-fed bees of several immune genes associated with regulatory functions, including a serine/threonine kinase (*inhibitor of nuclear factor kappa-B kinase subunit epsilon*; *Ikbke*), a serine/threonine-protein phosphatase (*serine/threonine-protein phosphatase 1 regulatory subunit GAC1-like*) and *acid phosphatase Acp3* (Figure 4.1). Conversely, machine learning of identified upregulation of mitogen-activated protein kinase 15 (*Map3k15*) in infected wildflower-fed bees. Upregulation of a single gene associated with detoxification (*aryl hydrocarbon receptor*) in infected wildflower-fed bees was identified by machine learning (Figure 4.3).

***Functional enrichment - Wildflower-fed bees: infected vs. un-infected.*** We did not find significant enrichment of any GO biological process or molecular function terms for either the DEGs identified in the DESeq2 model or the top 98 ranked genes identified by machine learning.

*IPA: Canonical Pathways*

A total of 39 out of the 98 top ranked optimal genes identified by machine learning were successfully mapped into IPA; 56 genes were uncharacterized and we were unable to identify human, rat or mouse orthologs for 3 genes. Six canonical pathways were enriched in infected wildflower-fed bees compared to un-infected wildflower-fed bees (Table S4.4, *Appendix F*). The top enriched canonical pathway was Choline Degradation I, driven solely by large downregulation of choline dehydrogenase (*Chdh*) in infected wildflower-fed bees. Several nucleotide metabolism pathways were enriched; upregulation of phosphoribosyl pyrophosphate synthetase 1 (*Prps1*) was associated with enrichment of the PRPP Biosynthesis I pathway, and downregulation of acid phosphatase 3 (*Acp3*) was associated with enrichment of the NAD Phosphorylzation and Dephosphorylzation, Urate Biosynthesis/Inosine 5-phosphate degradation, Guanasine Nucleotides Degradation III and Adenonsine Nucleotides Degradation II pathways.

**Discussion**

The whole transcriptome analysis of *B. impatiens* revealed upregulation of genes associated with multiple physiological processes in response to consumption of sunflower pollen. Namely, among infected bees, sunflower pollen upregulated genes associated with the Toll-mediated innate immune system, putative detoxification genes and genes associated with the repair and maintenance of gut epithelial cells. Similarly, among un-infected bees, sunflower

pollen upregulated similar detoxification genes and genes associated with repair and maintenance of gut epithelial cells, but not a Toll-mediated immune response. In control wildflower-fed bees, we did not detect upregulation of the same Toll-mediated immune response in infected bees or detoxification genes, but instead found signs of immune deactivation. Taken together, these results suggest that consumption of sunflower pollen causes a dissimilar immune response in infected bees compared to wildflower-fed bees, as well as a detoxification response. Moreover, gene expression patterns suggest that sunflower pollen may cause damage to the gut lining, which may feed back and enhance an effective immune response to *C. bombi* infection. Further research exploring the effects of plant defensive compounds found in sunflower pollen on bee physiology, as well as chemical or physical damage caused by sunflower pollen consumption, may provide functional insight into the mechanisms underlying the medicinal effects of sunflower pollen in bumble bees, as well as broaden our understanding of how diet influences disease ecology.

One major hypothesis that explains the mechanism underlying the medicinal effect of sunflower pollen is that it enhances the immune system. Indeed, studies have shown that metabolic and inflammatory pathways can converge at many levels, including at the level of cell-surface receptors, intracellular chaperones or nuclear receptors (Hotamisligil and Erbay 2008). In our study, we found concurrent upregulation of both a Toll-mediated immune response and several putative detoxification enzymes in infected sunflower-fed bees, but not in un-infected sunflower-fed bees or among wildflower-fed bees irrespective of infection treatment. In insects, CYPs are known to regulate both pathogen infection, by producing reactive oxygen radicals (e.g., nitric oxide), and detoxification of xenobiotics (Feyereisen 1999). CYP variants play a role in resistance to fungal infection in silkworms (Xing et al. 2017), varroa mite resistance in honey

bees (Jiang et al. 2016), metabolism of potent insecticides in bumble bees (Troczka et al. 2019) and *Crithidia* infection in bumble bees (Riddell et al. 2014). In honey bees, the *p*-coumaric acid found in pollen up-regulates both detoxification and immunity genes, including several CYPs and the AMP *abaecin* (Mao et al. 2013). Likewise, in honey bees, pesticides can regulate the expression of both detoxification genes and the bee immune response simultaneously, including expression of *hymenoptaecin* (Hu et al. 2017, Shi et al. 2018), which enhanced the immune response of honeybees against both a microsporidian pathogen and viral infections (Hu et al. 2017). In mammals, detoxification enzymes are also known to be an important determinant of the outcome of numerous drug therapies, including both inhibitory and inductive effects of anti-parasitic drugs (Bapiro et al. 2002, Li et al. 2003). Oxidoreductases, including FAD-GLD, and quinones produce superoxide radicals (Cavener 1992, Bolton et al. 2000) that directly destroy pathogens, or act as a messenger to induce immune-related genes (Stone and Yang 2006). In fact, Cox-Foster and Stehr (1994) suggested that FAD-GLDs interact with the phenoloxidase, and play an important role in the killing mechanism of pathogens by reducing quinone, which leads to the production of superoxide radicals that create a toxic environment for the pathogens. Further research is needed to determine if detoxification of phytochemicals in sunflower pollen is independent of the immune response to *C. bombi* infection in bumble bees.

A major challenge for living organisms is to maintain homeostasis in the face of multiple internal and external stressors, such as pathogen infection, variation in nutrient supply and exposure to toxins. In response, complex immune systems have evolved to eliminate the potential threat and re-establish homeostasis without causing excessive damage to healthy cells and tissues. Downregulation of an immune response can prevent the toxic accumulation of reactive oxygen species and the production of energetically costly immune effectors, such as

AMPs. We found signs of deactivation of an immune response in infected wildflower-fed bees 72 hours post-inoculation, including downregulation of functional communication along the gut-brain axis, serine kinases, serine protein phosphatases, acid phosphatase and a cysteine proteinase. This pattern agrees with another study that found expression of genes associated with Toll and melanization immune pathways in insects and several proteases occurred in a temporal manner, reducing expression at 72 hours post-inoculation (Jayaram et al. 2014). In both vertebrates and invertebrates, multiple glutamate receptors have been found in the gastrointestinal tract and nervous system, forming a complex bidirectional communication system between the gastrointestinal tract and the brain (Tomé 2018, Baj et al. 2019). Functional communication along the gut-brain axis via glutamate receptors is thought to play a key role in recognizing pathogen-associated molecular patterns and modulating innate immune responses (Ross et al. 1994, Qin et al. 2018). Downregulation of the gut-brain axis in infected bees may thus indicate deactivation of an innate immune response, including production of antimicrobial peptides and phagocytosis. Additionally, digestive cysteine proteinase 1 is homologous with mammalian cathepsin B, which is required for activation of inflammasomes (Jin and Flavell 2010) and phagosome maturation pathways. Acid phosphatases act as constitutive signals that alert the immune system to induce rapid release of proinflammatory mediators that facilitate pathogen killing, or downregulate inflammation and immune responses to prevent excess tissue damage. In fact, mice lacking acid phosphatase 5 activity secreted significantly greater levels of the proinflammatory cytokines in response to pathogen infection, which resulted in delayed clearance of the microbial pathogen, *Staphylococcus aureus* (Bune et al. 2001). Consequently, temporal variation in immune responses may obscure the underlying mechanism of medicinal sunflower pollen. We thus propose that follow-up studies track changes in host bumble bee

physiology over time to obtain an improved understanding of how different pollen or nectar diets mediate pathogen infection.

In addition to digestion and absorption of nutrients, the digestive tract plays an important role in protecting an organism from absorption of ingested xenobiotics that cause oxidative stress in consumers, such as plant defense compounds or pesticides. Sunflower pollen consumption strongly upregulated multiple enzymes in bumble bees that are involved in the primary metabolism of xenobiotics, including a P450 cytochrome (CYP), a quinone oxidoreductase, two glucuronosyltransferases, an ATP-binding cassette (ABC) transporter, thioredoxin reductase and E3 ubiquitinating proteins. A number of phenolic compounds that may cause oxidative stress in bees have been reported in sunflower pollen, including neochlorogenic acid (*5-O-Caffeoylquinic* acid) (Kostić et al. 2019). The oxidation of phenolic compounds, such as neochlorogenic acid, can form quinones that can inhibit protein digestion in herbivores (Felton et al. 1992) or have direct toxic effects on insects (Bolton et al. 2000). Quinones are commonly found in plants, environmental toxins and are generated through the metabolism of aromatic compounds (Thomson 2012). Two key P450 enzymes (3a4 and 2e1) and a quinone intermediate play a key role in the metabolism of neochlorogenic acid (Xie et al. 2012). Our BLAST results found that human CYP 3a4 is homologous to CYP 9e2 (e-value = 3.60E-75), the latter of which was strongly upregulated in infected and un-infected sunflower-fed bees. Along with the simultaneous upregulation of *glucose dehydrogenase [FAD, quinone]*-like (FAD GLD) in sunflower-fed bees, which catalyzes the reaction of D-glucose and a quinone to yield D-glucono-1,5-lactone and a quinol, suggesting detoxification of neochlorogenic acid found in sunflower pollen. Quinones, as either a by-product of neochlorogenic acid metabolism or as a natural component of sunflower pollen, may thus play a major role in the mechanism underlying the

medicinal effect of sunflower pollen. Thus, further research is needed to verify concentrations of plant secondary metabolites in sunflower pollen and their potential effects on bumble bee pathogens.

Pesticides are commonly used on sunflower crops to suppress weeds, herbivorous insects and plant pathogens (Elbert et al. 2008) and can pose a substantial risk for bees (Whitehorn et al. 2012). The upregulation of multiple detoxification enzymes in sunflower-fed bees may thus be an indication of pesticide contamination in sunflower pollen. However, while the pesticide residues in pollen used in this study were not measured, both the sunflower and wildflower pollen were sourced from the same suppliers as in Giacomini et al. (2018), which did measure pollen pesticide levels. In that study, a greater quantity of pesticide residues were found in wildflower compared to sunflower pollen, all but two of which were at trace levels. The two that were above trace levels were both miticides used to treat varroa mites in honey bee colonies. Sunflower pollen also contained a different miticide used to treat varroa in honey bees. Given that pesticide levels were low overall and greater in wildflower than sunflower pollen, it seems unlikely that pesticides are responsible for upregulation of detoxification genes in sunflower-fed bees.

In addition to detoxification and an immune response, gene expression patterns indicated wound healing activity in the abdominal gut tissues of *B. impatiens* in response to the consumption of sunflower pollen. The gut epithelium is a single layer of cells that forms the luminal surface of the digestive tract. Columnar cells (i.e., enterocytes) are the most abundant cell type that make up the gut epithelium, and are responsible for enzyme production and nutrient absorption. These columnar cells are covered in a brush-boarder membrane comprised of numerous microvilli that effectively increase surface area and facilitate highly efficient

absorption (Crawley et al. 2014). The cells of the epithelial layer are joined together by tight junctions composed of a branching network of transmembrane proteins, thus forming a contiguous and relatively impermeable membrane. Adherens junctions are specialized intercellular junctions, in which actin filaments are linked to cadherin molecules of adjacent cells via catenin molecules. These junctions perform multiple functions, including initiation and stabilization of cell-to-cell adhesion (Perez-Moreno et al. 2003, Hartsock and Nelson 2008). Wound healing in the digestive tract is a dynamic process that requires coordination between the proliferation of new cells, the reorganization of intracellular matrices and both inter- and intra-cellular signaling pathways that facilitate cell-to-cell adhesion (Bindschadler and McGrath 2007).

Consumption of sunflower pollen stimulated expression of genes associated with wound healing, including the enrichment of the Epithelial Adherens Junction Signaling pathway (EAJS), as well as genes associated with the formation of cellular surface protrusions, proliferation of fibroblasts and activation of signaling pathways in the brush border membrane. In infected bees, sunflower pollen consumption stimulated increased expression of α-catenin, actin and a par-3 family cell polarity regulator (*partitioning defective 3 homolog*), each of which play an important role in the regulation of adherens junctions (Hartsock and Nelson 2008). In un-infected bees, sunflower pollen consumption upregulated *tyrosine phosphatase* and *WD repeat domain-containing protein 83* (WD83), the former being an important regulator of cell motility and cell adhesion (Yan et al. 2006) and the latter a scaffold protein that regulates the Extracellular signal Related Kinase (ERK) cascade associated with an inflammatory response to wounding (Vomastek et al. 2004) and *jerky.* Specifically, WD83 is associated with β-catenin (Benchabane et al. 2011), which controls enterocyte (gut epithelial cells) cell proliferation

(Goessling et al. 2009). In addition, infected bees fed sunflower pollen increased expression of *chitinase-3-like protein 1* (CHI3L1) compared to wildflower-fed bees and increased expression of *endochitinase* compared to un-infected sunflower-fed bees. The midgut is lined by the peritrophic membrane (PM) consisting of a network of chitin molecules embedded within a mucin-based matrix of carbohydrates and proteins. The PM effectively functions to filter small molecules and aid in nutrient absorption, as well as protect the gut epithelium from damage from abrasive foods or pathogen invasion (Kuraishi et al. 2011, Riddell et al. 2014, Shibata et al. 2015, Vanderplanck et al. 2020). In insects, the PM is regularly shed and replaced for both growth and morphogenesis. This requires the well-regulated synthesis and turnover of chitin (Zhu et al. 2016). Thus, upregulation of enzymatic chitinases and chitin synthases may suggest an increased turnover of the PM in response to *C. bombi* infection, which may be a physiological response by host bumble bees that physically removes *C. bombi* cells from the digestive tract. Differential expression of host bumble bee chitinases in response to *Crithidia* infection has been demonstrated in previous work (Riddell et al. 2014), but an effect on *Crithidia* infection has not been detected. While many chitinases are glycosyl hydrolases that catalyze the hydrolysis of chitin, CHI3L1 in particular lacks chitinase/hydrolase activity, yet still binds strongly to chitin (Hakala et al. 1993). A recent review revealed that CHI3L1 plays a major role in tissue injury, inflammation, tissue repair, and remodeling responses (Zhao et al. 2020). Specifically, CHI3L1 influences tissue remodeling, cell adhesion and cell migration by affecting the enzymatic activity of matrix metalloproteinases (Iwata et al. 2009), and by suppressing the expression of E-cadherin (Scully et al. 2011).

While this study is unable to differentiate between chemically- or mechanically-induced damage caused by sunflower pollen consumption, upregulation of both a detoxification response

and a response to wound healing agrees with recent evidence that closely related *Taraxacum* pollen caused damage to the gut lining of *B. terrestris* bumble bees (Vanderplanck et al. 2020). Similarly, that study was unable to differentiate between a chemical or mechanical cause of damage since a non-*Taraxacum* pollen diet spiked with phytosterols found in *Taraxacum* pollen and crushed *Taraxacum* pollen both induced damage to the gut lining of the digestive tract. On one hand, crushed pollen could increase abrasiveness and cause mechanical damage to the gut lining. Alternatively, crushed pollen could release a greater quantity of phytochemicals that are otherwise trapped in undigested pollen grains, and thus increase exposure to toxins that cause damage to the gut lining. In our case, both sunflower pollen and the control wildflower pollen diets were provided to bees in the form of a paste, which required mechanical breakdown of honey-bee collected pollen pellets before adding water. During that process a small proportion of pollen grains are indeed fractured (JJG *personal observation*), but since both diets were treated the same we can rule out mechanical damage to the gut lining caused by fragmented pollen grains. However, *Helianthus sp*. pollen grains are much more echinate than *Taraxacum sp.* pollen, so we cannot rule out mechanical damage caused by intact pollen grains. If abrasiveness of echinate pollen causes damage to the gut lining, then pollen diets that contain a high proportion of pollen species with spines, regardless of plant family, or contain a proportion of empty (i.e., methanol extracted) pollen grains with spines, will cause damage. Alternatively, if removing the internal contents of sunflower pollen substantially reduces damage, this would suggest that chemical properties of sunflower pollen cause damage to the gut lining.

The data generated from this study provides an important foundation for future research that aims to disentangle the mechanism(s) that underly the medicinal effect of sunflower pollen in bumble bees. Chemical or mechanical properties of sunflower pollen may enhance the bumble

bee immune system, thus facilitating targeted destruction of *C. bombi* cells. Detoxification of phytotoxins found in sunflower pollen, such as neochlorogenic acid, may generate a toxic environment for *C. bombi* cells, or may stimulate and enhance a host immune response. Similarly, echinate sunflower pollen or phytosterols may cause damage to the gut lining, which may directly prevent growth and reproduction of *C. bombi*, or stimulate an effective host immune response. Future research should thus focus on disentangling effects of chemical and physical properties of sunflower pollen on host bumble bee physiology, and the implications *C. bombi* infection. Identifying plant traits that drive the medicinal effect of sunflower pollen in infected bumble bees may broaden our understanding of pollinator disease ecology and may provide opportunities for effective management of bee pathogens.

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**Author Contributions**

JJG, LSA, and REI designed research; JJG performed research; JJG and BJR designed the analysis pipeline and JJG performed the analyses; JJG and REI wrote the paper with feedback from all authors.

## Data Availability

All data generated from this project and custom scripts used for statistical analysis and plotting will be made publicly available upon publication.

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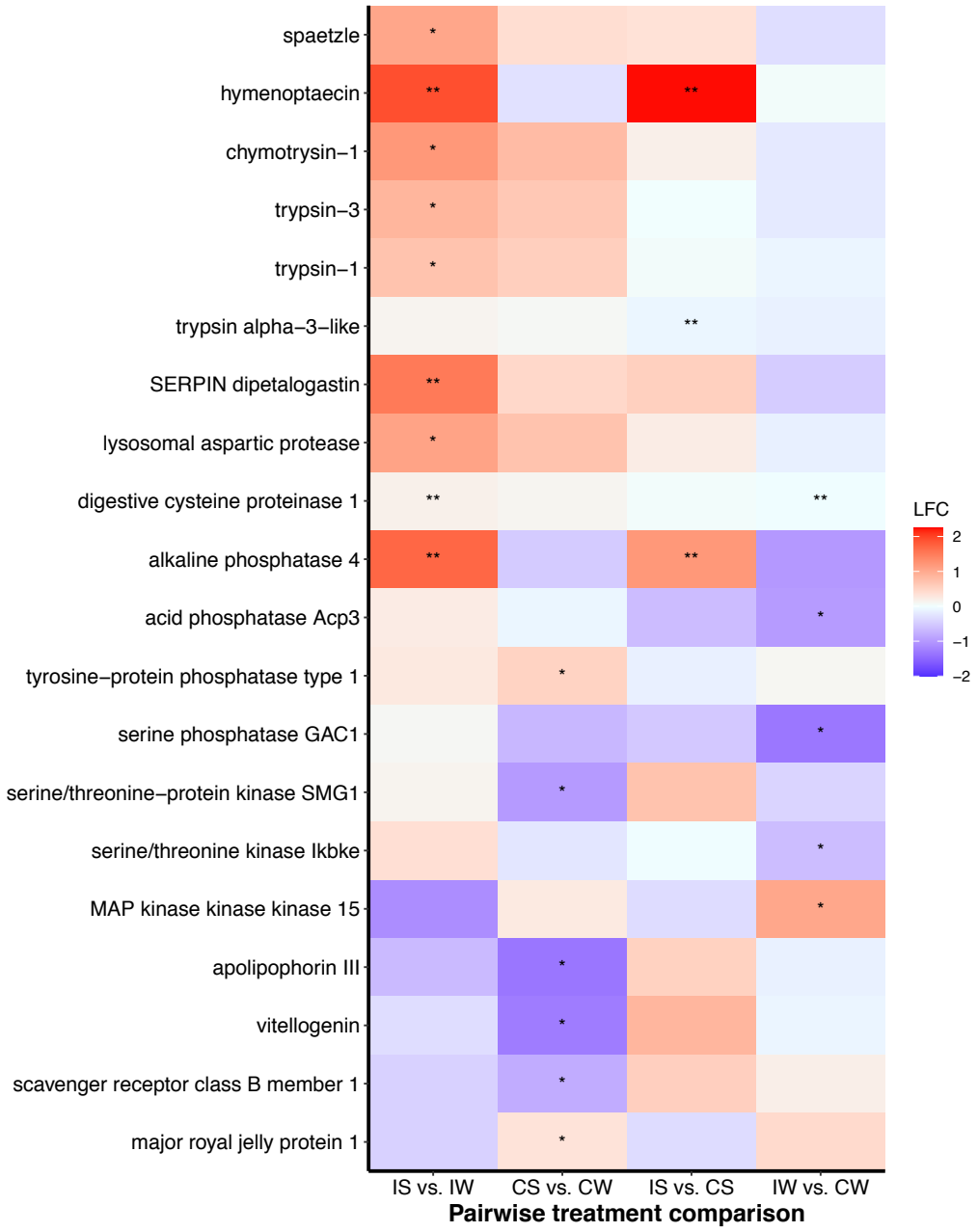


**Figure 4.1**. Gene expression profiles of putative immune genes in *Bombus impatiens* workers inoculated with the gut protozoan parasite *Crithidia bombi* (I) or inoculated with a sham control (C) and fed either sunflower (S) or wildflower (W) pollen. Colors indicate shrunken log fold changes estimated using a negative binomial model. Double asterisks indicate differentially expressed genes based on negative binomial DESeq2 model and FDR < 0.05. Single asterisk indicates important differentially expressed genes based on machine learning analysis.

