HERBIVORY AND PHENOLOGY SHAPE FLORAL REWARDS AND POLLINATOR- PATHOGEN INTERACTIONS

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Abstract-Herbivory influences plant-pollinator interactions by modifying floral traits and pollinator preferences, which can affect plant reproduction. However, effects of herbivory on pollinators beyond the plant-pollinator interaction are largely unknown. We assessed effects of leaf herbivory on nectar and pollen secondary chemistry in *Nicotiana tabacum*, and how herbivory-induced changes in nectar and pollen affect pollinator-pathogen interactions. We hypothesized that herbivory would induce higher concentrations of alkaloids in pollen and nectar, which would reduce pathogen levels in infected bees. We collected nectar and pollen and measured nicotine and anabasine concentrations from plants exposed to herbivory (i.e. damaged) compared to undamaged controls, from three collection periods (early, mid and late). Nectar and pollen were used for chemical analysis and for bioassays with bumblebees (*Bombus impatiens*) infected with the gut pathogen *Crithidia* to assess impacts on pathogen infection. We did not find alkaloids in nectar, and leaf damage did not alter the effect of nectar on *Crithidia* counts. In pollen, herbivory induced higher concentrations of anabasine but not nicotine, and alkaloid concentrations rose and then fell as a function of phenology. Within undamaged pollen treatments, bees fed pollen collected late had *Crithidia* counts 12 times lower than bees fed pollen collected early. Similarly, within pollen collected late, bees fed pollen from undamaged plants had 15 times less *Crithidia* than bees fed pollen from damaged plants, the opposite of our prediction. Our results emphasize the role herbivores can have in shaping floral reward chemistry that can modify interactions between pollinators and their pathogens.

Key Words-Multitrophic interactions, *Bombus impatiens*, *Crithidia*, Floral chemistry, Pollinators.

INTRODUCTION

Plant defenses modify interactions between plants and other organisms, and induced defenses in response to herbivory can mediate interactions over three or even four trophic levels (Harvey et al. 2003). For example, when the root herbivore *Delia radicum* damages *Brassica nigra*, induced defenses reduce not only the performance of the aboveground herbivore *Pieris brassicae*, but also performance of the herbivore's parasitoids and hyperparasitoids (Soler et al. 2005). Although such multitrophic effects are typically studied in herbivores, bottom-up multitrophic effects also have the potential to affect interactions with mutualists, such as pollinators, and their natural enemies.

Herbivory can shape interactions between plants and pollinators, but it is not currently known whether or how herbivore-induced changes in plant traits affect pollinator health and fitness. To date, studies have focused almost exclusively on how herbivory changes floral traits that affect pollinator behavior and plant reproduction. For

example, herbivory alters flower number (Lehtilä and Strauss 1997; Mothershead and Marquis 2000), morphology (Hoffmeister et al. 2016; Lehtilä and Strauss 1997; Strauss et al. 1996), phenology (Hanley and Fegan 2007; Hoffmeister et al. 2016; Jordan et al. 2015) and scent (Bruinsma et al. 2014; Kessler and Halitschke 2009; Kessler et al. 2011; Schiestl et al. 2014; Theis et al. 2009). Similarly, herbivory induces changes in floral reward quality and quantity (Bruinsma et al. 2014; Krupnick et al. 1999; Lehtilä and Strauss 1999). In turn, herbivore-induced changes in floral traits affect pollinator behavior and ultimately plant reproduction (Barber et al. 2011; Kessler et al. 2011; Poveda et al. 2003; Poveda et al. 2005). To understand how herbivory affects pollinators past the immediacy of a pollination event, we must investigate how changes in floral rewards affect pollinator performance after visiting plants.

