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Research

Preinfection Effects of Nectar Secondary Compounds on a Bumble Bee Gut Pathogen

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Abstract

Bumble bee pollinators can be exposed to pathogens when foraging on flowers previously visited by infected individuals. Infectious cells may be deposited in floral nectar, providing a site for pathogens to interact with nectar secondary compounds prior to infecting bees. Some nectar secondary compounds can reduce pathogen counts in infected bumble bees, but we know less about how exposure to these compounds directly affects pathogens prior to being ingested by their host. We exposed the trypanosomatid gut pathogen, *Crithidia bombi* (Lipa & Triggiani 1988) (Trypanosomatida:Trypanosomatidae), to six different compounds found in nectar (aucubin, catalpol, nicotine, thymol, anabasine, and citric acid) for 1-h prior to ingestion by *Bombus impatiens* (Cresson 1863) (Hymenoptera: Apidae) workers that were then reared for 1 wk on a control diet. All of these compounds except citric acid reduce pathogen levels when consumed in hosts after infection, and citric acid is a common preservative found in citrus fruits and some honeys. We found that both citric acid and aucubin reduced *Crithidia* cell counts compared with controls. However, catalpol, nicotine, thymol, and anabasine did not have significant effects on *Crithidia* levels. These results suggest that *Crithidia* exposure in some floral nectars may reduce cell viability, resulting in a lower risk to visiting pollinators, but this effect may not be widespread across all flowering species.

Key words: bee disease, floral traits, pollinator decline, secondary metabolites

Insects provide critical ecosystem services by pollinating crops and are the primary pollinators of many native flowering species (Willmer 2011). Loss of pollinators may spur local extinctions of flowering plants (Memmott et al. 2004), decreasing diversity in vulnerable habitats (Goulson et al. 2008). Bees in particular are important pollinators at a global scale, including both managed bees, such as bumble and honey bees, as well as wild bees (Potts et al. 2010). However, several bumble bee species have suffered significant declines in recent years due to a range of environmental and physiological challenges (Cameron et al. 2011). Although declines are likely due to an array of factors (Goulson et al. 2015), pathogens can increase individual and colony stress (Brown et al. 2000, 2003; Schmid-Hempel 2001), are associated with reduced reproduction in wild colonies (Goulson et al. 2017), and have been implicated in the decline of some bumble bee species (Cameron et al. 2011, 2016; Schmid-Hempel et al. 2014).

Shared flower use by foraging bees provides a mechanism for the spread of pathogens within and between colonies (Durrer and Schmid-Hempel 1994, Graystock et al. 2015). Social bees such as bumble bees (genus *Bombus (Hymenoptera: Apidae)*) are highly efficient foragers, with workers observed visiting 13.9 and 17.6 flowers per minute in two *Bombus* species (Aizen et al. 2011). Frequently visited flower resources could provide 'hotspots' for communicable pathogen transmission, resulting in the potential for widespread colony infections (Graystock et al. 2015). For example, foraging by uninfected *Bombus lucorum* (Linnaeus 1761) (Hymenoptera: Apidae) workers on contaminated flowers resulted in 39% of workers becoming infected by the trypanosomatid gut parasite, *Crithidia bombi* (Lipa & Triggiani 1988) (Trypanosomatida: Trypanosomatidae) (Durrer and Schmid-Hempel 1994). Similarly, shared flower use by *Apis* (Hymenoptera: Apidae) and *Bombus* species facilitated interspecific transmission of three pathogens (Graystock et al. 2015). Flowering plant species can vary in their capacity to act as transmission sites, with some species increasing the likelihood or severity of infection more than others (Durrer and Schmid-Hempel 1994, Graystock et al. 2015, Adler et al. 2018). For example, bees foraging on flowering species with more reproductive structures per inflorescence had up to four-fold higher pathogen cell counts than flowering species with fewer reproductive structures (Adler et al. 2018). These differences suggest that floral traits play a role in mediating bee pathogen transmission, but the mechanisms behind such trait-mediated effects are largely unexplored (McArt et al. 2014).