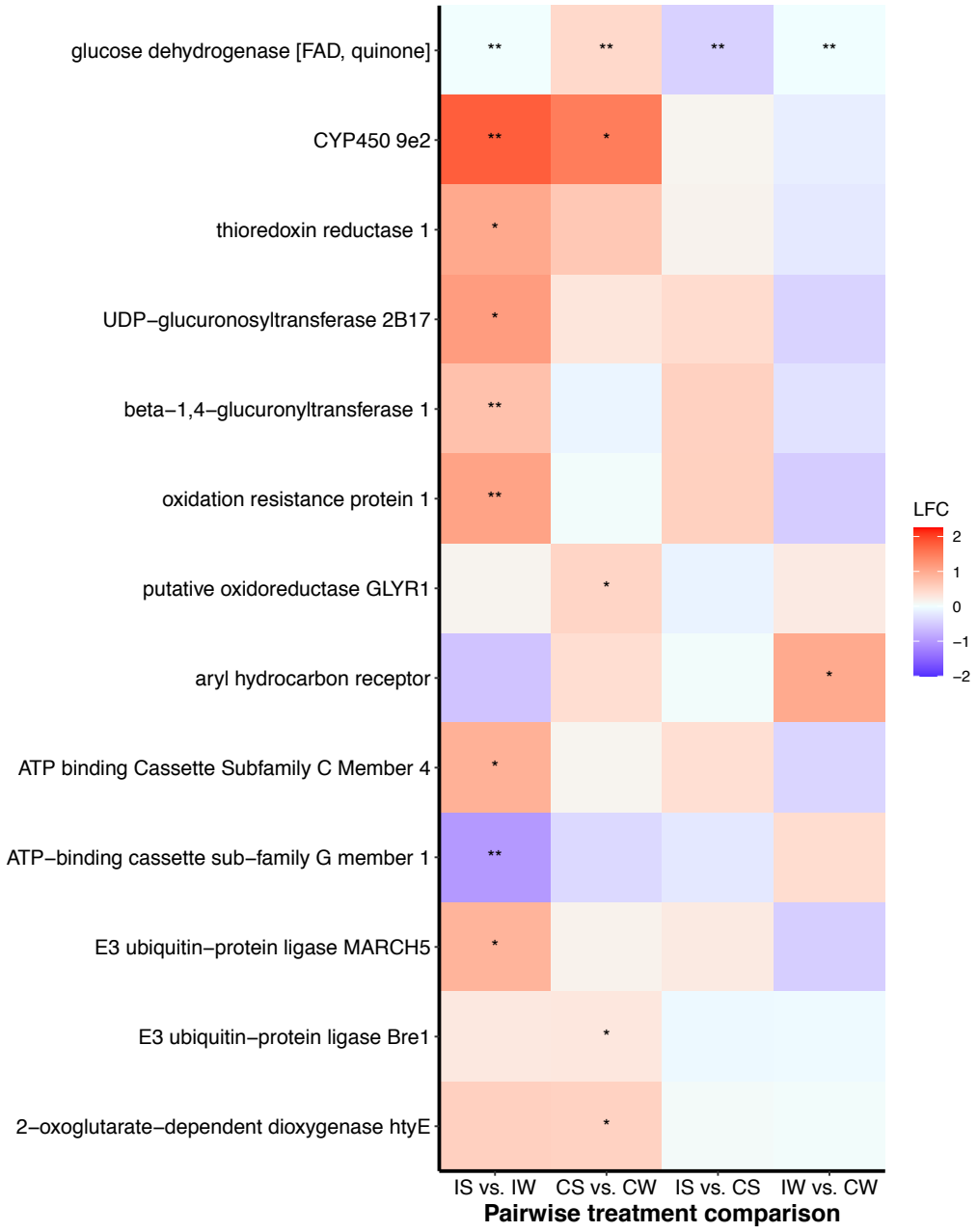


**Figure 4.2.** Gene expression profiles of putative detoxification genes in *Bombus impatiens* workers inoculated with the gut protozoan parasite *Crithidia bombi* (I) or inoculated with a sham control (C) and fed either sunflower (S) or wildflower (W) pollen. Colors indicate shrunken log fold changes estimated using a negative binomial model. Double asterisks indicate differentially expressed genes based on negative binomial DESeq2 model and FDR < 0.05. Single asterisk indicates important differentially expressed genes based on machine learning analysis.

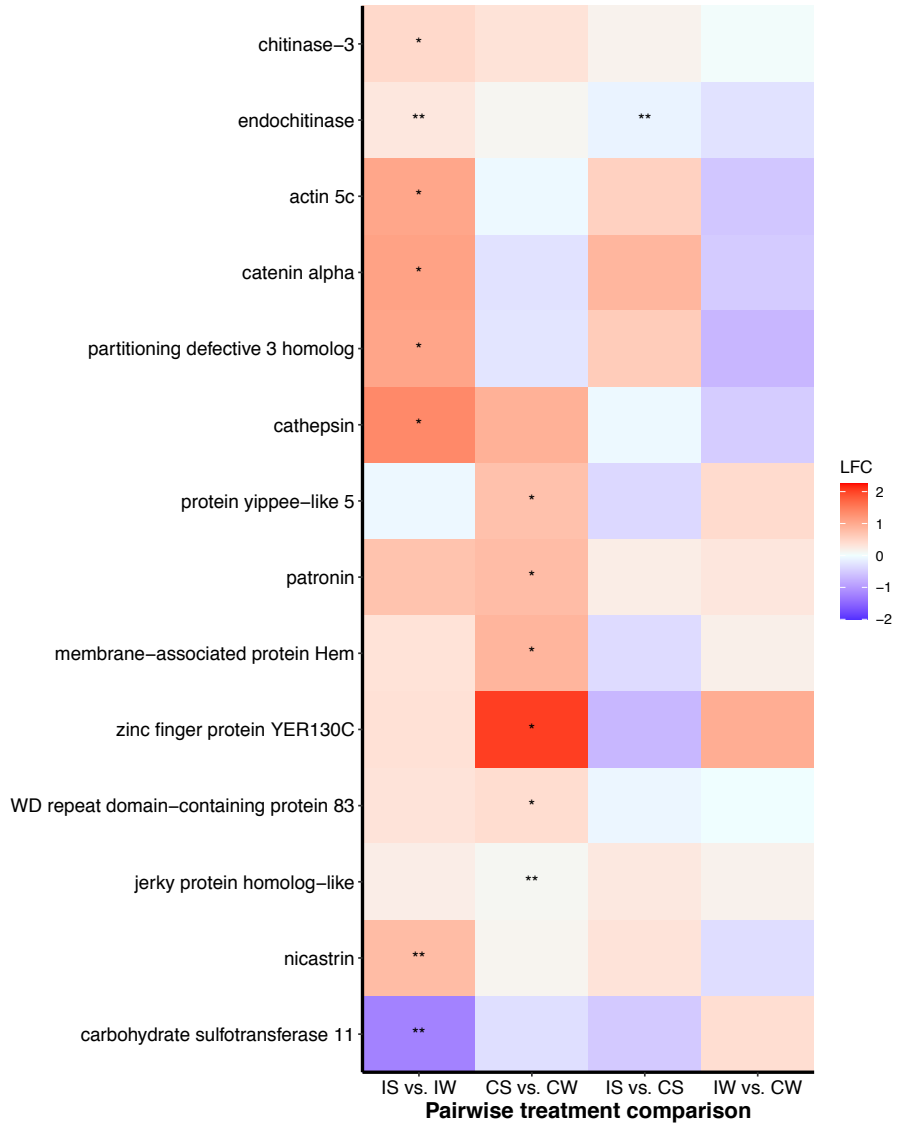


**Figure 4.3.** Gene expression profiles of gut morphology genes in *Bombus impatiens* workers inoculated with the gut protozoan parasite *Crithidia bombi* (I) or inoculated with a sham control (C) and fed either sunflower (S) or wildflower (W) pollen. Colors indicate shrunken log fold changes estimated using a negative binomial model. Double asterisks indicate differentially expressed genes based on negative binomial DESeq2 model and FDR < 0.05. Single asterisk indicates important differentially expressed genes based on machine learning analysis.

# APPENDICES

## Appendix A: Chapter 1: Supplementary Text

**1. Effects of pollen diet on *Crithidia* in bumble bees.** In all experiments, *Crithidia* populations grew rapidly in infected hosts after inoculation, reaching up to 5,777 cells $\mu L^{-1}$ in bees in wildflower control and buckwheat pollen diet treatments.

*Monofloral and mixed pollen*. Post-hoc analysis revealed all pairwise combinations of pollen diet had statistically different effects on *Crithidia* infection intensity (Tukey's HSD, $P<0.05$ in all comparisons; Fig. 1A). Additionally, almost two-thirds of the bees fed sunflower pollen and one-third of the bees fed the three-pollen polyfloral mix had no detectable *Crithidia* infection; fewer than 10% of bees fed canola and buckwheat pollen had no detectable infection. There was no significant difference in mortality for the different pollen diets (Fig. 1B, Fig. S1). The covariate bee size was significantly negatively related to *Crithidia* infection intensity ($\chi^2_{(1)} = 6.64$, $P = 0.010$).

*Effect of diet post-infection*. Post-hoc analysis revealed significant pairwise differences in the effect of sunflower pollen compared to wildflower and buckwheat pollen ($Z > 3.95$, $P < 0.05$ in both cases), but not between wildflower and buckwheat pollen ($Z = 0.68$, $P = 0.77$; Fig. 1C).

*Consistency with a different pathogen strain*. Sunflower pollen diet reduced *Crithidia* collected in North Carolina, USA compared to buckwheat pollen diet ($\chi^2_{(1)} = 30.7$, $P < 0.001$), with sunflower reducing infection 30-fold (Fig. S2). The covariate bee size was significantly positively associated with *Crithidia* infection intensity ($\chi^2_{(1)} = 10.17$, $P = 0.001$).

*Consistency using two sources of sunflower pollen*. Post-hoc analysis revealed a significant difference between the Chinese sunflower pollen diet and the wildflower pollen mix ($Z = 4.909$, $P < 0.001$), as well as a significant difference between the US sunflower pollen and

the wildflower pollen mix ($Z = 3.95$, $P < 0.001$). There was no difference between the US and Chinese sunflower pollen diets in terms of reduction in *Crithidia* infection intensity ($Z = 0.60$, $P = 0.82$; Fig. 1D).

**2. Costs and benefits of sunflower pollen on bee health, reproduction and *Crithidia*.**

*Crithidia* populations again grew in uninfected hosts receiving buckwheat pollen diet; counts of buckwheat-fed bees averaged 2000 cells $\mu L^{-1}$ in diluted gut homogenate after the termination of microcolonies. Sunflower pollen significantly reduced *Crithidia* infection intensity; bees that consumed sunflower pollen had 10-fold fewer *Crithidia* cells than bees that consumed buckwheat pollen (least squares means 4.3 cells $\mu L^{-1}$ for sunflower-fed bees and 40.0 cells $\mu L^{-1}$ for buckwheat-fed bees, pollen treatment: $\chi^2_{(1)} = 21.5$, $P < 0.001$).

Neither pollen diet nor *Crithidia* infection affected nectar consumption (pollen diet: $\chi^2_{(1)} = 0.0004$, $P = 0.98$; infection: $\chi^2_{(1)} = 1.63$, $P = 0.20$). However, there was a significant increase in nectar consumption over the course of the experiment ($\chi^2_{(1)} = 21.51$, $P < 0.001$). The consumption of sunflower pollen was stable over the course of the experiment, whereas consumption of buckwheat pollen declined over time (pollen x time interaction: $\chi^2_{(1)} = 38.92$, $P < 0.001$; Fig. S3). Averaged across all time points, sunflower pollen consumption was 2.3 times greater than buckwheat pollen ($\chi^2_{(1)} = 66.67$, $P < 0.001$; Mean ± SE g pollen, sunflower: 0.0607 g ± 0.0016 g, buckwheat: 0.026 g ± 0.0012 g; Fig. S3). *Crithidia* infection did not affect pollen usage and consumption ($\chi^2_{(1)} = 1.89$, $P = 0.17$).

Microcolonies in the sunflower pollen treatment laid eggs significantly more quickly than microcolonies fed buckwheat pollen, and were more than twice as likely to have laid eggs at any given time (hazard ratio = 2.13, $Z = 2.85$, $P = 0.004$). Similarly,

microcolonies in the sunflower treatment produced significantly more larvae, and had heavier larvae (number of larvae: $\chi^2_{(1)} = 8.37$, $P = 0.004$; larval mass: $\chi^2_{(1)} = 11.44$, $P < 0.001$; Fig. 2). Ten microcolonies that were provided sunflower pollen produced pupae or adults, whereas none of the microcolonies that were provided buckwheat pollen produced pupae or adults (pollen treatment $\chi^2_{(1)} = 7.89$, $P = 0.005$). *Crithidia* infection did not affect time to egg laying (hazard ratio = 0.78, $Z = -1.01$, $P = 0.31$), number of larvae produced ($\chi^2_{(1)} = 0.74$, $P = 0.39$), larval mass ($\chi^2_{(1)} = 0.017$, $P = 0.\ 0.89$), or pupae production ($\chi^2_{(1)} = 0.15$, $P = 0.69$).

Both *Crithidia* infection and sunflower consumption marginally increased mortality rate (infection: $\chi^2_{(1)} = 3.21$, $P = 0.07$, pollen diet: see main text). Bees that consumed sunflower pollen were 31% more likely to die than those that consumed buckwheat pollen (death hazard ratio = 1.31 ± 0.165 SE; Fig. S4). Moreover, infected bees were 33% more likely to die at any given point than uninfected bees (death hazard ratio = 1.33 ± 0.161 SE; Fig. S4).

**3. Effects of pollen diet on *Nosema* in honey bees**. Pollen diet and time had a significant effect on *Nosema* infection intensity in honey bees (pollen treatment x time interaction: $\chi^2_{(2)} = 48.2$, $P < 0.001$). At each time point, sunflower-fed bees had on average fewer *Nosema* spores than buckwheat-fed bees ($Z < -3.06$, $P < 0.01$ for both time points), but more than bees fed no pollen ($Z > 3.51$, $P < 0.01$, Fig. 3). Pairwise comparisons indicate significantly higher mortality on both the sunflower pollen and no pollen diets relative to the buckwheat pollen diet (sunflower: see main text; no pollen: $Z = 4.62$, $P < 0.001$; Fig. S5). There was no significant difference in mortality between the sunflower and no pollen diets ($Z = 0.75$, $P = 0.73$).

**4. Effect of sunflower plantings on *Crithidia* in bumble bees at the farm scale.** Collection date was a significant covariate in the *Crithidia* infection intensity model ($\chi^2_{(1)} = 14.46$, $P < 0.001$). Sunflower area tended to reduce the probability of *Crithidia* infection ($\chi^2_{(1)} = 2.92$, $P = 0.087$), with increasing sunflower area associated with reduced probability of *Crithidia* infection.

**Supplementary Methods - statistical analyses:**

**1. Effects of pollen diet on *Crithidia* in bumble bees.**

*Effect of diet post-infection*. Bee size and inoculation date were not statistically significant ($P > 0.2$ for both) and so were removed from the model. None of the experimental bees died before their dissection date, and so a survival analysis was not conducted.

*Consistency with a different pathogen strain*. All bees were inoculated on the same day, so inoculation date was not included as a random effect in this analysis. Only two bees died before their dissection date, and so a survival analysis was not conducted.

*Consistency using two sources of sunflower pollen*. Bee size was not statistically significant ($P = 0.95$), and so was dropped from the model. A survival analysis was not conducted because only 10 bees died before their dissection date.

**2. Costs and benefits of sunflower pollen on bee health, reproduction and *Crithidia*.** We excluded two microcolonies from the 'uninfected' treatment because one or more workers in the microcolony were infected with *Crithidia* upon dissection, and two microcolonies whose workers died within one week of inoculation. Significance of predictor variables was assessed with chi-squared tests (likelihood ratio tests for models fit by glmmTMB models, and Wald tests for all other models via the Anova function from package "car"[1]).

To model pollen and nectar consumption, a linear mixed effects model was employed using function lme in "lme4"[2] with maximum likelihood parameter estimation. This was a repeated measures analysis including infection treatment, pollen diet, and days elapsed since inoculation as predictor variables. Two extreme daily consumption outliers were removed from the analyses. Survival and time to egg laying were analyzed with a Cox Proportional Hazards mixed-effects model fit using "coxme"[3] with a maximum likelihood parameter estimation. Response variables for survival and egg laying were hazard rates, which incorporated both the event (survival/death, or eggs/no eggs) and time to the event. Egg laying occurred at the level of the microcolony, while death hazard rates were analyzed for each bee in the microcolony with bee nested inside microcolony. *Crithidia* infection intensity (cell counts) was analyzed for infected treatment colonies only, using a negative binomial error distribution and a log link function with Laplace approximation to likelihood by package "glmmTMB"[4]. Number of pupae/adults produced was low overall (only 11 out of 76 microcolonies produced any pupae or adults), and thus was recorded as a binary variable, analyzed with binomial error distribution in package "blme"[5] with function bglmer, which uses Bayesian estimation to account for complete separation (i.e., fitted probabilities of zero or one in some treatments). Frequentist models for analysis of pupae failed to converge. This analysis only included blocks 1 and 2, as blocks 3 and 4 produced only one pupa and no adults. The number of larvae produced was analyzed with a negative binomial distribution and larval mass with a gamma distribution, each with Laplace estimation within package "glmmTMB"[4]. The number of eggs produced was analyzed with a Poisson error distribution and Gauss-Hermite quadrature estimation in package "lme4"[2].

**3. Effects of pollen diet on *Nosema* in honey bees.** Significance of predictors was tested with

likelihood ratio chi-squared tests via the drop1() function in R. Post-hoc pairwise comparisons were made with the "lsmeans" package[6]; the Tukey method was used to adjust p-values for multiple comparisons. The no-pollen negative control had significantly lower *Nosema* infection than either of the pollen diets at both days 10 and 15 ($Z > 3.05$, $P < 0.01$), as predicted, suggesting that the bees were consuming the pollen diets in the other treatments. Analysis of Deviance (Type II Wald chi-squared tests) was used to calculate the p-value. Pairwise comparisons between pollen diets were made using general linear hypothesis testing with single-step p-value corrections for multiple comparisons[6,7].

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**Appendix B: Chapter 1: Supporting Figures and Tables**

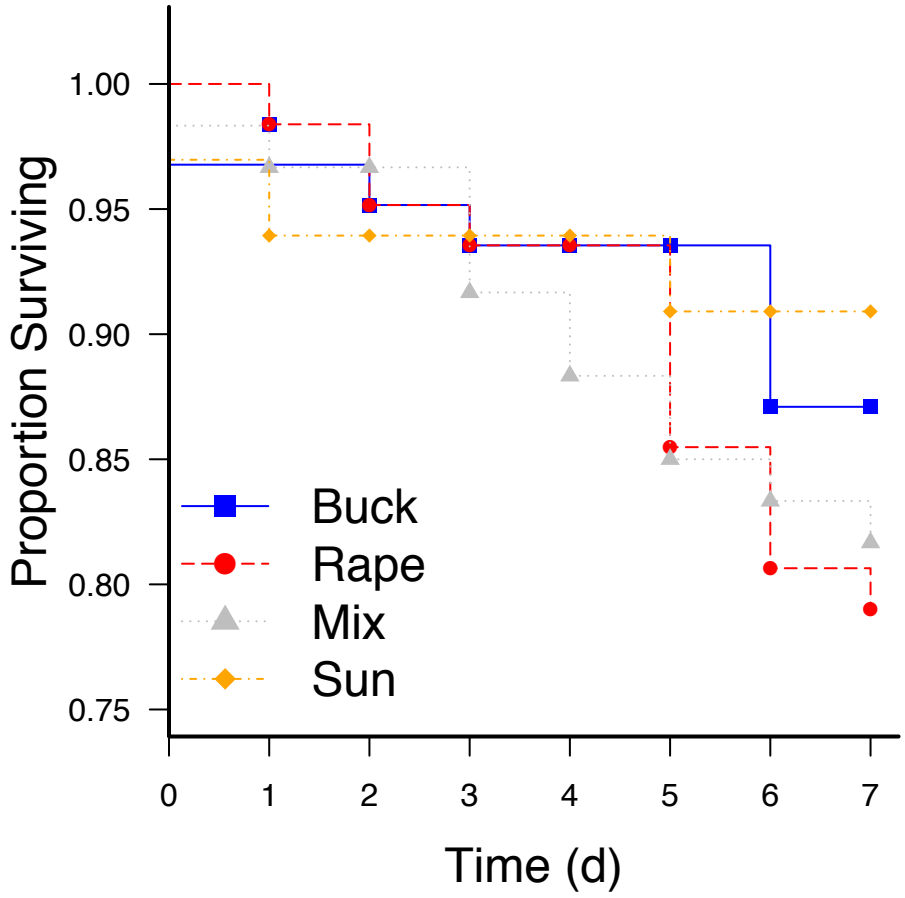


**Figure S1.1.** Proportion of *Bombus impatiens* workers infected with *Crithidia* surviving over time for each of four pollen diets. "Buck" refers to buckwheat, *Fagopyrum cymosum*, "Rape" to *Brassica campestris*, "Mix" to a pollen diet composed of equal weights of the three monofloral pollens, and "Sun" to sunflower, *Helianthus annuus*. Time was measured in days (d).