Herbivory-induced changes in plant vegetative chemistry are well studied (Kaplan et al. 2008; Karban and Baldwin 1997), but few studies have examined changes in floral tissues or rewards. Leaf damage induces changes in the concentration of secondary compounds in flowers (Euler and Baldwin 1996; McArt et al. 2013; McCall and Karban 2006) and the composition of floral volatile organic compound (VOC) emissions (Bruinsma et al. 2014; Kessler and Halitschke 2009; Kessler et al. 2011; Schiestl et al. 2014; Theis et al. 2009). A few studies have shown that concentrations of leaf and nectar or pollen secondary compounds are correlated (Adler et al. 2006; Adler et al. 2012; Kessler and Halitschke 2009; but see Manson et al. 2012) and some have found induced secondary compounds in nectar following leaf or flower damage (Adler et al. 2006; Halpern et al. 2010; Kaczorowski et al. 2014), suggesting that herbivory could lead to changes in pollen chemistry. However, to our knowledge no one has assessed whether pollen chemistry changes in response to herbivory. Given that nectar and pollen are the major sources of nutrients for many pollinators, a better understanding of how herbivory affects floral reward chemistry is needed.

Pollen is a particularly important resource for pollinators that has been understudied relative to nectar. This is surprising since pollen is the major source of protein and lipids for pollen-collecting pollinators and is thus critical for reproduction and survival (Roulston and Cane 2000). In addition to macronutrients, pollen and nectar also contain a myriad of secondary compounds. The presence of secondary compounds in nectar is puzzling given its sole role as a pollinator reward (Adler 2000) but nonetheless this occurs commonly (Palmer- Young et al. 2019; Stevenson et al. 2017). Pollen, on the other hand, is predicted to be more highly defended than nectar because it is the plant's male gamete and therefore tied to reproductive success (McKey 1974). This prediction of the optimal

defense theory is corroborated by recent studies showing higher concentrations and diversity of secondary compounds in pollen than nectar (Cook et al. 2013; Palmer- Young et al. 2019). Considering the importance of pollen for pollinator fitness, further studies are needed to understand how pollen secondary chemistry can be shaped by biotic factors such as herbivory.

In addition to directly affecting pollinator survival or reproduction, floral rewards can shape pollinator interactions with pathogens. Pollinators, like other herbivores, must cope with secondary compounds ingested through their diet (Irwin et al. 2014); if toxic, these compounds may make pollinators more susceptible to their natural enemies. On the other hand, secondary compounds could reduce pathogen infection by increasing host resistance or being directly toxic to pathogens (Stevenson et al. 2017). Thus far, a few studies have evaluated how secondary compounds in nectar affect pollinator pathogens (Anthony et al. 2015; Baracchi et al. 2015; Koch et al. 2019; Manson et al. 2010; Richardson et al. 2015). For instance, Richardson et al. (2015) tested the effects of eight nectar secondary compounds on the levels of *Crithidia*, a common gut endoparasite, in bumblebees and found that half of those compounds reduced infections, but other studies found that effects can be context-dependent (Palmer-Young et al. 2016; Thorburn et al. 2015). Moreover, studies so far have tested secondary compounds at the concentrations found in nectar, but pollinators could be exposed to higher concentrations via pollen consumption (Cook et al. 2013; Palmer- Young et al. 2019). For instance, certain types of pollen, such as sunflower (*Helianthus annuus*), can reduce pollinator pathogens more dramatically than nectar secondary compounds have (Giacomini et al. 2018; LoCascio et al. 2019).

Plant defenses may change in response to herbivore pressures throughout a plant's development (Boege and Marquis 2005); similarly, floral rewards can be under selection by pollinators, which may affect secondary compound concentrations during flowering (Adler et al. 2012). Nectar nicotine, for example, can reduce the volume of nectar consumed by pollinators per visit but increase pollinator visitation rates, increasing estimates of plant female and male reproduction (Kessler and Baldwin 2007). The "pollinator manipulation" hypothesis suggests that floral rewards change as the number of open flowers increases to deter pollinators from visiting too many flowers on the same plant (Biernaskie and Cartar 2004). Under this scenario, the concentrations of deterrent secondary compounds could be low in the first flowers and increase as the number of flowers on an individual plant increase. In addition to phenology-driven changes that occur over the flowering season, herbivory could induce higher concentrations of secondary chemicals in floral rewards over unknown but possibly shorter time scales. In

vegetative tissues, secondary compounds can be induced by herbivory over the course of hours to a few days (Karban 2011) and VOC's can similarly be induced within hours (Schaub et al. 2010). One study that measured induction in nectar due to nectar robbing found that secondary compound concentrations increased fivefold within 10 minutes after petal damage (Kaczorowski et al. 2014). To date we are aware of no study that has examined whether induction occurs in pollen.