Since flowers are sites of both pathogen and resource acquisition, it is crucial to understand the role that floral traits, including nectar chemistry, play in the dynamics of bumble bee pathogen transmission. Floral nectar often contains secondary metabolites with antiherbivory, antibiotic, or antifungal capabilities (Adler 2000, Heil 2011, McArt et al. 2014, Palmer-Young et al. 2018). Multiple manipulative studies have demonstrated that secondary metabolites in floral nectar can reduce *Crithidia* infections of *Bombus* spp. when consumed after bees are infected, although effects are not always consistent (Manson et al. 2010, Baracchi et al. 2015, Biller et al. 2015, Richardson et al. 2015, Thorburn et al. 2015). When mixed in sucrose solutions at ecologically relevant concentrations, thymol, anabasine, nicotine, and catalpol significantly reduced *Crithidia* infections in vivo in *Bombus impatiens* (Cresson 1863) (Hymenoptera: Apidae), and aucubin marginally reduced infection (Richardson et al. 2015). These results indicate the potential for secondary metabolites in nectar to reduce *Crithidia* post-infection.

Although nectar secondary metabolites may reduce pathogen levels post-infection (exposure after infection occurs), we know less about whether pre-infection exposure to secondary metabolites (exposure of *Crithidia* to secondary metabolites prior to ingestion and outside the bee gut) reduces the ability of *Crithidia* to infect bumble bees. Bumble bees can defecate during foraging, with the potential to deposit feces in floral nectar. For example, when bumble bees foraged on *Lythrum salicaria* L. (Myrtales: Lythraceae), a flowering species with short corolla tubes and a relatively flat corolla surface, 46% of fecal droplets were deposited inside flowers (Figueroa et al., unpublished data). In cases where feces are deposited in flowers, *Crithidia* cells may be exposed to secondary metabolites by mixing with nectar before consumption by pollinators. Secondary metabolites were variable in their ability to reduce *Crithidia* cell levels when cultured in vitro (Palmer-Young et al. 2016, Palmer-Young and Thursfield 2017), indicating that naturally occurring levels of some secondary compounds may be more effective than others at reducing pathogens outside the bee gut. Only two studies to our knowledge have examined how pre-exposure to nectar secondary compounds affects *Crithidia* ability to infect hosts. *Crithidia* pre-infection exposure to the alkaloids nicotine (Baracchi et al. 2015) and gelsemine (Manson et al. 2010) did not reduce infection at compound concentrations that were effective post-infection. However, compounds such as thymol and anabasine, which both reduced *Crithidia* growth in vitro (Palmer-Young et al. 2016), have never been assessed. More broadly, floral nectar contains dozens of secondary compounds across many compound classes (Palmer-Young et al. 2018), suggesting the need for a more extensive assessment of the potential for nectar secondary metabolites to reduce *Crithidia* before consumption by bumble bees.

To assess the potential pre-infection effects of nectar secondary metabolites, *Crithidia* cells were exposed to six secondary metabolites (thymol, anabasine, nicotine, catalpol, aucubin, or citric acid) prior to ingestion by uninfected *B. impatiens* workers. All of these compounds except citric acid had been shown to reduce *Crithidia* cell levels post-infection in previous work. Citric acid is present in chestnut and pine honey (Tezcan et al. 2011) and is a common preservative used in several honey bee pollen supplement recipes (Burns 2015). After 1 h of exposure to these secondary metabolites in a sucrose solution, *Crithidia* cells were used to infect *B. impatiens* workers that were then reared on a control diet for 1 wk before assessing infection levels. We predicted that subsequent *Crithidia* infections after 1 wk would be lower in workers fed *Crithidia* solutions exposed to secondary compounds than workers exposed to control solutions. A reduction in *Crithidia* cell levels would indicate floral secondary metabolites may mitigate the spread of pathogens by reducing pollinator exposure to infectious cells deposited in nectar.