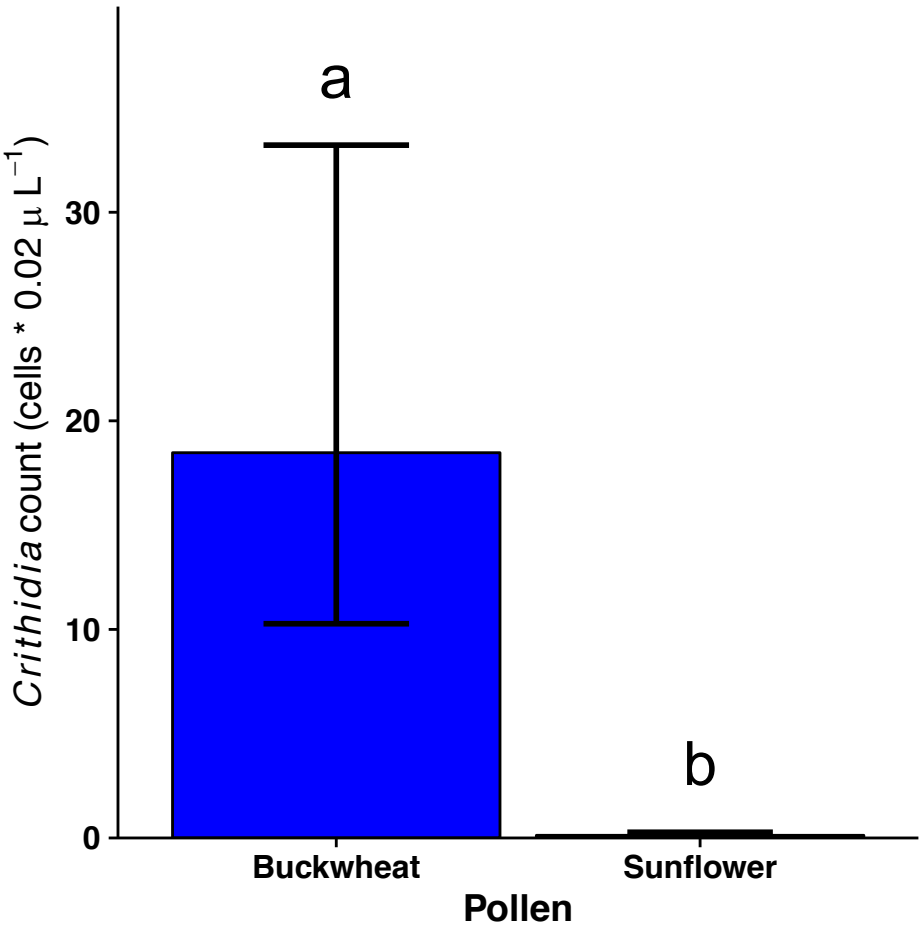


**Figure S1.2**. Effect of pollen diet on infection using individual *B. impatiens* workers that were inoculated with *Crithidia* sourced from J.C. Raulston Arboretum (35.793916 N, -78.698025 W), North Carolina, USA, and then fed either sunflower or buckwheat pollen. Bars and error bars indicate negative binomial model means and standard errors back-transformed (i.e., exponentiated) from the scale of the linear predictor. Different letters above each bar indicate significant differences.

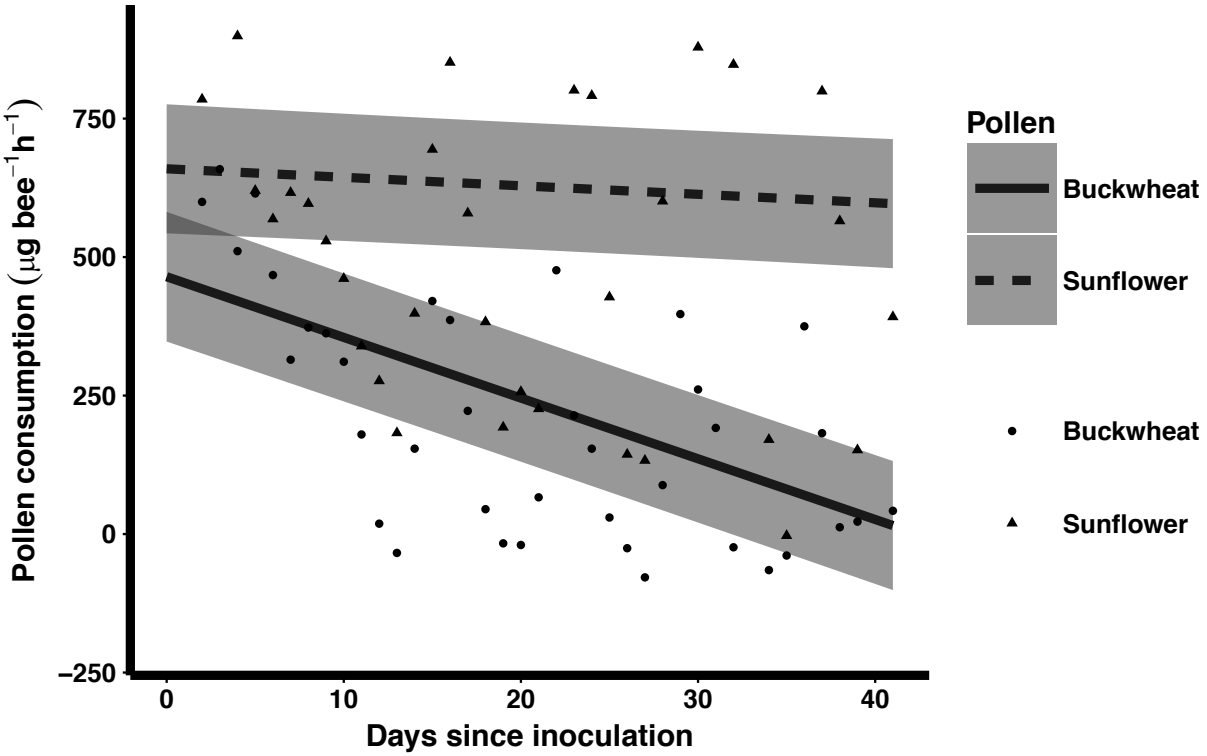


**Figure S1.3.** *Bombus impatiens* microcolony pollen consumption over time (days since inoculation) in $\mu$g bee$^{-1}$ h$^{-1}$ corrected for evaporation for buckwheat (solid line) and sunflower pollen (dashed line) treatments. Trend lines are overlaid over raw data; grey bands indicate ± one standard error.

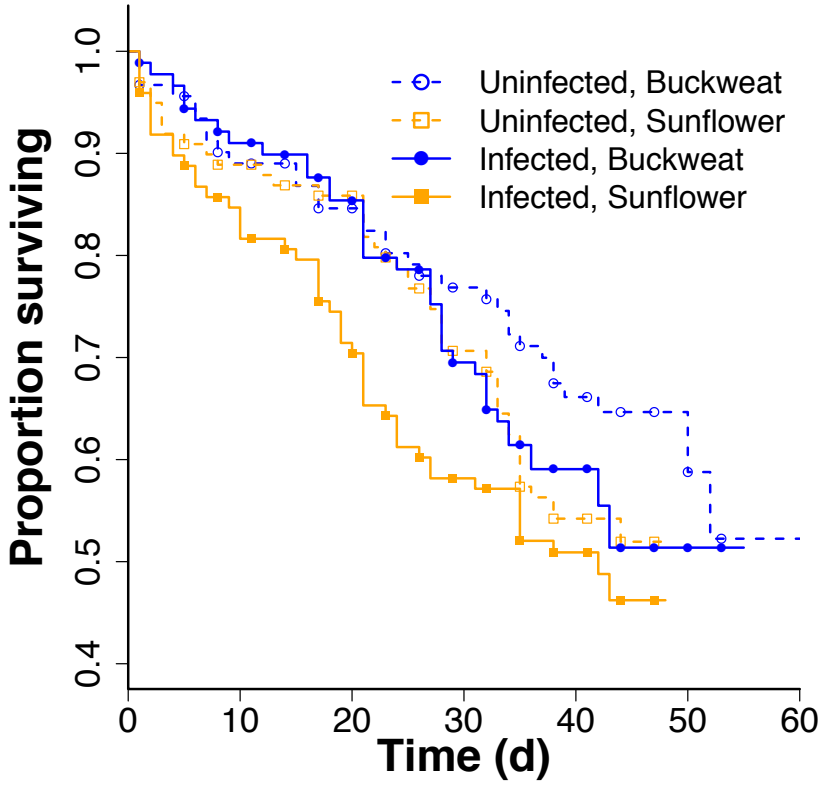


**Figure S1.4.** Proportion of *Bombus impatiens* workers in microcolonies surviving over time for each of four treatment combinations. Bees were either infected or uninfected with *Crithidia*, and fed sunflower pollen or buckwheat pollen. Time was measured in days (d).

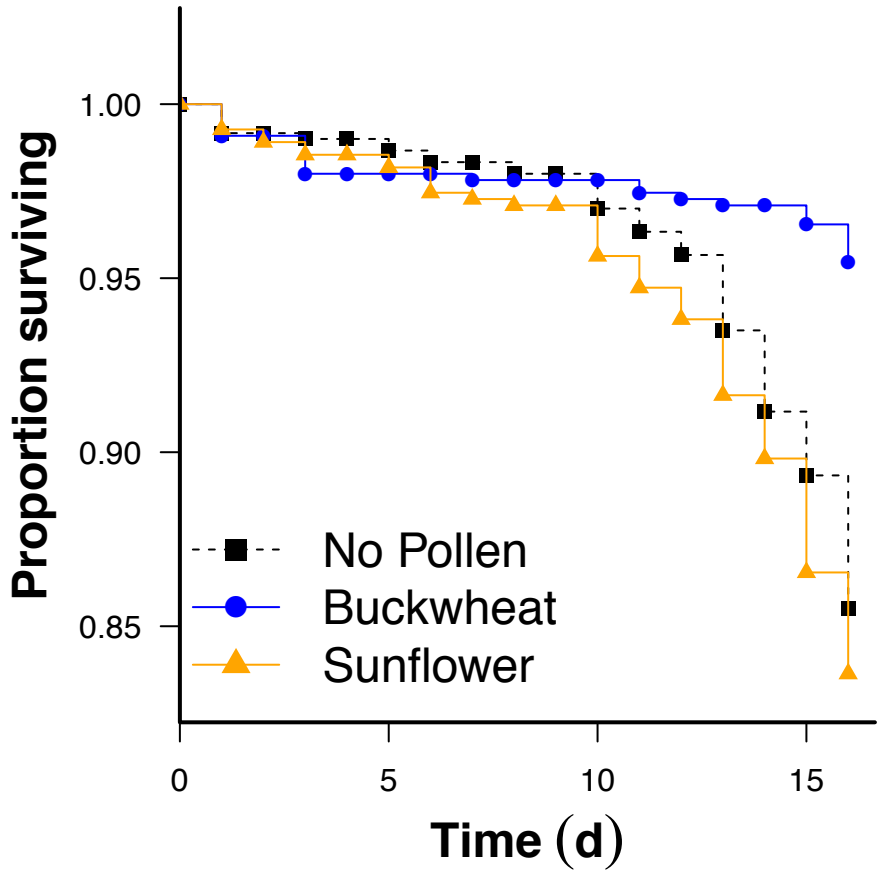


**Figure S1.5.** Proportion of individual honey bee workers infected with *Nosema* surviving over time for each of three pollen diets. Bees were provided sunflower pollen, buckwheat pollen or no pollen as a control. Time is measured in days.

**Table S1.1.** Pollen diets were screened for 213 pesticides and other agrochemicals (Agricultural Marketing Services' National Science Laboratories, United States Department of Agriculture, Gastonia, NC USA). We only report the chemicals detected in at least one of the pollen treatments and their concentrations in parts per billion (ppb). If concentrations were below detection limit, we report the limit of detection (LOD, in ppb) and list that the chemical was detected at trace concentrations. Not detected is listed as N.D. The highest pesticide concentrations were in the wildflower mix pollen, which also had relatively high *Crithidia* loads. Thus, pesticide contaminants do not explain the strong effect of sunflower pollen reducing *Crithidia* infection.

| Pesticide Residue | U.S.A. Sun | Buckwheat | China Sun | Wildflower |
|---|---|---|---|---|
| 2,4 Dimethylphenyl formamide (DMPF) | Trace | 19 | Trace | 110 |
| Pyraclostrobin | Trace | N.D. | N.D. | Trace |
| Thymol | Trace | N.D. | Trace | Trace |
| Fluvalinate | N.D. | 24 | 23 | N.D. |
| Fluopyram | N.D. | N.D. | N.D. | Trace |
| Pyrimethanil | N.D. | N.D. | N.D. | 35 |
| Methoxyfenozide | N.D. | N.D. | N.D. | Trace |
| Metolachlor | N.D. | N.D. | N.D. | Trace |

## Appendix C: Chapter 3: Supplementary Text

***Using imageJ to estimate volume and count of excretions.*** For each bee, we used imageJ software (Schindelin et al. 2012) to measure the area of excrement deposited onto each paper disc as an estimate of total volume of excrement and to measure the total count of non-overlapping excretion events. Overlapping excretion events were manually counted. For each still image, we first cropped out the background and identification code written on the border of each image to remove potential artifacts. Using the *Color Threshold* function in imageJ, with method set to default, threshold color set to red highlight and color space set to Lab, we then manually adjusted the L* filter such that noise was minimized while simultaneously highlighted as much of the fluorescent excrement as possible. We then used the *Analyze Particle*s function in imageJ, with minimum size set to 5 $mm^2$, pixel units left unchecked, circularity set from 0.00 – 1.00, show set to overlay and exclude on edges checked, which allowed us to measure the total area of highlighted excrement and total count of non-overlapping highlighted excretions with an area larger than 5 $mm^2$.

## Appendix D: Chapter 3: Supplementary Tables

**Table S3.1.** Correlation between excretion parameters. We estimated the total number of excretion events (No.), the bi-hourly excretion rate (Rate) and total volume (Vol.), each of which strongly correlated with each other. We used the corr.test function in the "psych" package in R to estimate Pearson's correlation coefficients (r) for each pairwise comparison, along with the lower and upper values of a 95% confidence interval.

| | lower | r | upper | p-value |
|---|---|---|---|---|
| **Rate-No.** | 0.254 | 0.422 | 0.564 | 4.49E-06 |
| **Rate-Vol.** | 0.253 | 0.420 | 0.563 | 4.89E-06 |
| **No.-Vol.** | 0.242 | 0.411 | 0.555 | 8.39E-06 |

## Appendix E: Chapter 4: Supplementary Text

**Timing of sunflower pollen effect methods:**

***Experimental Design***. The purpose of this experiment was to help determine the timing of the effect of sunflower pollen on *Crithidia bombi* infection within *Bombus impatiens* workers. The results of this experiment were used in conjunction with another study (LoCascio et al. 2019b) to determine when the abdomens of each experimental bee were dissected for RNA sequencing. Worker bumble bees were hand inoculated with a standardized dose of live *Crithidia* cells mixed into a sucrose solution and provided either sunflower pollen or control wildflower pollen for a period of time: 24 hours, 48 hours, 72 hours, 96 hours or 168 hours. Each time treatment corresponds to an amount of time in which a bee was allowed to consume pollen. For the remainder of the time (until 168 hours post-inoculation), a wildflower pollen mixture was provided to each bee. This protocol ensured that all bees were dissected on the same day, which allowed *Crithidia* infection intensity to reach potential asymptotic levels within all individual bees .

***Preparing pollen diets.*** We prepared two pollen diet treatments – sunflower and control wildflower. Honey bee-collected sunflower pollen pellets were obtained from Changge Hauding Wax Industry (China) and sorted by color to remove impurities. We verified a pure batch of sunflower pollen by staining five samples with basic fuschin dye (Kearns and Inouye 1983) and visually confirming only sunflower pollen was present with a compound microscope at 400X magnification. Honey bee-collected mixed wildflower pollen pellets were obtained from Koppert Biological Systems (Howell, MI, USA) and microscopically confirmed to contain < 5% Asteraceae pollen, identified by having spines on the exine (Blackmore et al. 2007).

Experimental pollen diets were provided to bees as a paste produced by mixing ground pollen pellets with distilled water to achieve a uniform consistency.

***Inoculation methods***. Three *Bombus impatiens* colonies were purchased from Koppert Biological Systems (Howell, MI, USA). We screened each colony for pre-existing *Crithidia* infection two days after receipt by dissecting five workers from each colony using previously established protocols (Richardson et al. 2015, Giacomini et al. 2018, etc.). Colonies were fed with 30% sucrose solution and mixed wildflower pollen throughout their lifetimes and housed in a dark room at 21 – 24ºC and ~50% rh. We removed 36 workers from two of the colonies and 60 workers from the third colony (total = 132 workers). Each bee was placed into a 7 dram snap cap vial and allowed to rest on the lab bench for approximately 4 hours. This resting period starves the bees and greatly helps facilitate consumption of the inoculum. During the starving period we made *Crithidia* inoculum using an established protocol (Manson et al. 2010, Richardson et al. 2015, Giacomini et al. 2018). Briefly, bee digestive tracts of 15 workers, excluding the honey crop, were removed with forceps, placed into 1.5 mL microcentrifuge tubes with 300 µL of distilled water, and ground with a pestle. We allowed each sample to rest at room temperature for 4-5 hours so that gut material settled and the *Crithidia* cells could ascend into the supernatant. *Crithidia* cells were counted from a 0.02 µL sample of supernatant per bee with a Neubauer hemacytometer under a compound light microscope at 400X magnification. We then mixed 150 µL of the supernatant with distilled water to achieve a concentration of 2400 cells $\mu L^{-1}$. The sample was then mixed with an equal volume of 50% sucrose solution to yield inoculum with 1200 cells $\mu L^{-1}$ in 25% sucrose. After the starving period, each bee was fed a 10uL drop of a 25% sucrose solution that contained 1200 cells per uL (Total cells = 12,000). Consumption of the inoculum was visually confirmed for each bee. Each bee was randomly assigned to a pollen

type (sunflower or wildflower pollen) and a timing treatment, then housed in a 3”x 4” plastic container with a mesh bottom for the remainder of the experiment. All bees were fed fresh sucrose and their respective pollen daily until dissection 168 hours post-inoculation.

***Pollen Consumption***. To ensure that all bees consumed pollen, the amount of daily pollen consumption (mg/day) was measured for each bee. Bees that consumed pollen, as determined by a net positive amount of pollen consumption after accounting for evaporation, were randomly assigned to a treatment or allowed to continue on to the experiment. To estimate net pollen consumption, we produced a set of evaporation controls for each feeding day by setting out 10 bee containers alongside the experimental bees that included 6 pollen feeders each (3 sunflower and 3 control wildflower) and a nectar feeder, but lacked a bee. We calculated evaporation-adjusted net consumption for each bee by fitting separate linear regressions for each day and pollen type, with initial weight of evaporation control pollen feeder regressed against the weight of the evaporation control pollen feeder 24-hr later. We then used the *predict* function in R to calculate an evaporation-adjusted feeder weight for feeders provided to each bee, thus yielding a net consumption estimate for each bee each day. Bees that did not consume pollen (net negative pollen consumption) at any point during their timing treatment were removed from the experiment. In other words, only bees that consumed a net positive amount of pollen for each day during their respective timing treatment were included in the final analysis. This method allows us to infer effects of sunflower pollen consumption and avoid confounding effects of staggered or missing consumption throughout the timing treatment period. We were unable to accurately estimate nectar consumption and thus excluded nectar consumption from the analysis.

***Measuring parasite load.*** Each bee was dissected as in ***Inoculation methods***, with the addition that all tools were washed with 70% ethanol and thoroughly dried between bees to

prevent cross-contamination. *Crithidia* cells from a 0.02 μL sample of supernatant per bee were counted with a Neubauer hemacytometer at 400X magnification with a compound light microscope (Manson et al. 2010, Richardson et al. 2015, Giacomini et al. 2018). We measured prevalence as the presence (1 or more *Crithidia* cells) or the absence of *Crithidia* cells per 0.02 μL of each sample, and *Crithidia* infection intensity as the number of flagellate *Crithidia* cells per 0.02 μL. We also removed the right forewing of each bee to measure marginal cell length, a proxy for bee size (Nooten and Rehan 2020).

***Statistical analyses***. Statistical analyses were conducted using R version 4.0.2 (R Core Team, 2015). We used generalized linear models to analyze how pollen diets and timing treatments affected *Crithidia* infection prevalence and intensity. The *Crithidia* prevalence model was fit with a binomial distribution, the infection intensity model was fit with a negative binomial distribution using the "MASS" package (Venables and Ripley 2002). Pollen type timing treatment and the interaction between pollen type and timing treatment were included as fixed effects in each model. Pairwise contrasts were evaluated separately for each combination of pollen type and timing treatment using the "emmeans" package (Lenth 2020). Due to low sample size (Table S4.11), we were unable to fit mixed-effect models with a random effect term for colony origin. In total 45 sunflower-fed and 42 wildflower-fed bees were included in the analysis, which resulted in 5 to 13 bees per pollen type per timing treatment.