We conducted a proof-of-concept study to examine whether herbivory can induce chemical changes in floral rewards, and thus influence interactions between a pollinator and its gut pathogen. Specifically, we asked whether herbivory by the solanaceous specialist, tobacco hornworm (*Manduca sexta* L., Sphingidae*)* induces higher levels of alkaloids in tobacco (*Nicotiana tabacum* L., Solanaceae) nectar and pollen, and we examined the time course of induction. Additionally, we asked whether herbivory-induced effects on tobacco nectar and pollen affected the gut pathogen *Crithidia bombi* (Trypanosomatidae) in the common eastern bumblebee, *Bombus impatiens* Cresson (Apidae). We hypothesized that herbivory would induce higher alkaloid concentrations in nectar and pollen, and that these changes will reduce pathogen counts in bumblebees.

MATERIALS AND METHODS

Study System. We assessed the effects of herbivory on nectar and pollen secondary compounds using domesticated tobacco (*Nicotiana tabacum*). Tobacco is an ideal study system because it produces copious amounts of easily accessible nectar and pollen. Tobacco also produces the alkaloids anabasine and nicotine in nectar (Adler et al. 2006) that when consumed by *Bombus impatiens*, reduced infection by the gut parasite *Crithida* in some studies (Anthony et al. 2015; Baracchi et al. 2015; Richardson et al. 2015), but not others (Palmer-Young et al. 2016; Thorburn et al. 2015). Furthermore, in tobacco these alkaloids are inducible in nectar following leaf herbivory (Adler et al. 2006). Tobacco is often damaged by the tobacco hornworm (*Manduca sexta*), a specialist herbivore of Solanaceae. The common eastern bumblebee (*Bombus impatiens*) is often used as a model system in pollinator disease ecology studies (Otterstatter and Thomson 2006; Otterstatter and Thomson 2007). *Crithidia bombi* (hereafter, *Crithidia*) is a common hindgut endoparasite of *Bombus* spp. that is transmitted fecal-orally on flowers and within the colony (Durrer and Schmid-Hempel 1994). *Crithidia* reduces individual survival, colony founding success, and foraging abilities in bumblebees (Brown et al. 2000; Gegear et al. 2006; Shykoff and Schmid-Hempel 1991).

Floral Induction: Herbivory Treatments. Two hundred and fifty tobacco plants were grown from seed in a greenhouse as described in Online Resource 1 (see *Plant Propagation*). We began applying herbivory treatments when the first flower buds appeared on each plant (April 14 until June 23, 2017). Three tobacco hornworms (*Manduca sexta*) (Great Lakes Hornworm, Romeo, Michigan, USA) were placed in mesh bags enclosing the three topmost, fully extended leaves. Caterpillars were removed when they had consumed the entire leaf, usually within three days. When leaves were not consumed within three days, another caterpillar was added until the whole leaf was consumed. Undamaged plants were similarly bagged (three topmost leaves for three days) but without caterpillars, to control for handling effects.

Floral Induction: Chemical Analysis. As plant began to flower, we collected nectar and pollen as described in Online Resource 1 (see *Nectar and Pollen Collection*). 53 pollen samples from 13 herbivore-damaged plants and 45 pollen samples from 11 undamaged plants were tested for anabasine and nicotine. On average, we assayed 4 pollen samples per plant; each 6-mg sample contained pollen pooled over 5 days. Samples were collected 9-59 days after treatment and samples from the same plant were separated by at least 10 days. We also tested 117 15-µL nectar samples from 29 plants for alkaloids, but none contained nicotine or anabasine.