Materials and Methods

Study System

Bombus impatiens, the common eastern bumble bee, is a native and abundant pollinator in the northeastern United States. Populations in western Massachusetts can be widely infected with the trypanosomatid intestinal parasite, *C. bombi*, with over 80% of bumble bees infected in one site in Massachusetts (Gillespie 2010). In wild nest sites of *Bombus terrestris* (Linnaeus 1758) (Hymenoptera: Apidae) in the United Kingdom, 49% of sampled workers were infected with *Crithidia* and all sites had at least one infected worker (Goulson et al. 2017). Although relatively benign under favorable nutritional conditions, *Crithidia* can increase worker mortality and reduce queen overwintering success, colony size, and male reproduction in nutrient-limited conditions (Brown et al. 2000, 2003; Schmid-Hempel 2001; Goulson et al. 2017). Moreover, wild colonies infected with *Crithidia* have a lower likelihood of producing new gynes, indicating wild infections can impact colony reproduction (Goulson et al. 2017). *Crithidia* cells are transmitted within colonies through fecal contamination (Schmid-Hempel 2001) or between individuals of different colonies during foraging (Durrer and Schmid-Hempel 1994).

We examined the individual effects of six different secondary metabolites: anabasine and nicotine, alkaloids from *Nicotiana* (Solanales: Solanaceae) nectar (Tadmor-Melamed et al. 2004, Adler et al. 2012), the terpenoid thymol from *Tilia* spp. honey (Malvales: Malvaceae) (Guyot et al. 1998) and *Thymus vulgaris* L. (Lamiales: Lamiaceae) nectar (Palmer-Young et al. 2016), the iridoid glycosides aucubin and catalpol from *Chelone glabra* L. (Lamiales: Plantaginaceae) nectar (Richardson et al. 2016), and citric acid from chestnut and pine honey (Tezcan et al. 2011).

Experimental Design

To assess the effects of pre-infection exposure to nectar secondary metabolites on *Crithidia* infection in *B. impatiens*, we conducted three experiments from December 2013 through April 2014 that each included two different secondary metabolite treatments and a control, for a total of six compounds tested (all from Sigma–Aldrich, St. Louis, MO). The first experiment included thymol (0.2 ppm; CAS no. 89-83-8) and anabasine (5 ppm; (+/–)-anabasine CAS no. 13078-04-1); the second included nicotine (2 ppm; (–)-nicotine-free base CAS no. 54-11-5) and catalpol (1417 ppm, CAS no. 2415-24-9), and the third included aucubin (1,600 ppm; CAS no. 479-98-1; Fluka) and citric acid (19,212 ppm; CAS no. 77-92-9). Apart from citric acid, these concentrations were chosen to match those used in a previous study of post-infection effects and were typically at the high end of the natural range (Richardson et al. 2015). We note that work conducted after this experiment found much higher thymol nectar concentrations than previously recorded (4.5–22 ppm; Palmer-Young et al. 2016), although only 0.2 ppm thymol was sufficient to reduce *Crithidia* growth post-infection in one experiment (Richardson et al. 2015) but not another (Biller et al. 2015). Thus, our test of thymol effects is conservative. For citric acid, we used a concentration within the range found in fresh fruit juice (9,100–48,000 ppm; Penniston et al. 2008), which we note is higher than that recorded for honey (chestnut and pine derived honey: 78.9–465 ppm; Tezcan et al. 2011).

Each experiment included callow (newly emerged) worker bees from at least three experimental *B. impatiens* colonies (BioBest LTD,

Leamington, Ontario, Canada) that were confirmed to be *Crithidia*-free via weekly dissections of five bees. Pupal clumps were harvested from each colony weekly, maintained in 473-ml deli cups in darkness at 28°C, and checked daily for callow emergence. We used callow bees as a precaution to avoid potential contamination with *Crithidia* due to contact with nest mates; however, we acknowledge that callows may also not obtain gut microbiota from nest mates that could provide resistance to *Crithidia* (Koch and Schmid-Hempel 2011). As bees emerged, they were individually placed in 18.5-ml plastic scintillation vials with 500 ml of 30% sucrose solution provided via a cotton dental wick, and with approximately 0.1–0.2 g of honey bee-collected wildflower pollen (Koppert Biological Systems, Howell, MI), mixed with 30% sucrose. Pollen was not tested for pathogens or viruses, but all bees were fed the same pollen, and *Crithidia* is not viable upon desiccation and so would not persist in the pollen (Figueroa et al., in preparation). Experimental bees were maintained at room temperature on a laboratory bench exposed to natural light from windows, and vials, pollen, and sucrose solutions were replaced daily. Callow bees were assigned to treatments as they emerged, alternating between treatments within colony. Separate colonies were used for each experiment, with three colonies each for the thymol/anabasine and aucubin/citric acid experiment and seven colonies for the catalpol/nicotine experiment. Callows emerged on 26 dates for the thymol/anabasine experiment between 5 December 2013 and 17 January 2014, on 43 dates for the nicotine/catalpol experiment between February 5 and 18 April 2014, and on 19 dates for the citric acid/aucubin experiment between March 14 and 11 April 2014. Bees were inoculated 2 d after emergence and were dissected to evaluate *Crithidia* cell counts 7 d after inoculation, when they have reached a representative level of infection (Otterstatter and Thomson 2006). We used *Crithidia* cell counts per 0.02 µl as our response, evaluated using the method described below for making inoculum. In a separate study, we demonstrated that the mean proportional error in *Crithidia* counts did not decrease with sample volume up to 0.1 µl, the largest volume we assessed, and that repeated samples from the same bee produced similar results (Supp. Material [online only]). The length of the radial cell of the right forewing was measured to use as a covariate of bee size (Harder 1982). We also recorded mortality as an additional response, including only bees that had died after they received their inoculation treatment.