**Timing of sunflower pollen effect results:**

We detected *Crithidia* infection in approximately 85% of bees fed control wildflower-fed throughout the entire experiment (168 hours post-inoculation; Figure S4.1a), indicating successful inoculation. In comparison, only 21% of sunflower-fed bees had detectable infection

by the end of the experiment. A generalized linear model indicated a significant effect of pollen type on *Crithidia* prevalence ($\chi^2 = 14.101$, df = 2, $p = 0.001$), but not timing period or their interaction ($\chi^2 < 7.1597$ $p > 0.210$; df = 5 and 4, respectively). Pairwise contrasts indicated a significant difference in prevalence between sunflower pollen and control wildflower pollen for the timing treatments of 96 hours and 168 hours post-inoculation ($t = -2.417$, $p = 0.018$; $t = -2.874$, $p = 0.005$; respectively), but not the timing treatments of 24 hours, 48 hours and 72 hours post-inoculation ($t < 0.001$, $p > 0.999$; for all). Similar to prevalence, infection intensity was greatest in bees fed control wildflower-fed bees throughout the entire experiment (168 hours post-inoculation; Figure S4.1b). The generalized linear model indicated a significant effect of pollen type and timing treatment on infection intensity ($\chi^2 > 30.549$, $p < 0.0001$ for both; df = 1 and 4, respectively), but not their interaction ($\chi^2 = 0.993$, df = 4, $p = 0.911$). Similar to prevalence, pairwise contrasts indicated a significant difference in infection intensity between sunflower pollen and control wildflower pollen for the timing treatments of 96 hours and 168 hours post-inoculation ($t = -2.890$, $p = 0.004$; $t = -3.979$, $p = 0.0001$; respectively), but not the timing treatments of 24 hours, 48 hours and 72 hours post-inoculation ($t < 0.004$, $p > 0.997$; for all). Taken together, these results suggest that *Crithidia* growth within host bumble bees fed sunflower pollen diverges from growth within host bumble bees fed control wildflower pollen between 72 and 96 hours post-inoculation.

**Appendix F: Chapter 4: Supporting Figures and Tables**

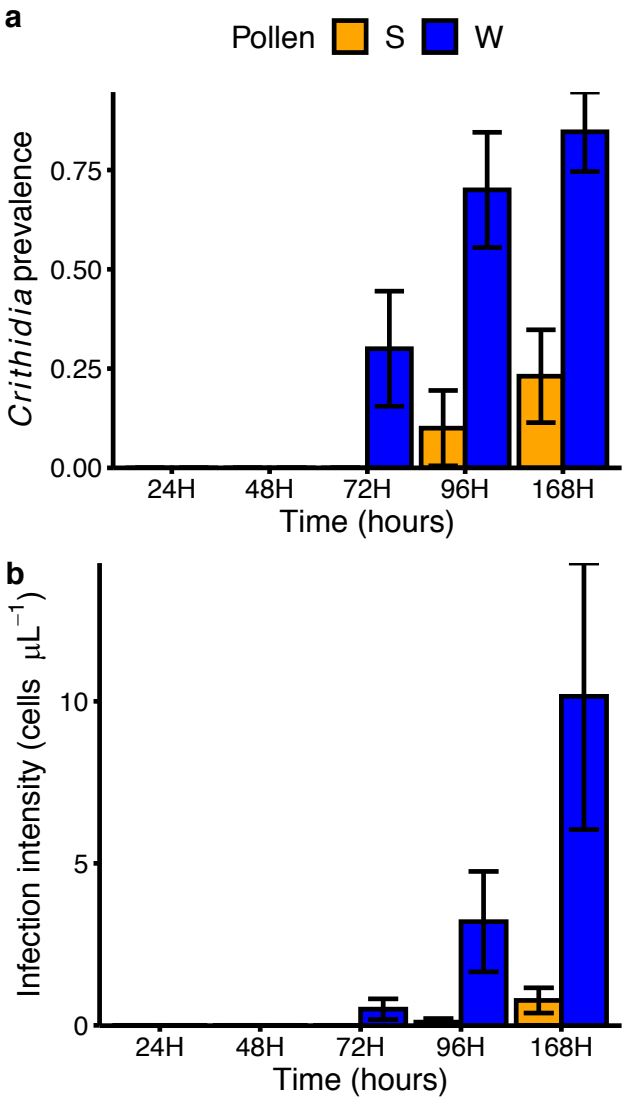


**Figure S4.1.** Mean proportion *Bombus impatiens* workers with detectable *Crithidia bombi* infection (**a**) and infection intensity (**b**). Bees were provided either sunflower pollen (S; orange bars) or control wildflower pollen (W; blue bars) for a period of either 24, 48, 72, 96 or 168 hours (H) post-inoculation. Bars and error bars represent model adjusted means and one standard error, respectively.

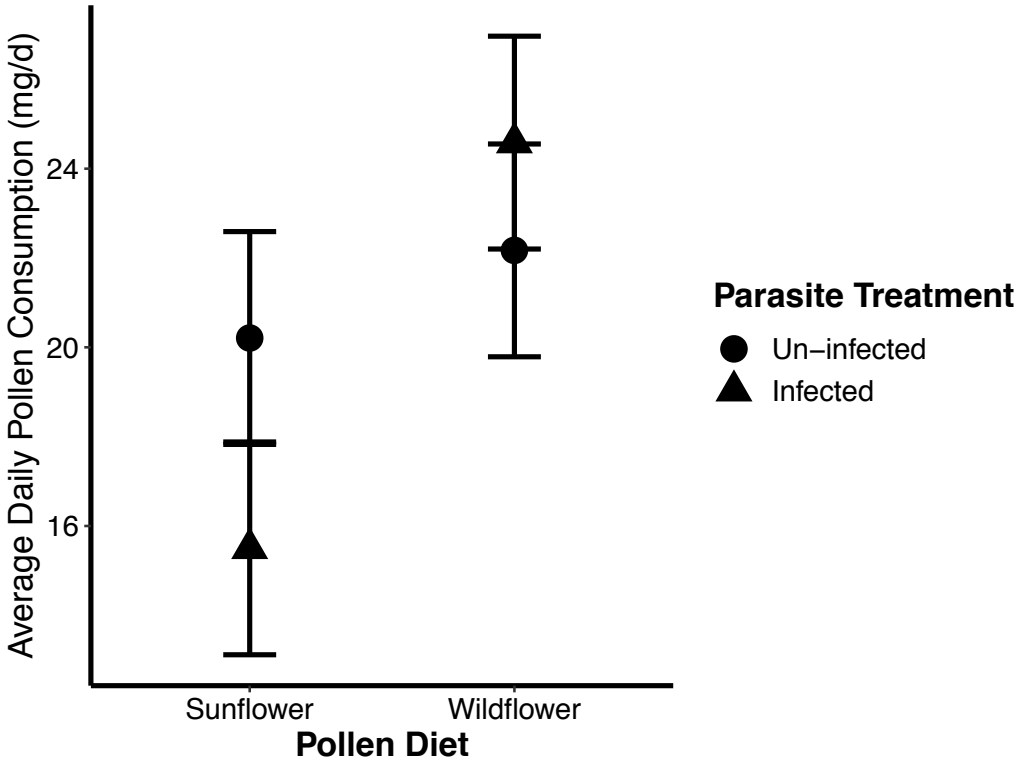


**Figure S4.2**. Average daily rate of sunflower or wildflower pollen consumption by *Bombus impatiens* workers either un-infected (circles) or infected with *Crithidia bombi* (triangles), and submitted for RNA sequencing. Points represent model adjusted means and errors bars 1 SE +/- of the mean.

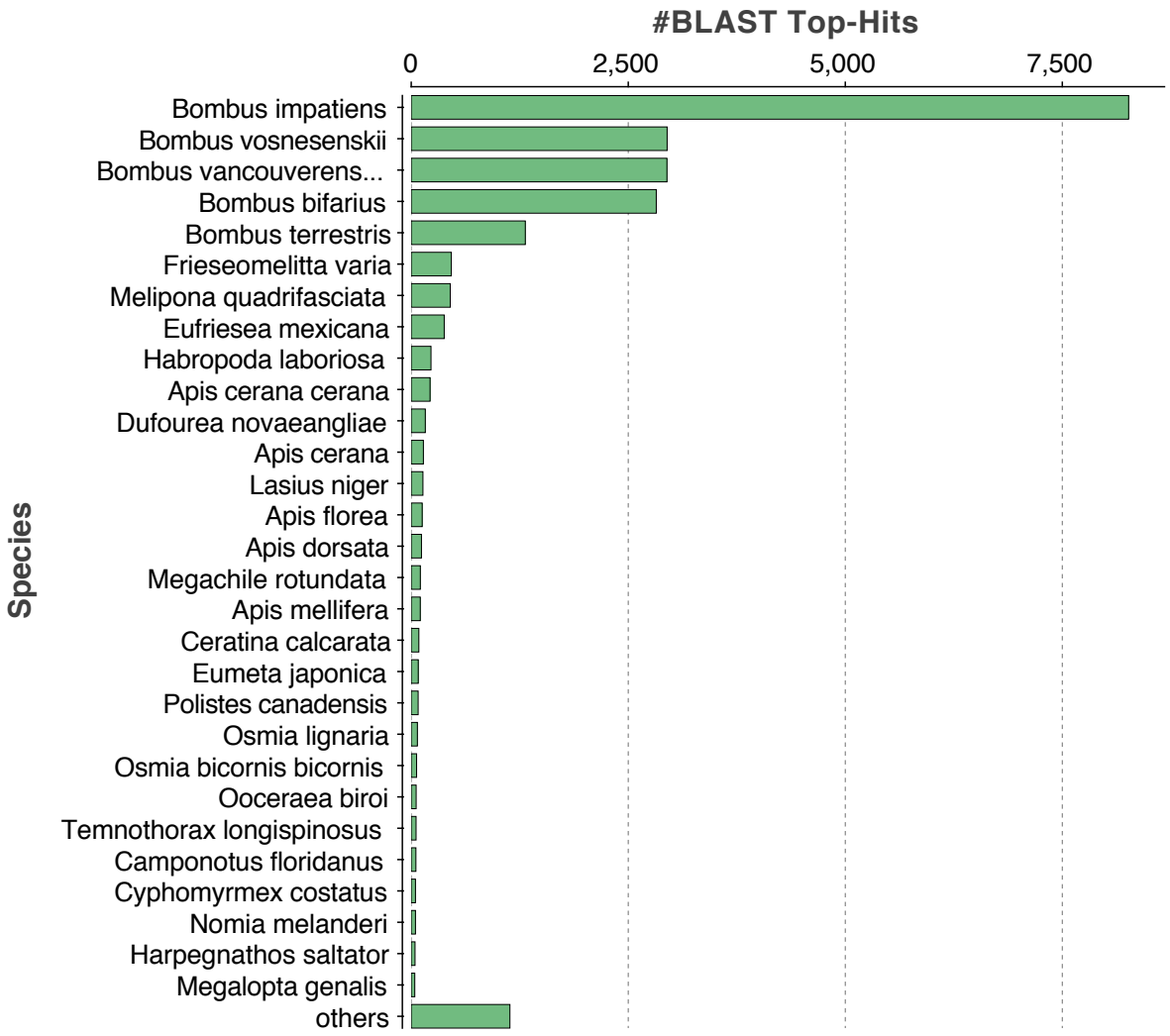


**Figure S4.3**. Species distribution of top BLAST hits from a blastx (OmicsBox) search using E-value of $10^{-25}$ against all arthropod sequences in the NCBI non-redundant database, with the number of hits restricted to 20. Out of 17077 hits the greatest number of top BLAST hits were found in *B. impatiens*, with the top five being *Bombus*, thus giving us confidence in our RNA sequence read quality.

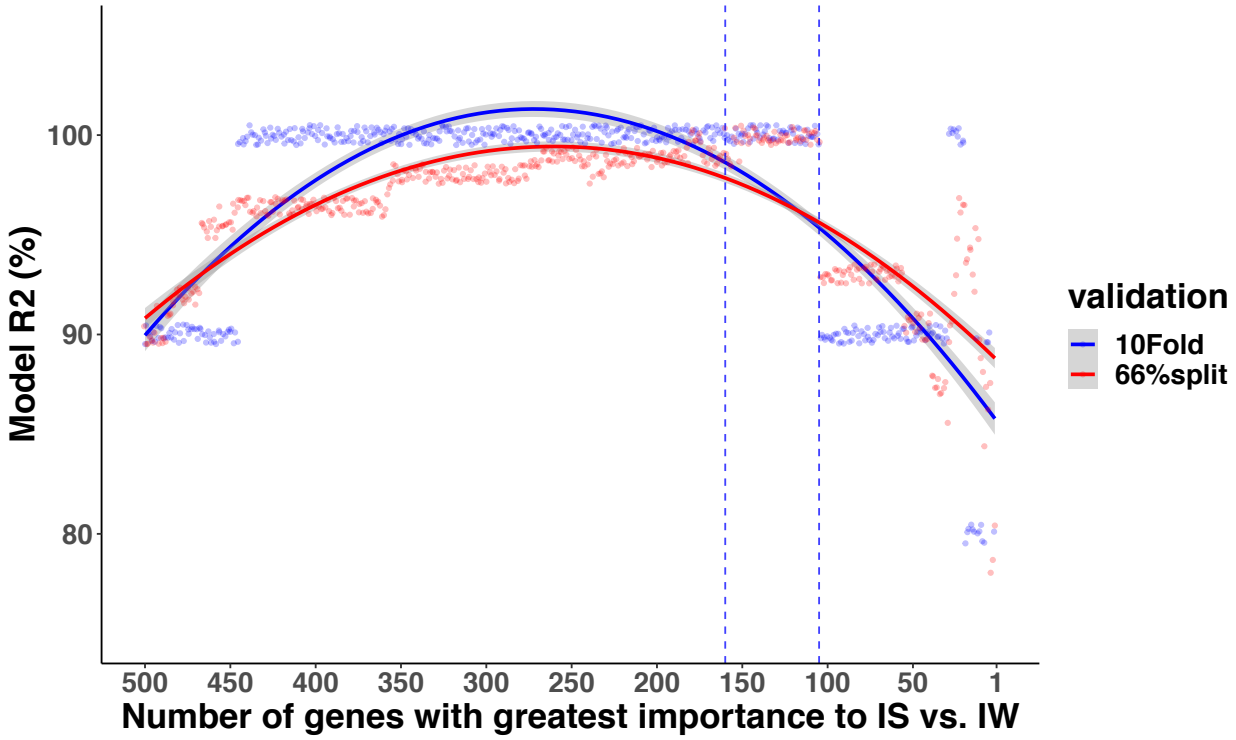


**Figure S4.4**. Machine learning model performance of support vector machine (SMO) ability to correctly classify pollen diet (sunflower or wildflower) based on transcriptomes of *Bombus impatiens* workers infected with *Crithidia bombi*. Two cross-validation methods were used: 10-fold stratified hold-out (blue) and 66% percent split (red). Vertical dashed blue lines indicate convergence between both validation methods in the number of genes that produced greatest model performance. Genes were selected from the DESeq2 models that were differentially expressed based on an un-corrected p-value < 0.05 and ranked based on Shannon's entropy using the InfoGain attribute evaluator and Ranker search method.

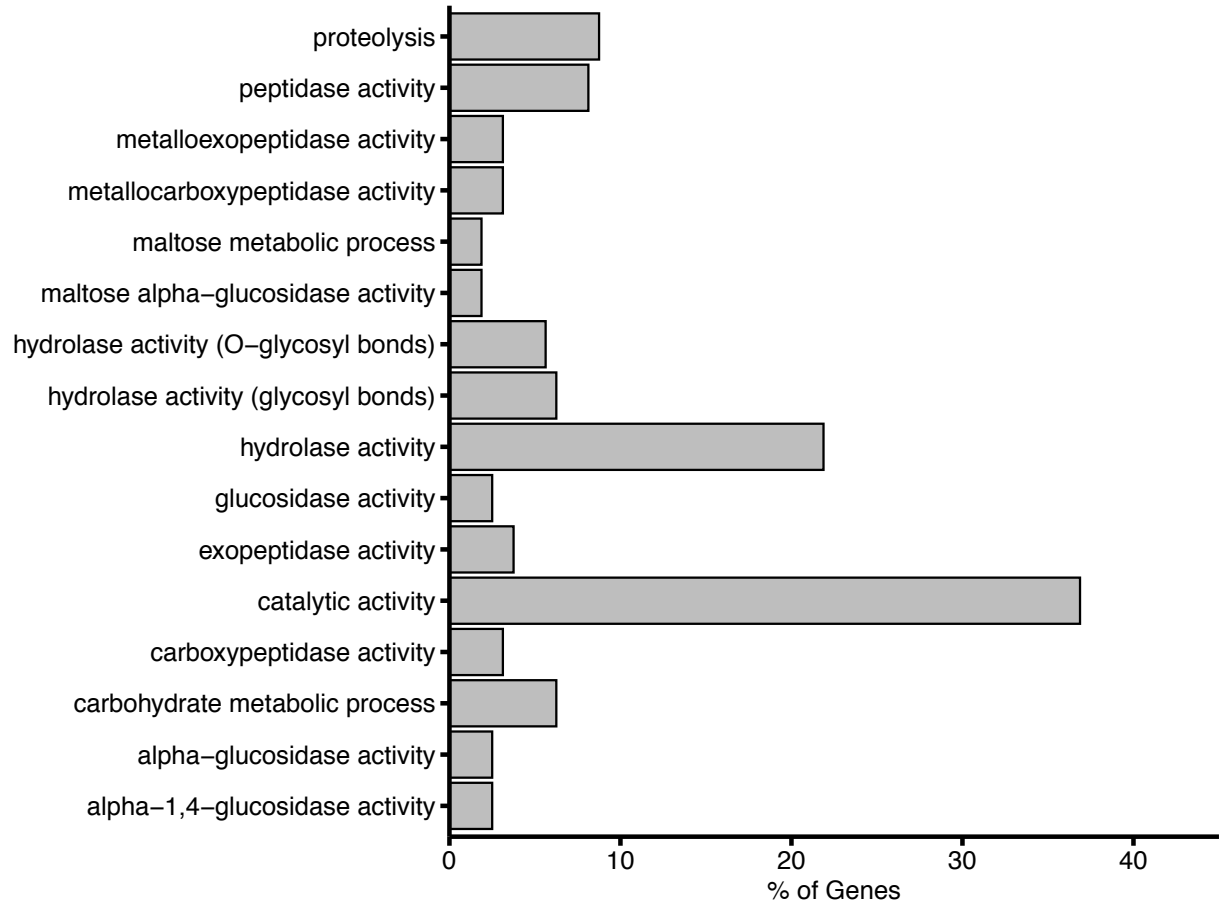


**Figure S4.5**. GO terms (biological processes and molecular activity) enriched in the machine learning DEG list for infected bees fed either sunflower or wildflower pollen. All terms are significant at an FDR < 0.05.

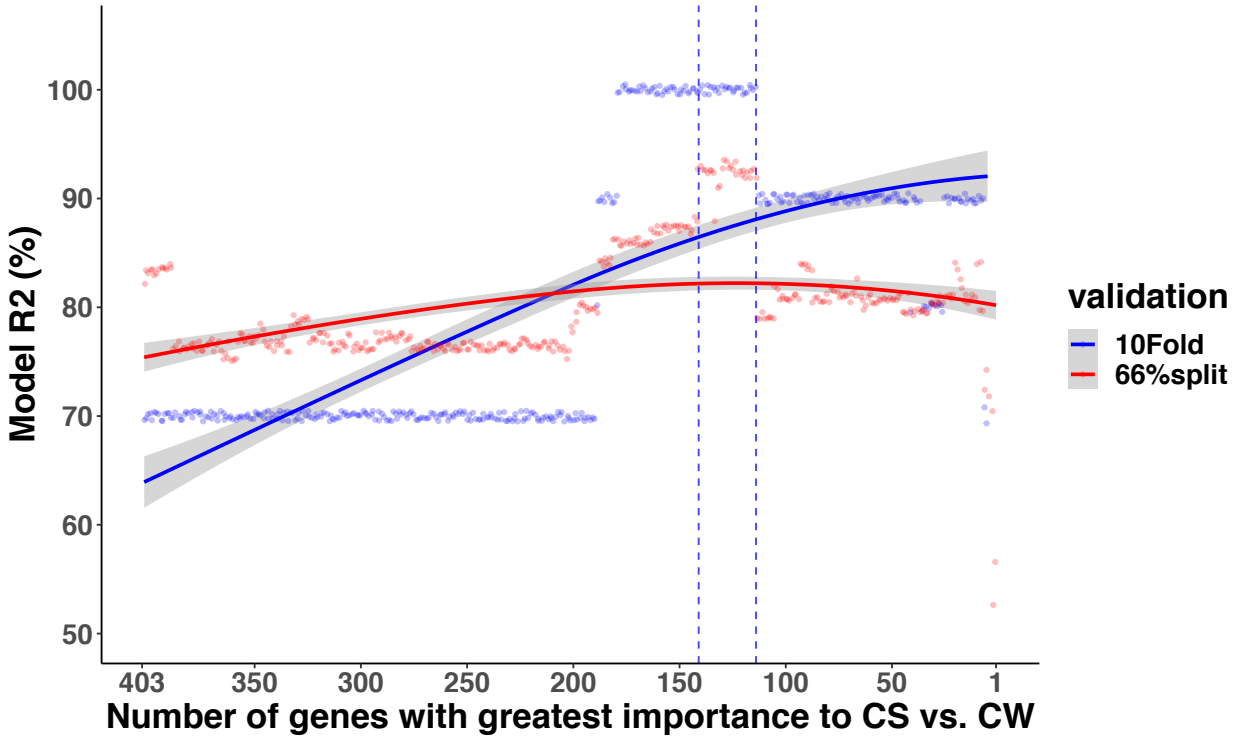


**Figure S4.6**. Machine learning model performance of support vector machine (SMO) ability to correctly classify pollen diet (sunflower or wildflower) based on transcriptomes of un-infected *Bombus impatiens*. Two cross-validation methods were used: 10-fold stratified hold-out (blue) and 66% percent split (red). Vertical dashed blue lines indicate convergence between both validation methods in the number of genes that produced greatest model performance. Genes were selected from the DESeq2 models that were differentially expressed based on an un-corrected p-value < 0.05 and ranked based on Shannon's entropy using the InfoGain attribute evaluator and Ranker search method.