Analyses of pollen and nectar samples was performed following the procedure in Davis et al (2019). Briefly, prior to analyses pollen samples were extracted in 500 µL of dichloromethane. Nectar samples were diluted with 250 µL of water and extracted with 250 µL of dichloromethane. Samples were injected into a gas chromatograph (Agilent 6890) coupled to a mass spectrometer (Agilent 5973) and fitted with a DB-5 capillary column (30 m length, 0.25 mm diameter, 0.25 µm film thickness; Agilent). Helium was used as the carrier gas at a constant flow of 1 mL/min. The column was initially held at 150ºC for 2 minutes, then increased by 6ºC/min until it reached 240ºC. The data was captured and analyzed using Chemstation (Agilent). The NIST Mass Spectral Database was used to identify the compounds, which were compared to authentic standards (Sigma Aldrich). Alkaloids were quantified against standard curves based on the abundance of the molecular ion (*m/z* 162).

Bee-Pathogen Bioassays: Diet Treatments. We used nectar and pollen collected from the floral induction experiment to assess effects on pollinator-pathogen interactions. After collecting enough nectar and pollen for chemical

analysis, the remaining nectar and pollen were pooled by treatment and collection periods in 2019. Because the chemical analysis did not detect anabasine in pollen collected before May 25, we pooled all nectar and pollen collected before May 25 into one period ('early') within both treatments. For samples collected after May 25, nectar and pollen were pooled within one month of the initial collection date ('mid'). The one-month period was chosen to account for potentially relaxed induction over time. Nectar and pollen collected after May 25 *and* one month after the initial collection date for each plant was also pooled ('late'). Thus, tobacco nectar and pollen were each pooled into six experimental groups (2 damage treatments x 3 periods). All six pollen treatments were mixed with a wildflower pollen mix (CC Pollen Co., Phoenix, Arizona, USA) in a 1:1 ratio by mass and supplemented with deionized water, as preliminary trials suggested that pure tobacco pollen was toxic to bees (J. K. Davis, unpublished data). Nectar treatments were not diluted. We also chemically analyzed one subsample from each pooled pollen treatment but did not detect any alkaloids. It is possible that after two years, although the samples were stored at -20°C, the alkaloids which were originally present at very low concentrations (see Results) had decomposed to below our detection threshold.

Bee-Pathogen Bioassays: Experimental Procedure. In 2019, we tested effects of the pooled pollen and nectar on the bumblebee gut parasite, *Crithidia bombi*. Nectar and pollen bioassays were conducted separately but identically, apart from the manipulation of pollen or nectar and the dates of inoculation. The nectar bioassays were conducted with 168 *B. impatiens* workers inoculated from April 26 to May 14, 2019 (9-27 bees per treatment combination; sample sizes in Online Resource 1; Table S1). The pollen bioassays were conducted with 190 *B. impatiens* workers inoculated from February 14 - 23 and from May 23 to June 7, 2019 (16-21 bees per treatment combination; sample sizes in Online Resource 1; Table S2).

Workers were taken from 4 commercial colonies (Biobest, Leamington, ON, Canada) for each bioassay. After a 2-hr starvation period, workers were inoculated with 6,000 cells of a lab-reared *C. bombi* strain originated from wild *B. impatiens* workers collected at Stone Soup Farm in 2015 (42°21'51.93"N, 72°33'55.88"W, Hadley, Massachusetts, USA). Bees were randomly assigned to a pollen or nectar treatment and fed their assigned diets for 7 days. In the pollen bioassay, bees were fed ~0.5 mg of their pollen treatment in 16x19 mm queen rearing cell cap cups and 1.5 mL of a 30% sucrose solution. In the nectar bioassay, bees were fed their nectar treatment in a 0.6 mL microcentrifuge tube and ~0.5 mg of a wildflower pollen mix (CC Pollen Co., Phoenix, Arizona, USA) in a 1.5mL