To create fresh inoculum for treatments, we dissected 10 bees daily from commercial source colonies that had originally been infected with *Crithidia* from local, wild *B. impatiens* collected from two sites (42°23′20″N 72°31′21″W and 42°24′31″N 72°31′43″W). We typically selected two to three bees with the highest counts to make inoculum. Guts from dissected bees were placed in 1.5-ml Eppendorf tubes, ground in 300-µl distilled water, vortexed briefly, and allowed to settle for 4 h at room temperature. We then placed a 10-µl sample of the supernatant onto a hemocytometer and counted moving *Crithidia* cells from the four corners and central square of the hemocytometer grid, a total of 0.02 µl volume, using a compound light microscope at 40× magnification. We used 150-µl samples from one to three bees to make a mixture diluted with distilled water to obtain a solution with 1,200 *Crithidia* cells/µl.

The diluted gut solution was then divided into three portions to comprise the three treatments for each experiment. One portion was mixed for 1 h with an equal amount of 50% sucrose solution as the control treatment. Because we tested multiple compounds that came from many different plant species, there was no single exposure period that would reflect the time period between deposition of *Crithidia* cells by one bee and the next flower visit by another bee for all plant species. Instead, we chose a 1-h exposure time to follow the average exposure time in Manson et al. (2010), the only other study we are aware of to expose *Crithidia* cells to nectar secondary compounds prior to infection. This study used no exposure, 1 h, or 2 h of exposure to the nectar alkaloid gelsemine to simulate natural delays in visitation by pollinators to the same flower (Manson et al. 2010).

The other two portions of *Crithidia* inoculum were mixed with 50% sucrose solution with one of the two treatment compounds in that experiment, each at twice the desired concentration (e.g., for the thymol treatment, we mixed the gut solution with 50% sucrose with 0.4 ppm thymol to result in a solution with 0.2 ppm thymol), to prepare final inoculum with 600 *Crithidia* cells/µl in 25% sucrose at the desired final secondary compound concentration. After 1 h, the resulting solutions were fed to experimental bees that had been starved for at least 2 h to motivate feeding. Bees that did not consume the entire droplet within 5 min were discarded. Ultimately, sample sizes were 110 bees in the aucubin/citric acid experiment that survived for *Crithidia* counts (38 aucubin, 35 citric acid, 35 control, excluding 72 that died prior to counting or were missing a radial cell length measurement), 212 bees in the nicotine/catalpol experiment (68 nicotine, 72 catalpol, 72 control, plus 91 that died or were missing a radial cell length measurement), and 109 bees in the thymol/anabasine experiment (41 thymol, 35 anabasine, 33 control, excluding 23 that died or were missing a radial cell length measurement).