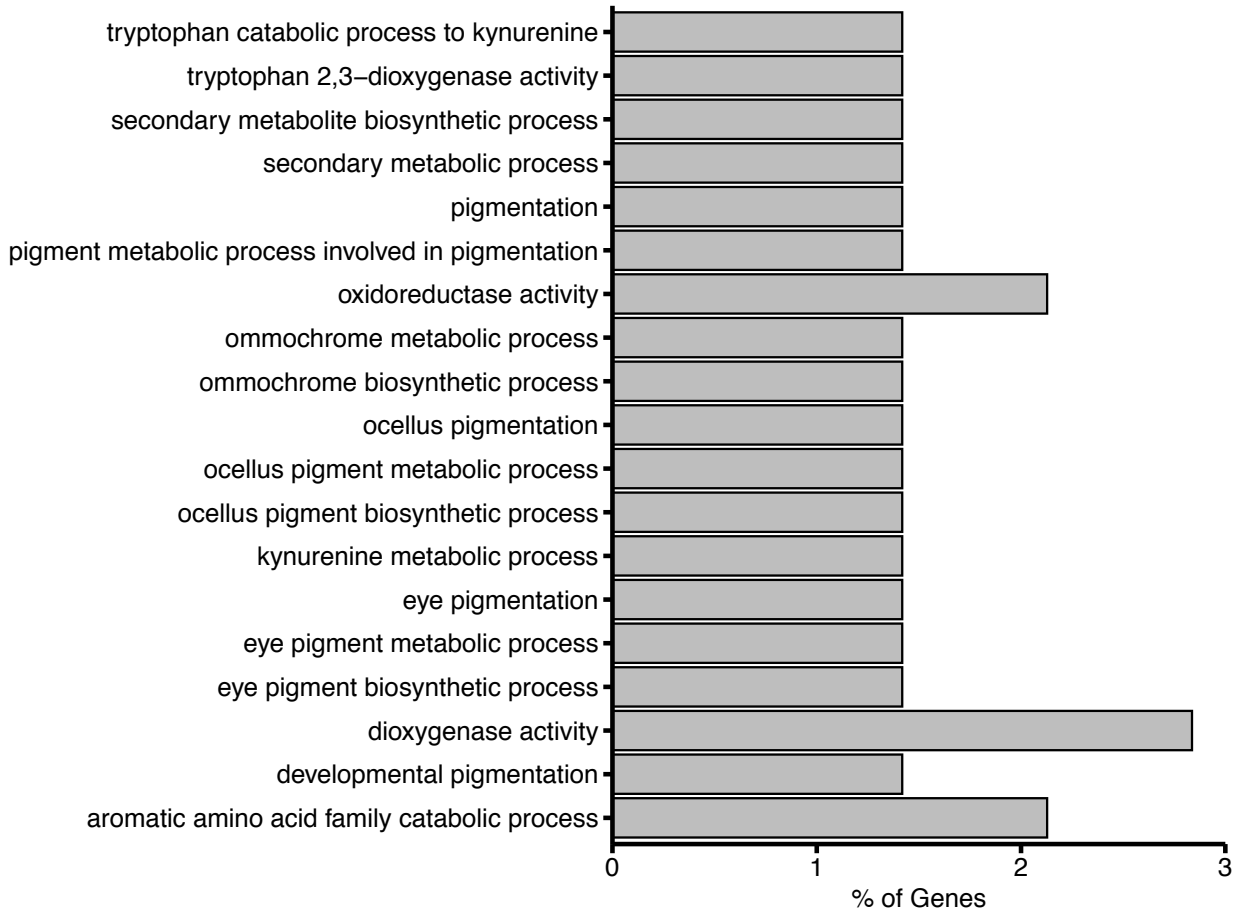


**Figure S4.7.** GO terms (biological processes and molecular activity) enriched in the machine learning DEG list for un-infected bees fed either sunflower or wildflower pollen. All terms are significant at an FDR < 0.05.

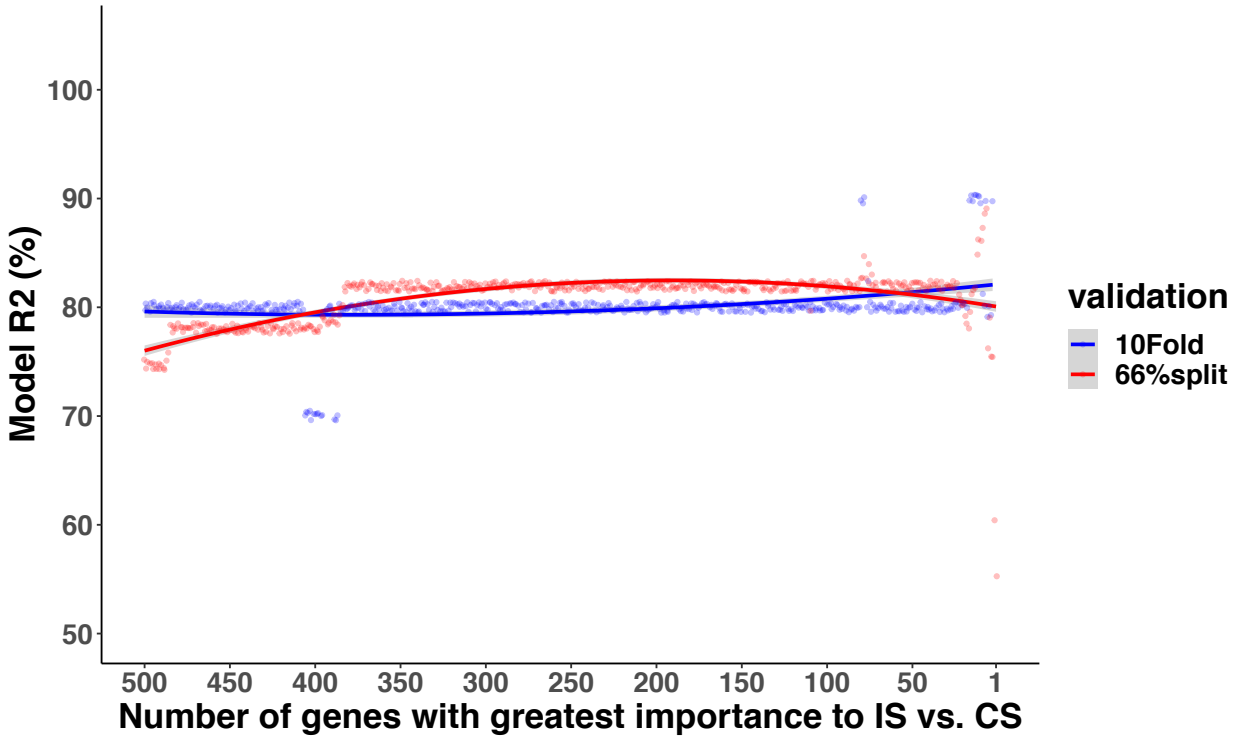


**Figure S4.8.** Machine learning model performance of support vector machine (SMO) ability to correctly classify infection treatment (inoculated with shame control or *Crithidia bombi*) based on transcriptomes of *Bombus impatiens* fed sunflower pollen. Two cross-validation methods were used: 10-fold stratified hold-out (blue) and 66% percent split (red). Genes were selected from the DESeq2 models that were differentially expressed based on an un-corrected p-value < 0.05 and ranked based on Shannon's entropy using the InfoGain attribute evaluator and Ranker search method.

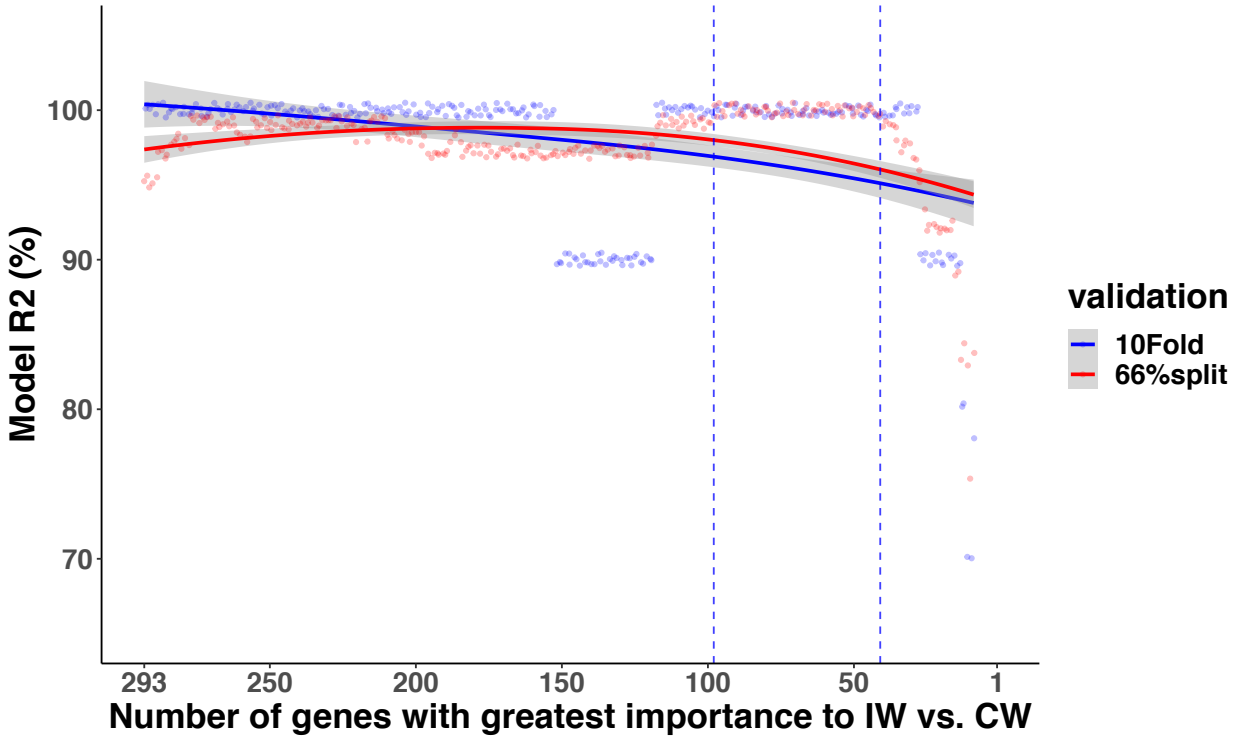


**Figure S4.9.** Machine learning model performance of support vector machine (SMO) ability to correctly classify infection treatment (inoculated with shame control or *Crithidia bombi*) based on transcriptomes of *Bombus impatiens* fed wildflower pollen. Two cross-validation methods were used: 10-fold stratified hold-out (blue) and 66% percent split (red). Vertical dashed blue lines indicate convergence between both validation methods in the number of genes that produced greatest model performance. Genes were selected from the DESeq2 models that were differentially expressed based on an un-corrected p-value < 0.05 and ranked based on Shannon's entropy using the InfoGain attribute evaluator and Ranker search method.

**Table S4.1**. Transcriptome assembly statistics for *Bombus impatiens* workers inoculated with either a shame control (C) or *Crithidia bombi* (I) and fed either sunflower pollen (S) or wildflower pollen (W). Clean reads were mapped to the *B. impatiens* genome (NCBI *B. impatiens* BIMP 2.2; GCA_000188095.4) with HiSat2 version 2.1.0.

| Inf. | Pollen | Clean reads | Uniquely mapped reads | Uniquely mapped rate | Reads mapped >1 times | Mapped >1 time rate | Un-mapped reads | Un-mapped rate | Overall map rate |
|---|---|---|---|---|---|---|---|---|---|
| C | S | 23335668 | 21316818 | 91.35% | 275766 | 1.18% | 1743084 | 7.47% | 92.53% |
| C | S | 22280940 | 20208002 | 90.70% | 240041 | 1.08% | 1832897 | 8.23% | 91.77% |
| C | S | 20563334 | 17855936 | 86.83% | 232087 | 1.13% | 2475311 | 12.04% | 87.96% |
| C | S | 21876134 | 19955912 | 91.22% | 274382 | 1.25% | 1645840 | 7.52% | 92.48% |
| C | S | 50201346 | 46094685 | 91.82% | 569847 | 1.14% | 3536814 | 7.05% | 92.95% |
| I | S | 23857146 | 21456697 | 89.94% | 319383 | 1.34% | 2081066 | 8.72% | 91.28% |
| I | S | 21081260 | 19202098 | 91.09% | 255888 | 1.21% | 1623274 | 7.70% | 92.30% |
| I | S | 29145053 | 26418761 | 90.65% | 317152 | 1.09% | 2409140 | 8.27% | 91.73% |
| I | S | 60011808 | 54555874 | 90.91% | 697392 | 1.16% | 4758542 | 7.93% | 92.07% |
| I | S | 15836186 | 14128277 | 89.22% | 173275 | 1.09% | 1534634 | 9.69% | 90.31% |
| C | W | 16426934 | 14658243 | 89.23% | 158521 | 0.97% | 1610170 | 9.80% | 90.20% |
| C | W | 41982749 | 38203936 | 91.00% | 421431 | 1.00% | 3357382 | 8.00% | 92.00% |
| C | W | 19376962 | 17621534 | 90.94% | 234904 | 1.21% | 1520524 | 7.85% | 92.15% |
| C | W | 31979220 | 29143727 | 91.13% | 362991 | 1.14% | 2472502 | 7.73% | 92.27% |
| C | W | 19639797 | 17274582 | 87.96% | 241821 | 1.23% | 2123394 | 10.81% | 89.19% |
| I | W | 25383002 | 22830985 | 89.95% | 264720 | 1.04% | 2287297 | 9.01% | 90.99% |
| I | W | 20691247 | 18770572 | 90.72% | 253799 | 1.23% | 1666876 | 8.06% | 91.94% |
| I | W | 42245908 | 38187736 | 90.39% | 463582 | 1.10% | 3594590 | 8.51% | 91.49% |
| I | W | 13702311 | 12166254 | 88.79% | 150068 | 1.10% | 1385989 | 10.12% | 89.88% |
| I | W | 22198707 | 20172087 | 90.87% | 265959 | 1.20% | 1760661 | 7.93% | 92.07% |
| Mean | | 27090786 | 24511136 | 90.23% | 308650 | 1.14% | 2270999 | 8.62% | 91.38% |
| Std. Dev. | | 12255014 | 112651623 | 1.25% | 137170 | 0.09% | 891882 | 1.27% | 1.27% |

**Table S4.2.** Differentially expressed genes between infected sunflower- and infected wildflower-fed bees based on the DESeq2 model (FDR < 0.05). Gene descriptions based on top BLAST hits (E-value of $10^{-25}$) against all arthropod sequences in the NCBI non-redundant database.

| StringTie ID | Fold Change (log2) | FDR | Description |
|---|---|---|---|
| MSTRG.21535 | 4.8157 | 0.0270 | hymenoptaecin |
| MSTRG.20285 | -2.4744 | 0.0355 | prohormone-3 |
| MSTRG.11358 | 21.2532 | 0.0000 | glutamate receptor 3.2-like |
| MSTRG.8149 | 30.1294 | 0.0000 | uncharacterized protein LOC117204693 |
| MSTRG.3654 | -2.0130 | 0.0153 | PREDICTED: uncharacterized protein LOC105662916 |
| MSTRG.14101 | 5.9837 | 0.0380 | uncharacterized protein LOC117233211 isoform X8 |
| MSTRG.8725 | -1.4158 | 0.0380 | uncharacterized protein LOC100748164 isoform X3 |
| MSTRG.9723 | 3.9849 | 0.0380 | uncharacterized protein LOC100749322 isoform X2 |
| MSTRG.3174 | 6.5770 | 0.0153 | cytochrome P450 9e2-like |
| MSTRG.11066 | 11.4536 | 0.0017 | probable endochitinase |
| MSTRG.5278 | -1.8675 | 0.0425 | TWiK family of potassium channels protein 7 |
| MSTRG.14467 | 44.3295 | 0.0000 | RNaN-directed DNaN polymerase from mobile element jockey-like |
| MSTRG.7865 | -36.7045 | 0.0000 | ejaculatory bulb-specific protein 3-like |
| MSTRG.19478 | 25.1746 | 0.0000 | glucose dehydrogeNaNse [FAD, quinone]-like |
| MSTRG.11147 | 8.0354 | 0.0000 | alkaline phosphatase 4-like |
| MSTRG.12016 | 4.7762 | 0.0110 | maltase A2 isoform X1 |
| MSTRG.6331 | -1.5704 | 0.0189 | enzymatic polyprotein endonuclease reverse |
| MSTRG.16362 | 1.2463 | 0.0355 | putative inorganic phosphate cotransporter |
| MSTRG.19383 | 23.0473 | 0.0078 | digestive cysteine proteinase 1 |
| MSTRG.872 | -2.8824 | 0.0001 | piggyBac transposable element-derived protein 4-like |
| LOC105680309 | 24.8898 | 0.0023 | probable ATP-dependent RNA helicase DDX46 |
| MSTRG.8718 | 6.6666 | 0.0056 | TM2 domain-containing protein |
| MSTRG.18222 | 0.8339 | 0.0149 | nicastrin isoform X2 |
| MSTRG.20614 | 5.8225 | 0.0149 | mitochondrial potassium channel ATP-binding subunit |
| MSTRG.14747 | 9.4394 | 0.0153 | beta-1,4-glucuronyltransferase 1 |
| MSTRG.4550 | -1.0866 | 0.0190 | ATP-binding cassette sub-family G member 1 |
| MSTRG.8519 | 7.3143 | 0.0223 | oxidation resistance protein 1 isoform X9 |
| MSTRG.8539 | 6.8131 | 0.0355 | NPC intracellular cholesterol transporter 2-like |
| MSTRG.8520 | 5.1741 | 0.0452 | oxidation resistance protein 1 isoform X6 |
| MSTRG.18428 | 0.6567 | 0.0467 | cholinephosphotransferase 1 isoform X3 |

**Table S4.2** (continued).

| | | | |
|---|---|---|---|
| MSTRG.9363 | -1.4573 | 0.0467 | carbohydrate sulfotransferase 11 |
| MSTRG.362 | 46.4711 | 0.0000 | NaN |
| MSTRG.12884 | 29.4999 | 0.0000 | NaN |
| MSTRG.996 | 15.2676 | 0.0002 | NaN |
| MSTRG.2541 | -3.2737 | 0.0153 | NaN |
| MSTRG.3872 | 5.8753 | 0.0153 | serine protease inhibitor dipetalogastin |
| MSTRG.21434 | 5.1708 | 0.0212 | NaN |
| MSTRG.16497 | 21.2627 | 0.0256 | hypothetical protein ALC62_09346 |
| MSTRG.20557 | 4.8864 | 0.0380 | NaN |
| MSTRG.7729 | -1.2714 | 0.0425 | NaN |

**Table S4.3.** Top-ranked 160 genes that differentiate infected sunflower- and infected wildflower-fed bees based on a 100% correct classification rate using Machine Learning. A subset of genes from the DESeq2 model with an un-corrected p-value < 0.05 were ranked (Rank) based on information gain with respect to the treatment using the InfoGain attribute evaluator and Ranker search method in Weka. Gene descriptions based on top BLAST hits (E-value of $10^{-25}$) against all arthropod sequences in the NCBI non-redundant database.