microcentrifuge tube cap. Nectar and pollen were replaced every other day in both bioassays. In the pollen bioassay, pollen was wetted with distilled water on days it was not replaced because it dried out within 24 hrs. In the nectar bioassay, we did not need to wet the pollen because wildflower pollen retained its moisture over 48 hrs. During the seven days, workers were kept at 27°C in darkness in 16-oz individual containers. On day 8, bees were dissected to assess infection levels using the protocol established in Richardson et al. (2015). Briefly, we ground the bees' hindguts in Ringer's solution, homogenized them with a vortex and left them standing for 4 hours to allow the tissues to settle. We micro-pipetted 10 μL of the supernatant onto a hemocytometer and counted the number of live *C. bombi* cells in a 0.02 μL volume. We also measured the radial cell length from the right forewing (hereafter referred to as "wing size" for simplicity), which serves as a proxy for bee size (Harder 1982) to account for variability in cell counts due to bee size.

Nectar consumption was measured for all surviving bees in the nectar bioassay during the last day of the experiment to assess whether treatments affected consumption. We weighed a 0.6-mL microcentrifuge tube of nectar at the start and end of a 23-hr consumption period. On one occasion we assessed consumption at 24 hrs; thus, we standardized by calculating consumption per hour. We were unable to measure pollen consumption due to logistical difficulties (e.g., frequent defecation in pollen and dislocation of the pollen from the cap). Lastly, we recorded deaths each day to assess whether the diet treatments affected bee mortality.

Statistical Analyses. All statistical analyses were performed using R ver. 3.3.3 (R Core Team 2017) and all plots were created using the graphics (base), emmeans, ggeffects and ggplot2 packages. For all analyses, we used Akaike information criterion (AIC) and parsimony to select the best model amongst all possible models. We fitted a global model with all possible factors and interactions between treatment and collection period. Then we selected the best model by removing interactions first, and then removed factors until simpler models did not yield lower AIC values. When term removals did not yield changes greater than 2 AIC units, we selected the simplest model. For zero-inflated compound Poisson models, the current R statistical packages did not calculate AIC and log-likelihood values. Hence, to select the best models we simply removed the least significant term until all factors remaining were significant at $P < 0.05$. Because calculating the uncertainty for the zero-inflated compound Poisson models using the available R statistical packages was not possible, we provide a complete list of model parameter estimated in Online Resource 1(Tables S3 and S4).

We assessed the effects of herbivory on pollen nicotine and anabasine concentrations in separate analyses. We used generalized linear mixed models with a binomial error distribution to determine whether herbivory affected the likelihood of detecting alkaloids in pollen at any time within our sampling period (i.e., a plant was scored as having alkaloids present if alkaloids were detected in at least one sample from that plant), since we did not detect nicotine and anabasine in 30% and 52% of plants, respectively. In the global models for each alkaloid, we included herbivory treatment, treatment date (Julian date) and number of pollen samples collected (i.e., higher probability of detection due to greater sampling effort) per plant as fixed effects. The top model only included treatment date as a predictor. We then analyzed the concentrations of nicotine and anabasine for plants whose pollen contained alkaloids in at least one sample. In this analysis, multiple pollen samples from the same plant were included as separate data points, and all pollen samples were included from a plant if that plant had alkaloids detected in any of its pollen. We fit generalized linear mixed models with Tweedie compound Poisson error distributions for each alkaloid using the cplm package. We chose a Tweedie distribution because our data were continuous, highly dispersed and included many zeros (Jørgensen 1992). The global model included herbivory treatment, the number of days after the first flower when sampling occurred (for simplicity, hereafter referred to as "days after first flower") and a scaled quadratic term for days after first flower as fixed effects, and the plant individual as a random effect. We included a quadratic term for number of days after first flower because an analysis of anabasine and nicotine concentrations over time by Davis et al. (2019) found a quadratic response, where concentrations peaked mid flowering season. We scaled the quadratic term by dividing it by the mean days after first flower to avoid model convergence issues. The top model for nicotine included days after first flower and its quadratic term. The top model for anabasine included treatment, days after first flower and its quadratic term. Both models excluded plant individual as a random effect.