Statistical Analysis

For all three experiments, we analyzed *Crithidia* cell counts per 0.02 µl using generalized linear mixed models with Poisson error distribution, log link function, and observation-level random factor to account for overdispersion, using glmer() in the lme4 package (Bates et al. 2015). Fixed factors were treatment (control and two secondary metabolites), radial cell length to control for bee size, and colony from which each bee was obtained (three experimental colonies per experiment). Inoculation date and colony by inoculation date were each treated as random effects in a random intercept model, although the colony by interaction date term was removed from all models because it did not improve model fit ($\chi^2 < 0.02$, $P > 0.9$ in all cases). Factors were tested using likelihood ratio tests comparing models with and without the factor included. Significant treatment effects were followed by Tukey contrasts to compare treatment levels using glht() in the multcomp package (Hothorn et al. 2008).

We analyzed bee death using generalized linear mixed effect models with binomial error structure and logit link function using glmer() in the R package lme4 (Bates et al. 2015). Treatment was a fixed factor, and colony was treated as a random intercept. Models with and without treatment were compared with likelihood ratio tests. We then analyzed time to death using Cox proportional hazard models in the survival package (Therneau 2015). Treatment was an independent variable, and we examined models both with and without colony included as a covariate, although the inclusion of colony did not qualitatively change results.

Results

Across all experiments and treatments, *Crithidia* grew rapidly in infected bees, reaching cell counts of 20.5 ± 2.8, 39.6 ± 4.0, and 41.1 ± 6.1 cells per 0.02 µl for the aucubin/citric acid, nicotine/catalpol, and thymol/anabasine experiments, respectively (mean ± SE, averaged across treatments within experiment). *Crithidia* exposure to aucubin and citric acid for one hour prior to infecting bumble bee workers significantly reduced *Crithidia* cell counts in infected bees ($\chi^2 = 9.80$, $P = 0.007$). Aucubin and citric acid treatments resulted in 62 and

68% lower counts on average, respectively, than the control counts (z = 2.51, P = 0.033 and z = 2.88, P = 0.011, respectively; Fig. 1A). The effect of aucubin and citric acid on *Crithidia* cell counts did not differ from each other (z = 0.43, P = 0.905; Fig. 1A). The covariates radial cell length (χ^2 = 1.32, P = 0.125) and colony (χ^2 = 5.70, P = 0.06) were not significant, but the random effect of inoculation date was (χ^2 = 6.69, P = 0.01). However, exposing *Crithidia* to nicotine and catalpol (χ^2 = 2.61, P = 0.271; Fig. 1B) or thymol and anabasine (χ^2 = 0.69, P = 0.707; Fig. 1C) had no effect on *Crithidia* cell counts in infected bees. In both the nicotine/catalpol experiment and thymol/anabasine experiment, neither radial cell length nor colony were significant covariates (all P > 0.1), but inoculation date was significant (P < 0.001 in both cases). In all experiments, treatments had no effect on the probability of death (binomial model; df = 2, χ^2 < 0.75, P > 0.6 for all experiments) or on death including time to death, with or without colony included (Cox proportional hazards model, Wald z-tests, P > 0.3 for all).

Discussion

The effect of pre-infection exposure of *Crithidia* to nectar secondary compounds on subsequent *B. impatiens* worker infections depended on the compound. When *Crithidia* was exposed to aucubin and citric acid for 1 h prior to bee consumption, infections were reduced by 62 and 68%, respectively, compared with infections of workers inoculated with control *Crithidia* solutions. These reductions were surprising considering that aucubin did not significantly reduce *Crithidia* infections when tested post-infection (Richardson et al. 2015). Interestingly, aucubin is at higher concentrations than catalpol in *Chelone glabra* nectar, but catalpol concentrations are higher than aucubin in leaf and corolla tissue (Richardson et al. 2016). In nature, *Crithidia* exposure to nectar compounds likely occurs from fecal deposition of cells within flowers during foraging (Figueroa et al., unpublished data). In laboratory conditions, *B. terrestris* workers avoided artificial flowers that were contaminated with *Crithidia* (Fouks and Lattorff 2011). Avoiding *Crithidia* exposure while foraging may increase visits to plants with secondary compounds that reduce *Crithidia* viability, providing reproductive benefits to those plants. For example, *C. glabra* inflorescences with experimentally increased concentrations of aucubin and catalpol had longer visits and more return visits by infected bumble bees, resulting in higher estimates of male plant fitness (Richardson et al. 2016). If aucubin but not catalpol can reduce *Crithidia* viability within floral nectar, then we speculate that it may be advantageous for *C. glabra* to produce higher nectar aucubin concentrations to attract pollinators (Richardson et al. 2016). Further exploration of reproductive success of flowering species with nectar secondary compounds effective at reducing *Crithidia* viability should be conducted to examine these potential interactions.