| StringTie ID | Fold Change (log2) | FDR | Rank | Description |
|---|---|---|---|---|
| MSTRG.8520 | 5.1741 | 0.0452 | 1 | oxidation resistance protein 1 isoform X6 |
| MSTRG.11543 | 2.2353 | 0.3857 | 2 | cyclin-A2 |
| MSTRG.5752 | -1.1313 | 0.3471 | 3 | ABC transporter G family member 23 |
| MSTRG.13004 | -1.2471 | 0.2864 | 4 | flexible cuticle protein 12-like |
| MSTRG.14101 | 5.9837 | 0.0380 | 5 | uncharacterized protein LOC117233211 isoform X8 |
| LOC105681892 | -1.4653 | 0.3503 | 6 | uncharacterized protein LOC117233964 isoform X1 |
| MSTRG.11112 | -2.6979 | 0.1358 | 7 | endocuticle structural glycoprotein SgAbd-4-like |
| MSTRG.9141 | 1.8054 | 0.3051 | 8 | solute carrier organic anion transporter family member 1A5 isoform X3 |
| MSTRG.5223 | -2.3864 | 0.3477 | 9 | NaN |
| MSTRG.8539 | 6.8131 | 0.0355 | 10 | NPC intracellular cholesterol transporter 2-like |
| MSTRG.1888 | 2.9456 | 0.3657 | 11 | thioredoxin reductase 1, mitochondrial isoform X3 |
| MSTRG.15603 | -1.8601 | 0.1575 | 12 | probable serine/threonine-protein kinase samkC isoform X3 |
| MSTRG.2194 | 3.7519 | 0.2785 | 13 | NaN |
| MSTRG.10445 | 1.6140 | 0.3948 | 14 | Neuropilin and tolloid-like protein 1 |
| MSTRG.20639 | 4.9580 | 0.2468 | 15 | transmembrane protease serine 9-like |
| MSTRG.15194 | 2.8636 | 0.1990 | 16 | putative inhibitor of apoptosis isoform X2 |
| MSTRG.5323 | -1.0620 | 0.1990 | 17 | Nose resistant to fluoxetine protein 6 |
| MSTRG.7877 | 2.1562 | 0.2441 | 18 | peptide transporter family 1 isoform X2 |
| MSTRG.3173 | 2.3805 | 0.0763 | 19 | cytochrome P450 9e2-like |
| MSTRG.1167 | 4.0506 | 0.2864 | 20 | homeobox protein ARX-like |
| MSTRG.20082 | 2.3136 | 0.2740 | 21 | protein regulator of cytokinesis 1-like |
| MSTRG.11252 | 2.7142 | 0.2984 | 22 | protein spaetzle 4 |
| MSTRG.5775 | 1.7092 | 0.3560 | 23 | max dimerization protein 1 isoform X2 |
| MSTRG.6180 | 5.1841 | 0.1805 | 24 | aminopeptidase N |
| MSTRG.10178 | 4.3293 | 0.2409 | 25 | farnesol dehydrogenase-like |
| MSTRG.328 | 0.9206 | 0.4595 | 26 | cytochrome P450 9e2-like |
| MSTRG.10176 | 3.2853 | 0.1679 | 27 | farnesol dehydrogenase-like |
| MSTRG.11845 | 2.4372 | 0.4031 | 28 | probable multidrug resistance-associated protein lethal(2)03659 |
| MSTRG.7691 | 2.0235 | 0.4729 | 29 | NaN |

**Table S4.3** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.3174 | 6.5770 | 0.0153 | 30 | cytochrome P450 9e2-like |
| MSTRG.10693 | 3.1739 | 0.2740 | 31 | Coiled-coil domain-containing protein 13 |
| MSTRG.5531 | -1.8260 | 0.4651 | 32 | uncharacterized protein LOC105189360 isoform X1 |
| MSTRG.128 | 1.6859 | 0.4363 | 33 | NaN |
| MSTRG.7209 | 2.3821 | 0.2639 | 34 | spectrin beta chain, non-erythrocytic 2 isoform X4 |
| MSTRG.20055 | 4.6991 | 0.2162 | 35 | Alpha-amylase 4N |
| MSTRG.4011 | 1.8716 | 0.4755 | 36 | sporozoite surface protein 2-like |
| MSTRG.3529 | -2.0199 | 0.4250 | 37 | homeobox protein rough |
| MSTRG.18721 | 2.7970 | 0.4063 | 38 | Haloacid dehalogenase-like hydrolase domain-containing protein 2 |
| MSTRG.16555 | 2.3924 | 0.4287 | 39 | NaN |
| MSTRG.20058 | 5.3531 | 0.2616 | 40 | Alpha-amylase-related protein |
| MSTRG.15815 | 1.3312 | 0.4646 | 41 | E3 ubiquitin-protein ligase MARCH5 isoform X1 |
| MSTRG.1625 | 4.3249 | 0.2785 | 42 | chymotrypsin-1 |
| MSTRG.18320 | 4.3598 | 0.3620 | 43 | maltase 2-like |
| MSTRG.8221 | 2.6658 | 0.0817 | 44 | probable ATP-dependent RNA helicase DDX28 |
| MSTRG.10703 | 4.9882 | 0.2409 | 45 | facilitated trehalose transporter Tret1-like |
| MSTRG.13224 | 1.8790 | 0.4421 | 46 | aconitate hydratase, mitochondrial |
| MSTRG.9658 | 1.9252 | 0.3182 | 47 | E3 ubiquitin-protein ligase MIB2 |
| MSTRG.13228 | 2.0740 | 0.2312 | 48 | piggyBac transposable element-derived protein 4-like |
| MSTRG.13229 | 3.0219 | 0.2740 | 49 | NaN |
| MSTRG.14832 | -1.2801 | 0.2035 | 50 | NaN |
| MSTRG.17560 | 3.0003 | 0.1791 | 51 | NaN |
| MSTRG.2166 | 1.5898 | 0.4171 | 52 | uncharacterized protein LOC100740206 |
| LOC100742005 | -3.0339 | 0.1232 | 53 | cytoplasmic dynein 2 heavy chain 1 |
| MSTRG.5369 | -1.8027 | 0.3725 | 54 | NaN |
| SWRh | 5.4940 | 0.4250 | 55 | opsin, ultraviolet-sensitive |
| MSTRG.8521 | 5.9837 | 0.1550 | 56 | oxidation resistance protein 1 isoform X7 |
| MSTRG.9087 | 2.8125 | 0.1861 | 57 | uncharacterized protein LOC100740339 isoform X3 |
| MSTRG.19203 | 1.2584 | 0.2188 | 58 | transmembrane protein KIAA1109 isoform X4 |
| MSTRG.7899 | 2.1301 | 0.3739 | 59 | actin-5C |
| MSTRG.17374 | 4.0633 | 0.2785 | 60 | zinc carboxypeptidase-like |
| MSTRG.16597 | 1.6762 | 0.3899 | 61 | nucleolysin TIAR |
| MSTRG.12194 | 5.5899 | 0.0699 | 62 | uncharacterized threonine-rich GPI-anchored glycoprotein PJ4664.02-like |
| MSTRG.2280 | -3.3144 | 0.1371 | 63 | hypothetical protein WH47_09040 |
| LOC105681602 | 2.4282 | 0.2434 | 64 | balbiani ring protein 3-like |
| MSTRG.1889 | 1.9904 | 0.4500 | 65 | thioredoxin reductase 1, mitochondrial isoform X2 |
| MSTRG.20788 | 1.5486 | 0.3310 | 66 | uncharacterized protein LOC117161787 |
| MSTRG.14270 | 0.7698 | 0.4488 | 67 | ADP-ribosylation factor 1 |
| MSTRG.13139 | 2.7488 | 0.1812 | 68 | 39S ribosomal protein S30, mitochondrial |
| MSTRG.1623 | 4.1955 | 0.2441 | 69 | NaN |
| MSTRG.3881 | 3.4611 | 0.4590 | 70 | Alpha-mannosidase 2 |
| MSTRG.5154 | 1.5403 | 0.4031 | 71 | ATP synthase subunit alpha, mitochondrial |

**Table S4.3** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.5150 | 3.2674 | 0.2125 | 72 | ubiquitin carboxyl-terminal hydrolase 14 |
| MSTRG.11968 | 4.2689 | 0.3222 | 73 | maltase 1 |
| MSTRG.10179 | 2.8962 | 0.2149 | 74 | farnesol dehydrogenase-like |
| MSTRG.4519 | 4.0014 | 0.0852 | 75 | protein FAM151B isoform X2 |
| MSTRG.11969 | 4.5654 | 0.2740 | 76 | maltase 1 |
| MSTRG.2068 | 3.4386 | 0.4076 | 77 | partitioning defective 3 homolog isoform X3 |
| MSTRG.11965 | 6.5062 | 0.1150 | 78 | alpha-glucosidase-like isoform X1 |
| MSTRG.15256 | 1.3959 | 0.3208 | 79 | NaN |
| MSTRG.4704 | 3.9405 | 0.1679 | 80 | Zinc finger CCHC domain-containing protein 9 |
| MSTRG.8718 | 6.6666 | 0.0056 | 81 | TM2 domain-containing protein |
| MSTRG.825 | 1.8657 | 0.3894 | 82 | Catenin alpha |
| MSTRG.11065 | 2.8331 | 0.0699 | 83 | probable WRKY transcription factor protein 1 |
| MSTRG.15789 | 2.7328 | 0.2526 | 84 | protein angel |
| MSTRG.8955 | 3.4081 | 0.4514 | 85 | SET and MYND domain-containing protein 4-like |
| MSTRG.8369 | 2.4431 | 0.2468 | 86 | Proteasome subunit alpha type-4 |
| MSTRG.10733 | 2.4446 | 0.4262 | 87 | hexosaminidase D-like |
| MSTRG.8362 | 3.0582 | 0.3822 | 88 | tRNA modification GTPase GTPBP3, mitochondrial |
| MSTRG.4863 | -1.4910 | 0.2188 | 89 | Myosin light chain alkali |
| MSTRG.17373 | 4.8870 | 0.1358 | 90 | zinc carboxypeptidase-like |
| MSTRG.10731 | -1.0858 | 0.3083 | 91 | F-box/LRR-repeat protein 2 isoform X1 |
| MSTRG.19715 | 3.0427 | 0.3003 | 92 | farnesol dehydrogenase-like |
| MSTRG.4264 | -2.3357 | 0.3619 | 93 | hypothetical protein WN51_00414 |
| MSTRG.21292 | 3.7904 | 0.1813 | 94 | UDP-glucuronosyltransferase 2B17-like |
| MSTRG.3872 | 5.8753 | 0.0153 | 95 | serine protease inhibitor dipetalogastin |
| MSTRG.12016 | 4.7762 | 0.0110 | 96 | maltase A2 isoform X1 |
| MSTRG.14663 | -3.9664 | 0.4147 | 97 | Polypyrimidine tract-binding protein 2 |
| MSTRG.8913 | 2.9344 | 0.1659 | 98 | RRP12-like protein |
| MSTRG.2534 | 1.5899 | 0.3476 | 99 | RNA-directed DNA polymerase from mobile element jockey |
| MSTRG.9637 | 3.2075 | 0.1812 | 100 | lysosomal aspartic protease |
| MSTRG.17231 | 1.0199 | 0.2713 | 101 | uridine diphosphate glucose pyrophosphatase |
| MSTRG.19349 | 2.6199 | 0.3857 | 102 | Dopamine N-acetyltransferase |
| MSTRG.413 | 3.6691 | 0.2795 | 103 | adenylate cyclase type 2 isoform X2 |
| MSTRG.14772 | 4.7662 | 0.3075 | 104 | NaN |
| MSTRG.20638 | 8.2439 | 0.0763 | 105 | transmembrane protease serine 9-like |
| MSTRG.4575 | 3.2980 | 0.1288 | 106 | NaN |
| MSTRG.16410 | 1.2762 | 0.4180 | 107 | uncharacterized protein LOC100747659 isoform X4 |
| MSTRG.75 | -1.5967 | 0.4352 | 108 | insulin-like peptide receptor isoform X1 |
| MSTRG.14907 | 2.4632 | 0.1679 | 109 | NaN |
| MSTRG.16918 | 1.7859 | 0.4312 | 110 | E3 ubiquitin-protein ligase AMFR-like |
| MSTRG.17464 | 1.0745 | 0.4590 | 111 | uncharacterized protein LOC117162246 |
| MSTRG.13552 | 4.6985 | 0.3332 | 112 | protein mesh isoform X1 |
| MSTRG.5826 | 1.6990 | 0.3136 | 113 | NaN |
| MSTRG.9723 | 3.9849 | 0.0380 | 114 | uncharacterized protein LOC100749322 isoform X2 |

**Table S4.3** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.13589 | 1.7596 | 0.2856 | 115 | NaN |
| MSTRG.7600 | -2.8205 | 0.4100 | 116 | NaN |
| MSTRG.14601 | 1.9655 | 0.2911 | 117 | NaN |
| MSTRG.13252 | 5.0504 | 0.2800 | 118 | carboxypeptidase B-like |
| MSTRG.502 | 1.2860 | 0.4500 | 119 | tyrosine-protein phosphatase non-receptor type 2 isoform X2 |
| MSTRG.3516 | 2.6846 | 0.4408 | 120 | NaN |
| MSTRG.9055 | -2.0090 | 0.4100 | 121 | NaN |
| MSTRG.1626 | 5.5920 | 0.0852 | 122 | chymotrypsin-1 |
| LOC105681186 | 2.8943 | 0.2785 | 123 | G-protein coupled receptor Mth2-like |
| MSTRG.8482 | 4.7322 | 0.3452 | 124 | chitinase-3-like protein 1 |
| MSTRG.890 | 4.8198 | 0.3112 | 125 | trypsin-1 isoform X1 |
| MSTRG.1577 | 2.0329 | 0.2468 | 126 | uncharacterized protein LOC117241145 isoform X2 |
| MSTRG.8481 | 6.3376 | 0.3657 | 127 | chitinase-3-like protein 1 |
| MSTRG.3866 | 2.2856 | 0.3012 | 128 | dnaJ homolog subfamily C member 11 |
| MSTRG.16385 | 2.0737 | 0.4683 | 129 | NaN |
| MSTRG.11414 | 4.8044 | 0.4702 | 130 | NaN |
| MSTRG.20549 | 3.5421 | 0.2820 | 131 | NaN |
| MSTRG.5529 | 1.6367 | 0.3343 | 132 | uncharacterized protein LOC102671994 |
| MSTRG.19259 | -1.2239 | 0.2964 | 133 | Retrovirus-related Pol polyprotein from transposon 17.6 |
| MSTRG.8211 | 1.9139 | 0.3081 | 134 | kxDL motif-containing protein CG10681 |
| MSTRG.12622 | 1.6176 | 0.4646 | 135 | NaN |
| MSTRG.695 | -3.7479 | 0.3051 | 136 | NaN |
| MSTRG.9636 | 3.3960 | 0.3332 | 137 | lysosomal aspartic protease |
| MSTRG.10504 | 1.9854 | 0.3551 | 138 | NaN |
| MSTRG.9634 | 4.2748 | 0.0577 | 139 | NaN |
| MSTRG.14366 | 1.2306 | 0.4514 | 140 | FACT complex subunit spt16 isoform X2 |
| MSTRG.9870 | 3.8966 | 0.2280 | 141 | cGMP-dependent protein kinase 1-like |
| MSTRG.6877 | 1.1398 | 0.2666 | 142 | cholinesterase isoform X1 |
| MSTRG.1099 | 4.3402 | 0.1581 | 143 | NaN |
| MSTRG.14600 | 1.0146 | 0.3973 | 144 | NaN |
| MSTRG.102 | 2.1415 | 0.3042 | 145 | V-type proton ATPase subunit D |
| MSTRG.18165 | 2.2897 | 0.3267 | 146 | uncharacterized protein LOC117152339 |
| MSTRG.20647 | -2.2205 | 0.4423 | 147 | NaN |
| MSTRG.424 | -1.6504 | 0.4512 | 148 | probable cytochrome P450 305a1 |
| MSTRG.1730 | 1.9570 | 0.4259 | 149 | NaN |
| MSTRG.9255 | 2.1475 | 0.2964 | 150 | carbonyl reductase [NADPH] 1-like |
| MSTRG.19626 | 5.0703 | 0.1328 | 151 | facilitated trehalose transporter Tret1 isoform X2 |
| MSTRG.18876 | 3.2078 | 0.4399 | 152 | meiosis regulator and mRNA stability factor 1 isoform X7 |
| MSTRG.1938 | -2.6216 | 0.0862 | 153 | circadian clock-controlled protein |
| MSTRG.4646 | 1.7799 | 0.3437 | 154 | NaN |
| MSTRG.889 | 4.6928 | 0.2468 | 155 | trypsin-3 |
| MSTRG.15486 | 1.7693 | 0.3940 | 156 | alanine--glyoxylate aminotransferase 2-like |
| MSTRG.9453 | 1.8858 | 0.4648 | 157 | complement component 1 Q subcomponent-binding protein, mitochondrial |

**Table S4.3** (continued)

| | | | | |
|---|---|---|---|---|
| MSTRG.20877 | -2.5394 | 0.2358 | 158 | mitotic apparatus protein p62 |
| MSTRG.5302 | 2.5046 | 0.3442 | 159 | 40S ribosomal protein S17 |
| MSTRG.4742 | -2.0975 | 0.4040 | 160 | sodium- and chloride-dependent GABA transporter 2-like |

**Table S4.4**. Enrichment of canonical metabolic and signaling pathways identified by Qiagen's IPA for pairwise treatment comparisons using the machine learning DEG lists. *Bombus impatiens* workers were inoculated with either a shame control (Un-infected) or *Crithidia bombi* (Infected) and fed either sunflower or wildflower pollen. Numeric values indicate log(p-value) for each gene set and a canonical pathway and red indicates significant enrichment based on a p-value of overlap calculated using a right-tailed Fisher's Exact Test.

| **Canonical Pathways** | **Wilflower**: Infected vs. Un-infected | **Infected**: Sunflower vs. Wildflower | **Un-infceted**: Sunflower vs. Wildflower |
|---|---|---|---|
| NRF2-mediated Oxidative Stress Response | 1.297 | 6.668 | 1.638 |
| SPINK1 Pancreatic Cancer Pathway | 0.000 | 5.571 | 0.000 |
| LPS/IL-1 Mediated Inhibition of RXR Function | 1.235 | 2.599 | 1.547 |
| Estrogen Biosynthesis | 0.000 | 4.311 | 0.880 |
| Epithelial Adherens Junction Signaling | 0.000 | 2.550 | 2.092 |
| Acetone Degradation I (to Methylglyoxal) | 0.000 | 3.410 | 1.021 |
| Aryl Hydrocarbon Receptor Signaling | 0.652 | 2.502 | 1.173 |
| Cellular Effects of Sildenafil (Viagra) | 0.000 | 2.531 | 1.187 |
| Neuroprotective Role of THOP1 in Alzheimer's Disease | 0.763 | 2.954 | 0.000 |
| Protein Ubiquitination Pathway | 0.459 | 2.446 | 0.795 |
| Nicotine Degradation III | 0.000 | 2.799 | 0.825 |
| Stearate Biosynthesis I (Animals) | 1.013 | 0.663 | 1.891 |
| Bupropion Degradation | 0.000 | 2.362 | 1.174 |
| Melatonin Degradation I | 0.000 | 2.633 | 0.772 |
| RhoA Signaling | 0.000 | 1.957 | 1.381 |
| Thioredoxin Pathway | 0.000 | 3.332 | 0.000 |
| Nicotine Degradation II | 0.000 | 2.566 | 0.751 |
| Vitamin-C Transport | 0.000 | 2.165 | 1.078 |
| Superpathway of Melatonin Degradation | 0.000 | 2.400 | 0.698 |

**Table S4.4** (continued).

| | | | |
|---|---|---|---|
| Dilated Cardiomyopathy Signaling Pathway | 0.000 | 1.770 | 1.256 |
| Unfolded protein response | 0.892 | 1.369 | 0.685 |
| Tight Junction Signaling | 0.000 | 1.562 | 1.116 |
| Regulation of Actin-based Motility by Rho | 0.000 | 1.160 | 1.433 |
| LXR/RXR Activation | 0.745 | 0.422 | 1.357 |
| Hepatic Fibrosis / Hepatic Stellate Cell Activation | 0.000 | 1.445 | 1.037 |
| Polyamine Regulation in Colon Cancer | 0.000 | 1.653 | 0.825 |
| Sertoli Cell-Sertoli Cell Junction Signaling | 0.000 | 2.105 | 0.373 |
| Estrogen-Dependent Breast Cancer Signaling | 0.000 | 2.457 | 0.000 |
| Glycogen Degradation III | 0.000 | 1.152 | 1.300 |
| Remodeling of Epithelial Adherens Junctions | 0.000 | 1.591 | 0.795 |
| Protein Kinase A Signaling | 0.000 | 0.716 | 1.663 |
| Gap Junction Signaling | 0.000 | 1.376 | 0.991 |
| Mitochondrial Dysfunction | 0.000 | 2.309 | 0.000 |
| eNOS Signaling | 0.636 | 1.600 | 0.000 |
| Actin Cytoskeleton Signaling | 0.000 | 0.638 | 1.582 |
| Phagosome Maturation | 0.000 | 1.640 | 0.459 |
| Apelin Cardiomyocyte Signaling Pathway | 0.000 | 0.500 | 1.533 |
| Hepatic Cholestasis | 0.572 | 1.412 | 0.000 |
| Choline Degradation I | 1.974 | 0.000 | 0.000 |
| Huntington's Disease Signaling | 0.000 | 1.678 | 0.281 |
| Calcium Signaling | 0.000 | 0.252 | 1.701 |
| PRPP Biosynthesis I | 1.917 | 0.000 | 0.000 |
| Sorbitol Degradation I | 0.000 | 0.000 | 1.894 |
| PAK Signaling | 0.000 | 0.449 | 1.419 |
| Gα12/13 Signaling | 0.000 | 0.411 | 1.333 |
| Neuroinflammation Signaling Pathway | 0.000 | 1.485 | 0.241 |
| MSP-RON Signaling Pathway | 0.000 | 1.706 | 0.000 |
| Glycine Biosynthesis III | 0.000 | 1.664 | 0.000 |