To assess the effect of herbivory on *Crithidia* counts in the nectar and pollen bioassays, we used generalized mixed linear models with negative binomial error distributions in the lme4 package. Because we only had enough pollen from the late period during the first two inoculation dates, we conducted two separate analyses for the pollen bioassay. The first analysis included only data from the first two inoculation dates but all six experimental treatments; the second analysis includes data from all inoculation dates but excludes data from the late periods. The global models for the pollen bioassay data included treatment, collection period (early, mid or late), wing size and their interactions as fixed effects, and colony of origin and date of inoculation as random effects. We

note that the global model for the second analysis included wing size but the first one did not because half of the observations in the first analysis had missing wing measurements. The top model for the first pollen bioassay analysis (all experimental treatments, two inoculation dates) included an interaction between treatment and collection period as fixed effects, and colony of origin and inoculation date as random effects. The top model for the second pollen bioassay (all inoculation dates; 'late' period excluded) only included collection period, and colony of origin and date of inoculation as random effects. The global model for the nectar bioassay included treatment, collection period, their interaction, and wing size as fixed effects, and colony of origin and inoculation date as random effects. The top model included collection period and wing size as fixed effects.

To assess whether pollen and nectar diets affected bee survival, we performed a Cox Proportional Hazards test for bees in the pollen and nectar bioassays. However, we did not perform this analysis on the first pollen bioassay, because it included too few deaths.

RESULTS

Floral Induction. When we analyzed the probability of detecting nicotine or anabasine in a plant (i.e. presence/absence), herbivory treatment was not included in either top model. Plants whose pollen was collected later after treatment were more likely to contain anabasine (z-value = 2.51, P = 0.01) but they were not more likely to have nicotine (z-value = 1.76, P = 0.08). When analyzing concentrations in plants that contained alkaloids, adding the herbivory term to the best model did not affect nicotine concentration (z-value = -1.26, P = 0.20; Fig. 1a). For plants that contained anabasine, in the best model herbivory increased anabasine concentrations (z-value = 2.29, P = 0.03; Fig. 1b). Days after first flower and its quadratic term significantly affected nicotine (z-value= 2.52, P = 0.01; z-value = -2.53, P = 0.01; respectively; Fig. 1a) and anabasine (z-value= 3.07, P = 0.01; z-value = -2.61, P = 0.01; respectively; Fig. 1b) concentrations, such that concentrations peaked on days 27 and 37 after first flower, respectively.

Bee Pathogen Bioassays. In the pollen bioassay, *Crithidia* counts were affected by the interaction of treatment and the pollen collection period. Within the undamaged treatments, bees fed late-period pollen had 12 times lower *Crithidia* counts than bees fed early period pollen (z-value = -3.03, P = 0.01; Fig. 2), indicating that pollen reduces *Crithidia* more when plants have been in bloom longer. However, in bees fed damaged plant pollen we did not

observe an effect of collection period on *Crithidia* counts (*z*-value = 0.12, *P* = 0.91; *z*-value = 0.55, *P* = 0.58; when comparing early collected pollen to mid and late collected pollen, respectively). These differences in the effect of time in damaged vs. undamaged plant pollen indicate that herbivory had little effect on the consequences of consuming pollen collected early, but when pollen was collected late (more than one month after blooming started), bees fed pollen from damaged plants had 15 times more *Crithidia* than bees fed pollen from undamaged plants (*z*-value = 2.63, *P* = 0.01; Fig. 2). In the second analysis, when data from all inoculation dates were included, but the "late" collection period was excluded, *Crithidia* counts were not affected by herbivory or collection period (*z*-value = 1.48, *P* = 0.14). We found no effect of pollen diet on mortality (*Wald*-test = 0.49, df = 2, *P* = 0.8).