We also found that pre-infection exposure to citric acid reduced *Crithidia* infection. Citric acid is sometimes used to reduce the pH of artificial nectar solutions fed to *Apis mellifera* (Linnaeus 1758) (Hymenoptera: Apidae) (Brighenti et al. 2017) as well as in commercial food production (Penniston et al. 2008). It is naturally occurring at high concentrations (up to 48,000 ppm) in citrus fruit juice (Penniston et al. 2008) and has been found at much lower concentrations in *Apis*-collected honey from chestnut and pine trees (78.9–465 ppm; Tezcan et al. 2011). Although concentrations in nectar are likely not as high as that used in our experiment (19,212 ppm), the observed reduction suggests citric acid could be effective as a potential additive to commercial bumble bee supplemental nectar. Future

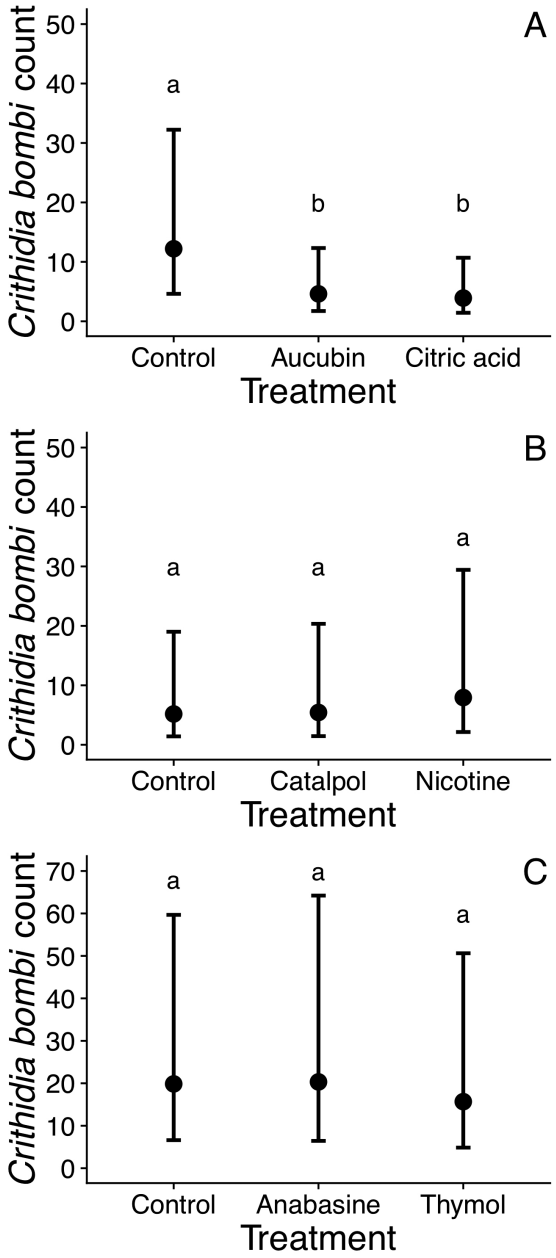

Fig. 1. Mean *Crithidia* cell count per 0.02-µl diluted hind gut sample when exposed to secondary compounds versus a sucrose solution control. Error bars indicate ± 95% CIs. Different letters above the means indicate significantly different means within each panel. Each panel depicts a separate experiment comparing (A) aucubin and citric acid, (B) catalpol and nicotine, or (C) anabasine and thymol against their respective control treatments.

work should determine if citric acid has similar pre- or post-infection effects at ecologically relevant concentrations, which would suggest that plant species with citric acid in nectar could be food resources with lower forager risk of contracting *Crithidia* infections. Further evaluation of post-infection effects on *Crithidia* infections in *B. impatiens* is a logical next step in determining which concentrations may reduce *Crithidia* infections at levels that are safe for bumble bee consumption.