**Table S4.4** (continued).

| | | | |
|---|---|---|---|
| Thyroid Hormone Biosynthesis | 0.000 | 1.598 | 0.000 |
| Tetrahydrofolate Salvage from 5,10-methenyltetrahydrofolate | 0.000 | 0.000 | 1.596 |
| Superoxide Radicals Degradation | 0.000 | 0.000 | 1.596 |
| SPINK1 General Cancer Pathway | 0.000 | 1.580 | 0.000 |
| NAD Phosphorylation and Dephosphorylation | 1.523 | 0.000 | 0.000 |
| Assembly of RNA Polymerase III Complex | 0.000 | 0.000 | 1.484 |
| Urate Biosynthesis/Inosine 5'-phosphate Degradation | 1.463 | 0.000 | 0.000 |
| Guanosine Nucleotides Degradation III | 1.463 | 0.000 | 0.000 |
| Tyrosine Degradation I | 0.000 | 0.000 | 1.452 |
| DNA Double-Strand Break Repair by Homologous Recombination | 0.000 | 0.000 | 1.452 |
| Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde | 0.000 | 0.000 | 1.395 |
| Adenosine Nucleotides Degradation II | 1.394 | 0.000 | 0.000 |
| Crosstalk between Dendritic Cells and Natural Killer Cells | 0.000 | 1.351 | 0.000 |
| Mismatch Repair in Eukaryotes | 0.000 | 0.000 | 1.345 |
| Glycogen Degradation II | 0.000 | 0.000 | 1.345 |
| 5-aminoimidazole Ribonucleotide Biosynthesis I | 0.000 | 0.000 | 1.345 |
| Death Receptor Signaling | 0.000 | 1.318 | 0.000 |
| FXR/RXR Activation | 0.000 | 0.000 | 1.316 |
| Fatty Acid Activation | 0.000 | 0.000 | 1.300 |

**Table S4.5**. Differentially expressed genes between uninfected sunflower- and uninfected wildflower-fed bees based on the DESeq2 model (FDR < 0.05). Gene descriptions based on top BLAST hits (E-value of $10^{-25}$ ) against all arthropod sequences in the NCBI non-redundant database.

| String Tie ID | Fold Change (log2) | FDR | Description |
|---|---|---|---|
| MSTRG.19477 | 29.9440 | 0.0000 | glucose dehydrogenase [FAD, quinone]-like |
| MSTRG.9983 | 29.9894 | 0.0000 | uncharacterized protein LOC112212842 |
| MSTRG.14467 | 29.5606 | 0.0000 | RNA-directed DNA polymerase from mobile element jockey-like |
| MSTRG.20466 | -22.4594 | 0.0006 | NaN |
| MSTRG.20478 | -22.2347 | 0.0008 | dynein beta chain, ciliary-like |
| MSTRG.10459 | 18.5702 | 0.0018 | NaN |
| MSTRG.362 | -20.8330 | 0.0030 | NaN |
| MSTRG.17182 | 20.8841 | 0.0035 | putative odorant receptor 92a |
| MSTRG.15434 | 14.9761 | 0.0055 | NaN |
| MSTRG.19495 | 13.5107 | 0.0265 | jerky protein homolog-like |

**Table S4.6.** Top-ranked 141 genes that differentiate uninfected sunflower- and uninfected wildflower-fed bees based on a 100% correct classification rate using Machine Learning. A subset of genes from the DESeq2 model with an un-corrected p-value < 0.05 were ranked (Rank) based on information gain with respect to the treatment using the InfoGain attribute evaluator and Ranker search method in Weka. Gene descriptions based on top BLAST hits (E-value of $10^{-25}$) against all arthropod sequences in the NCBI non-redundant database.

| String Tie ID | Fold Change (log2) | FDR | Rank | Description |
|---|---|---|---|---|
| MSTRG.235 | -1.1252 | 1.0000 | 1 | lamin Dm0-like isoform X1 |
| MSTRG.7221 | -1.3343 | 1.0000 | 2 | NaN |
| MSTRG.9187 | 3.2432 | 1.0000 | 3 | NaN |
| MSTRG.502 | -1.2114 | 1.0000 | 4 | tyrosine-protein phosphatase non-receptor type 2 isoform X2 |
| MSTRG.4864 | -2.1114 | 1.0000 | 5 | myosin light chain alkali isoform X2 |
| MSTRG.17055 | 0.7114 | 1.0000 | 6 | NaN |
| MSTRG.15488 | -2.7218 | 1.0000 | 7 | vitellogenin |
| MSTRG.15843 | 1.9997 | 1.0000 | 8 | Transposon Tf2-9 polyprotein |
| MSTRG.14903 | -4.3002 | 1.0000 | 9 | distal membrane-arm assembly complex protein 2 |
| MSTRG.9958 | -1.0144 | 1.0000 | 10 | trifunctional purine biosynthetic protein adenosine-3 isoform X1 |
| MSTRG.21068 | 1.4029 | 1.0000 | 11 | uncharacterized protein LOC105666051 |
| MSTRG.16550 | -1.5799 | 1.0000 | 12 | Nucleolar and coiled-body phosphoprotein 1 |
| MSTRG.19724 | -1.9075 | 1.0000 | 13 | myosin regulatory light chain 2 |
| MSTRG.1019 | -1.7131 | 1.0000 | 14 | Tubulin alpha-1 chain |
| MSTRG.19252 | -3.3987 | 1.0000 | 15 | uncharacterized protein LOC100642617 isoform X3 |
| MSTRG.20047 | -3.0857 | 1.0000 | 16 | PAN2-PAN3 deadenylation complex catalytic subunit PAN2 isoform X3 |
| MSTRG.15344 | 1.8679 | 1.0000 | 17 | gag-pol polyprotein |
| MSTRG.15387 | -2.2987 | 1.0000 | 18 | ankyrin repeat and KH domain-containing protein 1 isoform X4 |
| MSTRG.17583 | -3.8835 | 1.0000 | 19 | NaN |
| MSTRG.4852 | 2.1813 | 1.0000 | 20 | membrane-associated protein Hem |
| MSTRG.8766 | 2.7234 | 1.0000 | 21 | balbiani ring protein 3-like |
| MSTRG.5796 | -1.3817 | 1.0000 | 22 | NaN |
| MSTRG.15482 | -1.8351 | 1.0000 | 23 | zinc finger SWIM domain-containing protein 8 isoform X1 |
| MSTRG.18933 | 0.7795 | 0.4627 | 24 | odorant receptor 46a-like |
| MSTRG.21015 | -3.5586 | 1.0000 | 25 | NaN |
| MSTRG.12442 | 3.6998 | 0.0759 | 26 | zinc finger protein YER130C isoform X2 |
| MSTRG.7733 | 0.8942 | 1.0000 | 27 | protein yippee-like 5 |
| MSTRG.9564 | 2.5118 | 1.0000 | 28 | uncharacterized protein LOC110119883 |

**Table S4.6** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.21342 | 2.0539 | 1.0000 | 29 | NaN |
| MSTRG.8780 | 1.8734 | 1.0000 | 30 | uncharacterized protein LOC112212704 |
| MSTRG.10709 | -4.0532 | 1.0000 | 31 | NaN |
| MSTRG.14066 | -3.7618 | 1.0000 | 32 | 4-coumarate--CoA ligase 1-like |
| MSTRG.18133 | -2.4614 | 1.0000 | 33 | NaN |
| LOC105681602 | 1.3488 | 1.0000 | 34 | balbiani ring protein 3-like |
| MSTRG.20562 | -2.1733 | 1.0000 | 35 | RNA-directed DNA polymerase from mobile element jockey |
| MSTRG.2074 | 2.0966 | 1.0000 | 36 | NaN |
| MSTRG.2249 | -2.7250 | 1.0000 | 37 | putative uncharacterized protein DDB_G0277057 |
| MSTRG.18073 | -1.5528 | 1.0000 | 38 | E3 ubiquitin-protein ligase SIAH1-like |
| MSTRG.3573 | -2.7096 | 1.0000 | 39 | cartilage oligomeric matrix protein |
| MSTRG.11468 | 1.5309 | 1.0000 | 40 | ectonucleoside triphosphate diphosphohydrolase 5 isoform X2 |
| MSTRG.1825 | 1.4801 | 1.0000 | 41 | NaN |
| LOC105681186 | 2.5126 | 1.0000 | 42 | G-protein coupled receptor Mth2-like |
| MSTRG.15013 | 2.2747 | 1.0000 | 43 | NaN |
| MSTRG.11652 | -1.7649 | 1.0000 | 44 | NaN |
| MSTRG.9455 | -2.5856 | 1.0000 | 45 | replication protein A 70 kDa DNA-binding subunit |
| MSTRG.14915 | -3.1361 | 1.0000 | 46 | LIM/homeobox protein Lhx3 isoform X1 |
| MSTRG.7774 | -2.7167 | 1.0000 | 47 | NaN |
| MSTRG.4585 | -1.7744 | 1.0000 | 48 | receptor-type guanylate cyclase gcy-4-like isoform X2 |
| MSTRG.9773 | -1.0251 | 1.0000 | 49 | NaN |
| MSTRG.9630 | 1.1200 | 1.0000 | 50 | lysoplasmalogenase-like protein TMEM86A isoform X2 |
| MSTRG.3173 | 1.7870 | 0.8383 | 51 | cytochrome P450 9e2-like |
| MSTRG.13662 | -3.0904 | 1.0000 | 52 | proteasome activator complex subunit 4-like isoform X2 |
| MSTRG.16794 | 0.7455 | 1.0000 | 53 | NaN |
| MSTRG.21207 | -1.4786 | 1.0000 | 54 | serine/threonine-protein kinase SMG1 isoform X2 |
| MSTRG.18616 | -1.0445 | 1.0000 | 55 | NaN |
| MSTRG.4982 | -2.3372 | 1.0000 | 56 | transferrin |
| MSTRG.12519 | 3.3959 | 1.0000 | 57 | NaN |
| MSTRG.6801 | 1.5653 | 1.0000 | 58 | NaN |
| MSTRG.726 | -2.4791 | 1.0000 | 59 | NaN |
| MSTRG.18858 | 2.4604 | 1.0000 | 60 | NaN |
| MSTRG.18797 | -2.7311 | 1.0000 | 61 | coronin-1C-A |
| MSTRG.19964 | 3.2008 | 1.0000 | 62 | thyrotropin-releasing hormone-degrading ectoenzyme isoform X1 |
| MSTRG.2170 | -3.1263 | 1.0000 | 63 | hypothetical protein WH47_01332 |
| MSTRG.10517 | 3.5554 | 1.0000 | 64 | NaN |
| MSTRG.19220 | -3.3635 | 1.0000 | 65 | fatty acyl-CoA reductase wat-like |
| LOC100746040 | -3.2034 | 1.0000 | 66 | uncharacterized protein LOC100746040 |
| MSTRG.15012 | 3.9300 | 1.0000 | 67 | uncharacterized protein LOC100742276 |
| MSTRG.4831 | 1.0353 | 1.0000 | 68 | tachykinin-like peptides receptor 99D |
| MSTRG.14849 | 1.3630 | 1.0000 | 69 | NaN |
| MSTRG.9706 | 0.2902 | 1.0000 | 70 | gem-associated protein 2 |

**Table S4.6** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.8508 | 0.5304 | 1.0000 | 71 | N-acetylgalactosaminyltransferase 6-like |
| MSTRG.21143 | -2.1638 | 1.0000 | 72 | carcinine transporter isoform X1 |
| MSTRG.3257 | -0.9088 | 1.0000 | 73 | Alpha-tocopherol transfer protein-like |
| MSTRG.21140 | -1.7618 | 1.0000 | 74 | NaN |
| MSTRG.21147 | -2.4463 | 1.0000 | 75 | 4-coumarate--CoA ligase 1 |
| MSTRG.5822 | -1.7236 | 1.0000 | 76 | NaN |
| MSTRG.21367 | 3.3807 | 1.0000 | 77 | glucose dehydrogenase [FAD, quinone]-like |
| MSTRG.16294 | 3.3230 | 1.0000 | 78 | band 7 protein AGAP004871 isoform X2 |
| MSTRG.15870 | -1.7701 | 1.0000 | 79 | (11Z)-hexadec-11-enoyl-CoA conjugase-like isoform X2 |
| MSTRG.8040 | 0.3449 | 1.0000 | 80 | pre-mRNA-splicing factor Syf2 |
| MSTRG.19005 | -3.6169 | 1.0000 | 81 | hypothetical protein WN51_02805 |
| MSTRG.13659 | -0.6371 | 1.0000 | 82 | proteasome activator complex subunit 4-like isoform X2 |
| MSTRG.10306 | -0.2700 | 1.0000 | 83 | ATP-dependent RNA helicase dbp2-like isoform X1 |
| MSTRG.4191 | -0.6581 | 1.0000 | 84 | NaN |
| MSTRG.1654 | -0.7423 | 1.0000 | 85 | sprT-like domain-containing protein Spartan |
| MSTRG.500 | 0.5563 | 1.0000 | 86 | tyrosine-protein phosphatase non-receptor type 1-like |
| MSTRG.2679 | -6.0332 | 1.0000 | 87 | NaN |
| MSTRG.20466 | -22.4594 | 0.0006 | 88 | NaN |
| MSTRG.10750 | -1.3781 | 1.0000 | 89 | hrp65 protein isoform X2 |
| MSTRG.18718 | -1.9180 | 1.0000 | 90 | WD repeat-containing protein 48 |
| MSTRG.14760 | 2.9057 | 1.0000 | 91 | uncharacterized protein LOC100743799 |
| MSTRG.15016 | 0.5677 | 1.0000 | 92 | 2-oxoglutarate-dependent dioxygenase htyE isoform X2 |
| MSTRG.19334 | -1.4883 | 1.0000 | 93 | NaN |
| MSTRG.11504 | 1.5113 | 1.0000 | 94 | NaN |
| MSTRG.13194 | 5.8735 | 0.8383 | 95 | uncharacterized protein LOC117237235 isoform X1 |
| MSTRG.2038 | -0.6181 | 1.0000 | 96 | NaN |
| MSTRG.7590 | 0.3070 | 1.0000 | 97 | transcription initiation factor TFIID subunit 6-like |
| MSTRG.6059 | -1.8860 | 1.0000 | 98 | Homogentisate 1,2-dioxygenase |
| MSTRG.15298 | 1.7787 | 1.0000 | 99 | NaN |
| MSTRG.14539 | 14.4897 | 0.1562 | 100 | NaN |
| MSTRG.3500 | -1.0535 | 1.0000 | 101 | uncharacterized protein LOC100747314 |
| MSTRG.18601 | -1.7324 | 1.0000 | 102 | synaptic vesicle membrane protein VAT-1 homolog-like |
| MSTRG.7659 | -0.3846 | 1.0000 | 103 | Protein disulfide-isomerase A6 |
| MSTRG.11065 | 1.2769 | 1.0000 | 104 | probable WRKY transcription factor protein 1 |
| MSTRG.18840 | -1.9928 | 1.0000 | 105 | ubiquitin carboxyl-terminal hydrolase 4-like isoform X3 |
| MSTRG.436 | 2.1747 | 1.0000 | 106 | forkhead box protein I1 |
| MSTRG.12434 | -1.0282 | 1.0000 | 107 | hypothetical protein WN48_10360 |
| MSTRG.9240 | -0.6025 | 1.0000 | 108 | hypoxia up-regulated protein 1 isoform X1 |
| MSTRG.12416 | -1.7447 | 1.0000 | 109 | tryptophan 2,3-dioxygenase |
| MSTRG.12777 | -1.1247 | 1.0000 | 110 | elongation of very long chain fatty acids protein AAEL008004 |
| MSTRG.19526 | 1.9357 | 1.0000 | 111 | GDP-D-glucose phosphorylase 1-like |
| MSTRG.12415 | -1.0023 | 1.0000 | 112 | tryptophan 2,3-dioxygenase |
| MSTRG.21242 | -1.1534 | 1.0000 | 113 | ATP-binding cassette sub-family G member 4 |

**Table S4.6** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.362 | -20.8330 | 0.0030 | 114 | NaN |
| MSTRG.10890 | -2.0671 | 1.0000 | 115 | protein amnionless-like |
| LOC100744673 | -2.0474 | 1.0000 | 116 | green-sensitive opsin-like isoform X2 |
| MSTRG.11307 | 0.4356 | 1.0000 | 117 | methionine aminopeptidase 1 |
| MSTRG.487 | 0.9258 | 1.0000 | 118 | NaN |
| MSTRG.4977 | -1.7477 | 1.0000 | 119 | putative glutamate receptor |
| MSTRG.4784 | 0.2956 | 1.0000 | 120 | transcription factor IIIB 90 kDa subunit isoform X1 |
| MSTRG.8818 | -1.6297 | 1.0000 | 121 | uncharacterized protein LOC100740289 isoform X3 |
| MSTRG.4093 | 0.2892 | 1.0000 | 122 | E3 ubiquitin-protein ligase Bre1 isoform X2 |
| MSTRG.5949 | -2.1720 | 1.0000 | 123 | cell division cycle and apoptosis regulator protein 1-like |
| MSTRG.435 | 1.7094 | 1.0000 | 124 | NaN |
| MSTRG.12382 | 0.5350 | 1.0000 | 125 | Putative oxidoreductase GLYR1 like protein |
| MSTRG.10145 | -1.7454 | 1.0000 | 126 | uncharacterized protein LOC117162488 |
| MSTRG.19183 | 2.8764 | 1.0000 | 127 | patronin isoform X1 |
| MSTRG.4714 | -1.6354 | 1.0000 | 128 | Sorbitol dehydrogenase |
| MSTRG.209 | -2.2832 | 1.0000 | 129 | scavenger receptor class B member 1-like |
| MSTRG.12778 | -1.5820 | 1.0000 | 130 | elongation of very long chain fatty acids protein AAEL008004 |
| MSTRG.12847 | -1.6736 | 1.0000 | 131 | uncharacterized protein LOC100643622 isoform X3 |
| MSTRG.69 | 1.4398 | 1.0000 | 132 | uncharacterized protein LOC100744309 isoform X3 |
| MSTRG.360 | -1.4387 | 1.0000 | 133 | uncharacterized protein LOC117236781 isoform X2 |
| MSTRG.4262 | -6.6602 | 0.0818 | 134 | NaN |
| MSTRG.12190 | -2.0753 | 1.0000 | 135 | apolipophorin III-like protein |
| MSTRG.20782 | -1.3227 | 1.0000 | 136 | uncharacterized protein LOC110120041 |
| MSTRG.8423 | 0.4228 | 1.0000 | 137 | WD repeat domain-containing protein 83 |
| MSTRG.9880 | 1.9316 | 1.0000 | 138 | NaN |
| MSTRG.3256 | -2.3439 | 1.0000 | 139 | NaN |
| MSTRG.18758 | 4.3435 | 1.0000 | 140 | major royal jelly protein 1 |
| MSTRG.1056 | -2.3511 | 1.0000 | 141 | NaN |

**Table S4.7**. Differentially expressed genes between infected sunflower- and uninfected sunflower-fed bees based on the DESeq2 model (FDR < 0.05). Gene descriptions based on top BLAST hits (E-value of $10^{-25}$ ) against all arthropod sequences in the NCBI non-redundant database.

| String Tie ID | Fold Change (log2) | FDR | Description |
|---|---|---|---|
| MSTRG.19477 | -29.5358 | 0.0000 | glucose dehydrogenase [FAD, quinone]-like |
| MSTRG.20478 | 24.3540 | 0.0004 | dynein beta chain, ciliary-like |
| MSTRG.20557 | 5.6167 | 0.0010 | NA |
| MSTRG.21434 | 5.6302 | 0.0010 | NA |
| MSTRG.11066 | 10.8113 | 0.0010 | probable endochitinase |
| MSTRG.9983 | -22.9533 | 0.0010 | uncharacterized protein LOC112212842 |
| MSTRG.20466 | 22.1348 | 0.0015 | NA |
| MSTRG.21535 | 5.1220 | 0.0015 | hymenoptaecin |
| MSTRG.872 | -2.2853 | 0.0033 | piggyBac transposable element-derived protein 4-like |
| MSTRG.11147 | 5.9210 | 0.0046 | alkaline phosphatase 4-like |
| MSTRG.13521 | 12.0889 | 0.0167 | trypsin alpha-3-like |
| MSTRG.21576 | 10.6097 | 0.0479 | P-loop NTP hydrolase |

**Table S4.8**. Top-ranked 80 genes that differentiate infected sunflower- and uninfected sunflower-fed bees based on an 80% correct classification rate using Machine Learning. A subset of genes from the DESeq2 model with an un-corrected p-value < 0.05 were ranked (Rank) based on information gain with respect to the treatment using the InfoGain attribute evaluator and Ranker search method in Weka. Gene descriptions based on top BLAST hits (E-value of $10^{-25}$) against all arthropod sequences in the NCBI non-redundant database.