In the nectar bioassay, *Crithidia* counts did not differ between bees fed damaged and undamaged plant nectar treatments, but they were affected by the collection period and bee size (Online Resource 1; Fig. S1). Bees fed mid-period nectar had 33% and 45% fewer *Crithidia* cells than bees fed early period (*z*-value= -1.988, *P* = 0.047) and late period (*z*-value= -1.77, *P* = 0.08) nectar, respectively. For bees in the nectar bioassay, we also observed that larger bees had lower infections (*z*-value = -1.99, *P* = 0.05). Nectar consumption was not affected by herbivory or date of collection, but larger bees consumed more nectar (*Pearson's* correlation: r = 0.23, df = 130, *P* = 0.01). We found no effect of nectar diet on mortality (*Wald*-test = 0.71, df = 3, *P* = 0.9).

DISCUSSION

We provide the first example of multitrophic effects of herbivory on pollinators and their natural enemies via changes in floral rewards. Herbivory-induced responses often mediate bottom-up multitrophic effects on herbivores and their natural enemies (Soler et al. 2005), but studies have not considered effects on pollinators. It has been long recognized that herbivory can affect interactions between plants and pollinators, but work has focused on how herbivory changes floral traits, pollinator behavior and plant reproduction (reviewed in Lucas-Barbosa 2016). We found that bumblebees that consumed pollen from tobacco plants damaged by herbivores had *Crithidia* infections that were more severe than bees that consumed pollen from undamaged plants when the pollen was collected at least one month after flowering began (Fig. 2). Thus, we have demonstrated that consequences of herbivory can extend beyond plant-pollinator interactions to affect trophic levels above plant mutualists.

While herbivory altered pollen composition enough to modify interactions between bees and their pathogens, this was not mediated by changes in alkaloids. Although previous studies showed that anabasine is

inducible in nectar (Adler et al. 2006; Kaczorowski et al. 2014) and that anabasine and nicotine can reduce *Crithidia* cell counts in *B. impatiens* (Anthony et al. 2015; Baracchi et al. 2015; Richardson et al. 2015), we did not detect alkaloids in nectar, or in the pollen fed to bees in the bioassays (see *Bee-Pathogen Bioassays: Diet Treatments* in Materials and Methods). Furthermore, herbivory increased anabasine in our original pollen samples but decreased *Crithidia* counts, the opposite of our prediction and what was found in prior work studying the effect of these alkaloids on *Crithidia*. Nonetheless, the differences in *Crithidia* levels between bees that consumed control and damage pollen indicate that herbivory induced some change that mediated interactions between bees and their pathogens, although we do not know whether the changes occurred in nutritive or nonnutritive components of pollen. This is an exciting area for future research.

This work emphasizes the role that biotic and abiotic environmental factors can play in shaping pollinator diet. We found that herbivory can impact pollinators by increasing pathogen infection. Given the detrimental effects of *Crithidia* on their hosts, such as cognitive reductions, higher mortality and lower colony founding success (Brown et al. 2000; Gegear et al. 2006; Shykoff and Schmid-Hempel 1991), the negative indirect effects of herbivory can potentially further reduce pollinator fitness. Environmental impacts on pollinator-pathogen interactions via changes in floral rewards may extend well beyond effects of herbivory. Other recent work in the same system found that other biotic and abiotic environmental factors can shape pollinator diet quality (Davis et al. 2019). Soil fertilizer increased pollen alkaloids in *N. tabacum*, while mycorrhizal association decreased them. These soil treatments affected nectar and pollen quality, which in turn influenced bee-*Crithidia* interactions, but effects did not correspond with alkaloid concentrations. Similarly, we found that herbivory affected floral alkaloids, but that the effect of pollen and nectar diets on *Crithidia* was not mediated by alkaloids. Our findings, in conjunction with Davis et al. (2019), highlight the importance of a plant's ecological context in shaping interactions with pollinators through changes in floral rewards.