In contrast to the significant effects of citric acid and aucubin, the four compounds that significantly reduced *Crithidia* infections post-infection in Richardson et al. (2015), anabasine, nicotine, catalpol,

and thymol, did not reduce infection when exposed to *Crithidia* pre-infection. A similar result was found for the nectar alkaloid gelsemine, in which post-infection exposure reduced *Crithidia* infections, but pre-infection *Crithidia* exposure to the same alkaloid concentration did not (Manson et al. 2010). This suggests that some compounds may not reduce *Crithidia* viability outside of the bee gut, but instead have indirect effects on *Crithidia* mediated through the host via a wide range of potential mechanisms. These mechanisms include upregulation of the bee's immune response, which is involved in protecting against a wide array of parasites (Schmid-Hempel 2005). For example, consumption of p-coumaric acid, a monomer of sporopollenin that is a principle component of pollen cell walls, upregulated honey bee antimicrobial peptide genes involved in parasite resistance (Mao et al. 2011). Gut microbiota can also be responsible for specific immune phenotypes (Koch and Schmid-Hempel 2012), and diet can alter bumble bee gut microbiota (Billiet et al. 2016). Additionally, secondary metabolites may increase excretion rates as a potential flushing mechanism to remove pathogen cells post-infection (Wink and Theile 2002, Tadmor-Melamed et al. 2004). Although the effects of secondary compounds on bee immune function, the gut environment and excretion rates are largely unknown, any of these mechanisms could make the host environment less tolerable for *Crithidia* cells after infection but would not influence pre-infection *Crithidia* viability.

Crithidia response to secondary compounds may also depend on strain susceptibility (Palmer-Young et al. 2016). We utilized *Crithidia* originating from the same source over the course of the three experiments, but it is possible that our genotypes may have had a higher tolerance to anabasine, thymol, nicotine, and catalpol than to aucubin or citric acid. It is notable that anabasine, nicotine, and thymol have all exhibited variable post-infection effects (Baracchi et al. 2015, Biller et al. 2015, Thorburn et al. 2015), that were inconsistent with the findings of Richardson et al. (2015). Inconsistency in *Crithidia* responsiveness to compounds due to variation in strain susceptibility may have consequently played a role in our result that *Crithidia* infection was reduced in response to exposure to citric acid and aucubin but not thymol, anabasine, nicotine, or catalpol. Examination of multiple *Crithidia* strains may reveal variation in interactions with plant secondary compounds and bee physiological responses, providing a more complete understanding of the diversity present within wild *Crithidia* populations.

Crithidia strain susceptibility may also have been related to the timing of pathogen exposure to secondary metabolites. *Crithidia* were exposed 1 h prior to worker inoculation to be consistent with the methodology of Manson et al. (2010) that compared gelsemine pre- and post-infection effects on infection. One hour was chosen to reflect natural breaks in pollinator visitation, but Manson et al. (2010) also tested 2-h pre-infection exposure and found that neither time frame had a significant effect on subsequent pathogen levels for gelsemine. It is possible that 1 h was not sufficient exposure for anabasine, thymol, nicotine, and catalpol to be effective at reducing *Crithidia* viability and that more significant reductions could have been observed with longer exposure time. However, we found in other work that *Crithidia* was not viable for >3 h on flowers of several species due to desiccation (Figueroa et al., unpublished data), suggesting that 3 h is the maximum ecologically relevant exposure time. Within this range, timing of pre-infection reductions in *Crithidia* viability may be compound specific, and the relevant exposure time (i.e., time between when *Crithidia* is likely to be deposited by one bee and consumed by another) may vary substantially depending on plant species and pollinator behavior. This relationship should be evaluated using plant species-specific timing of floral longevity and nectar availability of species containing potentially medicinal secondary metabolites.

Evaluating the role of timing of pathogen exposure to secondary compounds provides a more complete understanding of how floral chemistry influences pollinator disease acquisition and transmission. These interactions are not always consistent across nectar secondary compounds, and our study indicates the sometimes unpredictable ways that floral chemistry may mediate interactions between floral visitors and their pathogens. Ultimately, a comprehensive understanding of floral chemistry effects on pathogens will need to encompass both preinfection effects of nectar secondary compounds on pathogen viability and postinfection impacts within the host gut environment.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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Data Availability Statement

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.4gg142s (Michaud et al. 2019).

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