| String Tie ID | Fold Change (log2) | FDR | Rank | Description |
|---|---|---|---|---|
| MSTRG.10385 | -1.5601 | 1.0000 | 1 | NaN |
| MSTRG.13547 | 3.6600 | 1.0000 | 2 | NaN |
| MSTRG.357 | 1.7713 | 1.0000 | 3 | hypothetical protein WN51_13669 |
| MSTRG.2188 | 1.1836 | 1.0000 | 4 | E3 ubiquitin-protein ligase ZNF598 |
| MSTRG.10693 | 2.7794 | 0.9421 | 5 | Coiled-coil domain-containing protein 13 |
| MSTRG.4069 | 2.3744 | 1.0000 | 6 | angiotensin-converting enzyme-like |
| MSTRG.21015 | 2.9713 | 0.9409 | 7 | NaN |
| MSTRG.16171 | 1.2401 | 1.0000 | 8 | NaN |
| MSTRG.9133 | -1.6655 | 1.0000 | 9 | NaN |
| MSTRG.17084 | -1.4333 | 0.8988 | 10 | Probable RNaN-directed DNaN polymerase from transposon X-element |
| MSTRG.17231 | 0.9306 | 0.8868 | 11 | uridine diphosphate glucose pyrophosphatase |
| MSTRG.21291 | -1.0694 | 1.0000 | 12 | NaN |
| MSTRG.17222 | 1.3303 | 1.0000 | 13 | dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B isoform X1 |
| MSTRG.8981 | 1.1631 | 1.0000 | 14 | glycogen phosphorylase |
| MSTRG.16822 | -1.1778 | 1.0000 | 15 | NaN |
| MSTRG.13989 | 1.6588 | 1.0000 | 16 | leucine-rich repeat-containing protein 19 isoform X1 |
| MSTRG.7691 | 1.8210 | 1.0000 | 17 | NaN |
| MSTRG.12016 | 3.3784 | 0.2777 | 18 | maltase A2 isoform X1 |
| MSTRG.17560 | 2.2373 | 0.9201 | 19 | NaN |
| MSTRG.12597 | 1.3021 | 1.0000 | 20 | NaN |
| MSTRG.20336 | -1.5421 | 0.8868 | 21 | katanin p60 ATPase-containing subunit A1-like |
| MSTRG.3573 | 2.8266 | 1.0000 | 22 | cartilage oligomeric matrix protein |

**Table S4.8** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.5135 | -0.9204 | 1.0000 | 23 | NaN |
| MSTRG.18933 | -0.5069 | 1.0000 | 24 | odorant receptor 46a-like |
| MSTRG.8881 | -2.3558 | 0.8868 | 25 | NaN |
| MSTRG.2074 | -3.3456 | 0.6639 | 26 | NaN |
| MSTRG.19080 | 1.0317 | 1.0000 | 27 | RNaN-binding protein 5-like isoform X1 |
| MSTRG.17603 | 3.4766 | 0.6639 | 28 | NaN |
| MSTRG.18344 | 1.8623 | 1.0000 | 29 | uncharacterized protein LOC117207620 |
| MSTRG.9723 | 2.3227 | 0.8988 | 30 | uncharacterized protein LOC100749322 isoform X2 |
| MSTRG.7554 | 1.3987 | 1.0000 | 31 | Low-density lipoprotein receptor-related protein 2 |
| MSTRG.14740 | -5.8033 | 0.9409 | 32 | NaN |
| MSTRG.4810 | 3.9253 | 1.0000 | 33 | NaN |
| MSTRG.707 | -1.4494 | 0.8868 | 34 | dynein heavy chain 8, axonemal |
| MSTRG.14903 | 5.0391 | 0.3783 | 35 | distal membrane-arm assembly complex protein 2 |
| MSTRG.388 | -1.1395 | 0.9895 | 36 | katanin p60 ATPase-containing subunit A1-like |
| MSTRG.20762 | -2.7651 | 0.8988 | 37 | uncharacterized protein LOC112212704 |
| MSTRG.502 | 1.2064 | 1.0000 | 38 | tyrosine-protein phosphatase non-receptor type 2 isoform X2 |
| MSTRG.3171 | -0.8400 | 1.0000 | 39 | NaN |
| MSTRG.8877 | 3.8289 | 1.0000 | 40 | NaNtterin-4-like isoform X1 |
| MSTRG.15843 | -3.1405 | 0.6639 | 41 | Transposon Tf2-9 polyprotein |
| MSTRG.20532 | 1.9928 | 0.9026 | 42 | polyubiquitin-B |
| MSTRG.7483 | 1.2665 | 1.0000 | 43 | RNaN-binding protein cabeza-like isoform X3 |
| MSTRG.19397 | -1.8033 | 1.0000 | 44 | alpha-tocopherol transfer protein-like |
| MSTRG.6121 | 1.7780 | 1.0000 | 45 | G-protein coupled receptor Mth2-like |
| MSTRG.18057 | 1.6273 | 1.0000 | 46 | NaN |
| MSTRG.8291 | -4.4958 | 0.3197 | 47 | protein hairy |
| MSTRG.4520 | 2.3034 | 1.0000 | 48 | protein FAM151B isoform X2 |
| MSTRG.13756 | -0.7557 | 1.0000 | 49 | titin isoform X4 |
| MSTRG.11824 | -1.3117 | 1.0000 | 50 | uncharacterized protein LOC117177545 |
| MSTRG.10505 | -0.9930 | 1.0000 | 51 | uncharacterized protein LOC100745137 |
| MSTRG.8298 | 1.3425 | 0.8868 | 52 | NaN |
| MSTRG.1008 | 5.0477 | 0.8825 | 53 | carbohydrate sulfotransferase 11-like |
| MSTRG.7122 | 3.1141 | 0.9501 | 54 | NaN |
| MSTRG.15344 | -2.7529 | 0.8825 | 55 | gag-pol polyprotein |
| MSTRG.4646 | 1.4957 | 1.0000 | 56 | NaN |
| MSTRG.20507 | 2.4118 | 1.0000 | 57 | uncharacterized protein LOC100750217 |
| MSTRG.7358 | -1.7270 | 0.9499 | 58 | uncharacterized protein LOC105680532 |

**Table S4.8** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.6080 | 2.3889 | 1.0000 | 59 | protein obstructor-E-like |
| MSTRG.14101 | 4.6054 | 0.3706 | 60 | uncharacterized protein LOC117233211 isoform X8 |
| MSTRG.8779 | -3.0158 | 0.8868 | 61 | gag-pol polyprotein |
| MSTRG.21481 | -1.7375 | 0.8868 | 62 | Copia protein |
| MSTRG.15256 | 1.0433 | 1.0000 | 63 | NaN |
| MSTRG.9880 | -2.0864 | 1.0000 | 64 | NaN |
| MSTRG.10022 | -1.2107 | 1.0000 | 65 | NaN |
| MSTRG.21583 | 2.5511 | 1.0000 | 66 | reverse transcriptase |
| MSTRG.5446 | 1.7112 | 1.0000 | 67 | NaN |
| MSTRG.901 | -1.1467 | 1.0000 | 68 | hypothetical protein WN48_03057 |
| MSTRG.14495 | -1.8503 | 1.0000 | 69 | uncharacterized protein LOC112212704 |
| MSTRG.1182 | -1.2470 | 1.0000 | 70 | Ly6/PLAUR domain-containing protein 6B |
| MSTRG.4982 | 2.6530 | 1.0000 | 71 | transferrin |
| MSTRG.4114 | -1.9552 | 0.9992 | 72 | monocarboxylate transporter 12 isoform X1 |
| MSTRG.18601 | 1.5015 | 1.0000 | 73 | syNaNptic vesicle membrane protein VAT-1 homolog-like |
| MSTRG.5689 | 3.4669 | 1.0000 | 74 | NaN |
| MSTRG.7747 | 1.5130 | 1.0000 | 75 | uncharacterized protein LOC100740617 isoform X5 |
| MSTRG.11803 | 2.8612 | 1.0000 | 76 | glycine receptor subunit alpha-3-like isoform X1 |
| MSTRG.11112 | -1.9007 | 0.9477 | 77 | endocuticle structural glycoprotein SgAbd-4-like |
| MSTRG.2986 | -2.0885 | 0.8868 | 78 | hypothetical protein RF55_3628 |
| MSTRG.3431 | 2.4782 | 1.0000 | 79 | NaN |
| MSTRG.13494 | 4.4347 | 0.8965 | 80 | NaN |

**Table S4.9**. Differentially expressed genes between infected wildflower- and uninfected wildflower -fed bees based on the DESeq2 model (FDR < 0.05). Gene descriptions based on top BLAST hits (E-value of $10^{-25}$ ) against all arthropod sequences in the NCBI non-redundant database.

| String Tie ID | Fold Change (log2) | FDR | Description |
|---|---|---|---|
| MSTRG.362 | -50.8282 | 0.0000 | NaN |
| MSTRG.11358 | -19.1894 | 0.0000 | glutamate receptor 3.2-like |
| MSTRG.7865 | 36.4587 | 0.0000 | ejaculatory bulb-specific protein 3-like |
| MSTRG.996 | -16.3274 | 0.0000 | NaN |
| MSTRG.15434 | 19.6215 | 0.0000 | NaN |
| LOC105680309 | -26.1608 | 0.0000 | probable ATP-dependent RNA helicase DDX46 |
| MSTRG.19495 | 17.7464 | 0.0000 | jerky protein homolog-like |
| MSTRG.12884 | -24.8345 | 0.0000 | NaN |
| MSTRG.8149 | -24.2671 | 0.0000 | uncharacterized protein LOC117204693 |
| MSTRG.9983 | 24.0341 | 0.0000 | uncharacterized protein LOC112212842 |
| MSTRG.14539 | 20.3251 | 0.0001 | NaN |
| MSTRG.19478 | -19.9946 | 0.0001 | glucose dehydrogenase [FAD, quinone]-like |
| MSTRG.18410 | -17.6068 | 0.0049 | NaN |
| MSTRG.19383 | -19.1270 | 0.0120 | digestive cysteine proteinase 1 |
| MSTRG.12886 | -18.5406 | 0.0219 | LOW QUALITY PROTEIN: uncharacterized protein LOC117236038 |
| LOC100747481 | -18.4691 | 0.0219 | LIM/homeobox protein Awh-like isoform X1 |
| MSTRG.15744 | 16.6987 | 0.0267 | uncharacterized protein LOC112213491 |

**Table S4.10**. Top-ranked 98 genes that differentiate infected wildflower- and uninfected wildflower-fed bees based on an 100% correct classification rate using Machine Learning. A subset of genes from the DESeq2 model with an un-corrected p-value < 0.05 were ranked (Rank) based on information gain with respect to the treatment using the InfoGain attribute evaluator and Ranker search method in Weka. Gene descriptions based on top BLAST hits (E-value of $10^{-25}$) against all arthropod sequences in the NCBI non-redundant database.

| String Tie ID | Fold Change (log2) | FDR | Rank | Description |
|---|---|---|---|---|
| MSTRG.2970 | -0.9920 | 1.0000 | 1 | striatin-interacting protein 1 |
| MSTRG.4529 | 1.0832 | 1.0000 | 2 | NaN |
| MSTRG.6215 | 1.0238 | 1.0000 | 3 | lachesin-like isoform X2 |
| MSTRG.2011 | 1.3924 | 1.0000 | 4 | protein BTG2-like |
| MSTRG.20954 | 0.6861 | 1.0000 | 5 | uncharacterized protein LOC117157497 isoform X2 |
| MSTRG.8475 | 1.3709 | 1.0000 | 6 | proclotting enzyme-like |
| MSTRG.19449 | 1.1841 | 1.0000 | 7 | NaN |
| MSTRG.12964 | 0.5895 | 1.0000 | 8 | NaN |
| MSTRG.15486 | -2.2736 | 0.7421 | 9 | alanine--glyoxylate aminotransferase 2-like |
| MSTRG.15384 | -0.9837 | 1.0000 | 10 | NaN |
| MSTRG.3256 | -1.9381 | 1.0000 | 11 | NaN |
| MSTRG.6710 | -1.5980 | 1.0000 | 12 | rho GTPase-activating protein 44 isoform X3 |
| MSTRG.298 | 3.7106 | 1.0000 | 13 | RNA-binding protein squid |
| MSTRG.16875 | 1.6594 | 1.0000 | 14 | elongation of very long chain fatty acids protein 6-like |
| MSTRG.4708 | 1.0848 | 0.8668 | 15 | anoctamin-4 isoform X1 |
| MSTRG.10982 | 1.5617 | 1.0000 | 16 | NaN |
| MSTRG.3512 | -2.6495 | 1.0000 | 17 | NaN |
| MSTRG.18718 | -3.3143 | 1.0000 | 18 | WD repeat-containing protein 48 |
| MSTRG.19948 | -2.1623 | 1.0000 | 19 | SH3 domain-binding protein 5 homolog |
| MSTRG.12451 | 0.8816 | 1.0000 | 20 | uncharacterized protein LOC117162246 |
| MSTRG.6794 | -1.1977 | 1.0000 | 21 | NaN |
| MSTRG.6618 | 1.1992 | 1.0000 | 22 | transposase |
| MSTRG.9655 | 1.0977 | 1.0000 | 23 | NaN |
| MSTRG.8015 | -1.8438 | 1.0000 | 24 | ras-related GTP-binding protein A |
| LOC100740223 | 2.2893 | 1.0000 | 25 | homeobox protein B-H2-like |
| MSTRG.695 | 2.7745 | 1.0000 | 26 | NaN |
| MSTRG.5529 | -1.3283 | 1.0000 | 27 | uncharacterized protein LOC102671994 |
| MSTRG.5302 | -2.6243 | 1.0000 | 28 | 40S ribosomal protein S17 |
| MSTRG.14346 | 0.7129 | 1.0000 | 29 | ATP-binding cassette sub-family G member 4-like isoform X3 |

**Table S4.10** (continued).

| | | | | |
|---|---|---|---|---|
| MSTRG.12432 | 1.1147 | 1.0000 | 30 | Zinc finger protein Xfin |
| MSTRG.16211 | -0.8547 | 1.0000 | 31 | NaN |
| MSTRG.2498 | -2.5501 | 1.0000 | 32 | HAUS augmin-like complex subunit 3 |
| MSTRG.7174 | -2.7355 | 1.0000 | 33 | NaN |
| MSTRG.8538 | -1.3196 | 1.0000 | 34 | NPC intracellular cholesterol transporter 2-like |
| MSTRG.16762 | 1.4006 | 1.0000 | 35 | hexamerin-like |
| MSTRG.4948 | -1.0546 | 1.0000 | 36 | Pupal cuticle protein C1B |
| MSTRG.3866 | -2.0170 | 1.0000 | 37 | dnaJ homolog subfamily C member 11 |
| MSTRG.15012 | 4.0038 | 1.0000 | 38 | uncharacterized protein LOC100742276 |
| MSTRG.2919 | -1.5749 | 1.0000 | 39 | mitochondrial import receptor subunit TOM20 homolog |
| MSTRG.8766 | 4.1261 | 0.7299 | 40 | balbiani ring protein 3-like |
| LOC105681602 | -1.5017 | 1.0000 | 41 | balbiani ring protein 3-like |
| MSTRG.1111 | -2.5279 | 1.0000 | 42 | NaN |
| MSTRG.12405 | -2.1173 | 1.0000 | 43 | multidrug resistance protein homolog 49 isoform X1 |
| MSTRG.17808 | -1.3422 | 1.0000 | 44 | asparagine--tRNA ligase, cytoplasmic |
| MSTRG.13109 | -1.0957 | 1.0000 | 45 | DDB1- and CUL4-associated factor 12 |
| MSTRG.14625 | -2.2510 | 1.0000 | 46 | NaN |
| MSTRG.6962 | 2.8354 | 1.0000 | 47 | NaN |
| MSTRG.7220 | -3.9030 | 1.0000 | 48 | serine/threonine-protein phosphatase 1 regulatory subunit GAC1-like |
| MSTRG.19964 | 4.2505 | 1.0000 | 49 | thyrotropin-releasing hormone-degrading ectoenzyme isoform X1 |
| MSTRG.19027 | 1.5203 | 1.0000 | 50 | uncharacterized protein C553.10-like, partial |
| MSTRG.13943 | -1.3350 | 1.0000 | 51 | reverse transcriptase |
| MSTRG.10598 | -1.7071 | 1.0000 | 52 | TBC1 domain family member 25 |
| MSTRG.15604 | -3.4310 | 1.0000 | 53 | uncharacterized protein LOC100746190 |
| MSTRG.20722 | 1.1636 | 1.0000 | 54 | Retrovirus-related Pol polyprotein from transposon TNT 1-94 |
| MSTRG.119 | 1.5195 | 1.0000 | 55 | conserved hypothetical protein |
| MSTRG.14073 | -2.7443 | 1.0000 | 56 | luciferin 4-monooxygenase-like isoform X6 |
| MSTRG.10435 | -1.5915 | 1.0000 | 57 | NaN |
| MSTRG.1380 | -2.2248 | 1.0000 | 58 | NaN |
| MSTRG.11539 | -2.6621 | 1.0000 | 59 | nuclear fragile X mental retardation-interacting protein 1-like |
| MSTRG.7048 | 2.0188 | 1.0000 | 60 | piggyBac transposable element-derived protein 4-like |
| MSTRG.8362 | -3.5896 | 1.0000 | 61 | tRNA modification GTPase GTPBP3, mitochondrial isoform X2 |
| LOC112213846 | -3.4330 | 1.0000 | 62 | Protein lozenge |
| MSTRG.20983 | 5.7743 | 1.0000 | 63 | NaN |
| MSTRG.8229 | 4.3588 | 1.0000 | 64 | hypothetical protein WN48_05001 |
| MSTRG.13254 | 1.3363 | 1.0000 | 65 | zinc finger CCHC domain-containing protein 24-like |
| LOC100747736 | 1.6070 | 1.0000 | 66 | circadian locomoter output cycles protein kaput |
| MSTRG.14055 | 2.4815 | 1.0000 | 67 | NaN |
| MSTRG.15013 | 1.9031 | 1.0000 | 68 | NaN |
| MSTRG.4355 | -0.7193 | 1.0000 | 69 | inhibitor of nuclear factor kappa-B kinase subunit epsilon |
| MSTRG.15018 | -0.2317 | 1.0000 | 70 | translation initiation factor IF-2, mitochondrial |
| MSTRG.14908 | -1.5226 | 1.0000 | 71 | NaN |

**Table S4.10** (continued).

| | | | | |
|---|---|---|---|---|
| LOC100747481 | -18.4691 | 0.0219 | 72 | LIM/homeobox protein Awh-like isoform X1 |
| MSTRG.20614 | -2.6889 | 1.0000 | 73 | mitochondrial potassium channel ATP-binding subunit |
| MSTRG.9393 | 1.3862 | 1.0000 | 74 | NaN |
| MSTRG.8499 | -0.3870 | 1.0000 | 75 | growth hormone-regulated TBC protein 1 |
| MSTRG.4751 | -0.7321 | 1.0000 | 76 | COP9 signalosome complex subunit 4 |
| MSTRG.2588 | 1.7677 | 1.0000 | 77 | NaN |
| MSTRG.3654 | 0.7925 | 1.0000 | 78 | PREDICTED: uncharacterized protein LOC105662916 |
| MSTRG.8149 | -24.2671 | 0.0000 | 79 | uncharacterized protein LOC117204693 |
| MSTRG.6434 | 0.9958 | 1.0000 | 80 | ribose-phosphate pyrophosphokinase 1 isoform X2 |
| MSTRG.16362 | -0.6239 | 1.0000 | 81 | putative inorganic phosphate cotransporter |
| MSTRG.1744 | 0.9534 | 1.0000 | 82 | NaN |
| MSTRG.1112 | 3.0768 | 1.0000 | 83 | cytochrome P450 307a1-like |
| MSTRG.8141 | 1.1538 | 1.0000 | 84 | NaN |
| MSTRG.10228 | 2.8567 | 1.0000 | 85 | NaN |
| MSTRG.2597 | -0.2316 | 1.0000 | 86 | conserved oligomeric Golgi complex subunit 5 |
| MSTRG.19478 | -19.9946 | 0.0001 | 87 | glucose dehydrogenase [FAD, quinone]-like |
| MSTRG.435 | 1.7020 | 1.0000 | 88 | NaN |
| MSTRG.2368 | -0.8815 | 1.0000 | 89 | NaN |
| MSTRG.15256 | -0.9675 | 1.0000 | 90 | NaN |
| MSTRG.21259 | 2.0608 | 1.0000 | 91 | putative uncharacterized protein DDB_G0289041 |
| MSTRG.6137 | 0.9310 | 1.0000 | 92 | aquaporin AQPAn.G isoform X3 |
| MSTRG.2534 | -1.2250 | 1.0000 | 93 | RNA-directed DNA polymerase from mobile element jockey-like |
| MSTRG.16044 | -2.4720 | 1.0000 | 94 | uncharacterized protein LOC105681292 |
| MSTRG.1526 | 1.8967 | 1.0000 | 95 | NaN |
| MSTRG.2166 | -1.2922 | 1.0000 | 96 | uncharacterized protein LOC100740206 |
| MSTRG.10306 | -0.3938 | 1.0000 | 97 | ATP-dependent RNA helicase dbp2-like isoform X1 |
| MSTRG.868 | 1.5115 | 1.0000 | 98 | Mitogen-activated protein kinase kinase kinase 15 |

**Table S.4.11**. Final sample sizes for **Timing of sunflower pollen effect** pilot experiment. Numeric values indicate the number of bees that consumed a net positive amount of pollen per each pollen type (sunflower or wildflower) per each timing treatment (24H, 48H, 72H, 96H and 168H).

| POLLEN TYPE | 24H | 48H | 72H | 96H | 168H |
|---|---|---|---|---|---|
| **SUNFLOWER** | 5 | 7 | 10 | 10 | 13 |
| **WILDFLOWER** | 5 | 4 | 10 | 10 | 13 |