We found that herbivory did not induce higher concentrations of nicotine but did increase anabasine (Fig. 1). This result corroborates previous work showing that herbivory induces higher concentrations of alkaloids in floral rewards (Adler et al 2006; Halpern et al. 2010; Kaczorowski et al. 2014), but it is the first to demonstrate this outcome in pollen. Understanding how pollen secondary chemistry is shaped is crucial because its role in plant-pollinator interactions has largely been unexplored (Parachnowitsch and Manson 2015; Stevenson 2019) relative to nectar chemistry. Optimal defense theory predicts that pollen should be more heavily defended than nectar, and

several studies have shown that pollen contains more abundant secondary compounds than nectar (Cook et al. 2013; Davis et al. 2019), that are also more diverse (Palmer- Young et al. 2019). We found alkaloids only in pollen, corroborating this prediction. Pollen is necessary for pollinator survival and reproduction, but typically has higher constitutive secondary compounds than nectar, and our work shows that these compounds are inducible. Thus, pollen secondary chemistry could be a major mediator in the effects of herbivory on pollination. Furthermore, because pollinators can quickly assess pollen quality (i.e. taste) (Muth et al. 2016; Ruedenauer et al. 2016) pollinators may be sensitive to variation and rapid changes in pollen chemistry.

Several studies have examined the secondary chemistry of floral rewards (Palmer- Young et al. 2019; Stevenson et al. 2017), but little is known about how secondary compounds vary with plant phenology. This knowledge gap is crucial because temporal variation in secondary compound production can have repercussions for plant-animal interactions, such as reduced digestive efficiency and growth rate (Quintero and Bowers 2018). To our knowledge only one study has evaluated secondary compound concentrations in pollen over time and found that in greenhouse-grown *N. tabacum*, alkaloid concentrations for an experimental population increased early in the season, before reaching a peak midseason and falling as the flowering period progressed (Davis et al. 2019). In our study we also evaluated alkaloid concentrations over time, but as a function of time since each plant began flowering (i.e. days since after first flower) rather than general seasonality. We found a similar rise and fall in the concentrations of nicotine and anabasine, with anabasine concentrations peaking 10 days later than nicotine (Fig. 1).

We postulate a corollary to the "pollinator manipulation" hypothesis proposed by Biernaskie and Cartar (2004) to explain the temporal variation of alkaloid production in pollen. This hypothesis states that plants modify pollinator reward production to limit the number of flowers probed on the same plant during times of high flower production to reduce within-plant self-pollen transfer (geitonogamy). Nicotine could drive such a mechanism to deter pollinators, as has been shown in another *Nicotiana* species (Kessler and Baldwin 2007). We hypothesize that the rise and fall of alkaloid concentrations in individual plants coincides with the production of flowers and could limit pollinator visits to flowers on the same plant. Future work should explicitly quantify flower numbers in relation to alkaloid concentrations in pollen and nectar and measure impacts on pollination service to test this hypothesis.

Diets composed of floral rewards collected at distinct periods had different effects on pollinator-pathogen interactions. Although collection period was important in both nectar and pollen bioassays, the relationship between collection period and *Crithidia* counts was different for each. Bees fed control pollen collected early had

considerably higher *Crithidia* cell counts than bees fed pollen collected later. By contrast, bees with the highest *Crithidia* infections in the nectar bioassay were those fed nectar collected early and late, rather than from the 'mid' period. Thus, the effect of phenology on pollinator-pathogen dynamics may differ for pollen and nectar. However, it appears that the temporal effects of nectar and pollen on pollinator-pathogen interactions were not driven by alkaloids because we did not detect them in our treatment diets. In order to understand the mechanisms driving the effect of differences due to collection period and herbivory, surveys of how broader arrays of primary and secondary compounds vary in time and in ecological contexts will be essential.

In conclusion, we demonstrate that herbivore damage can affect pollen quality and modify interactions between pollinators and their pathogens, a novel example of multitrophic effects resulting from herbivore induction. Thus, biotic and abiotic environmental factors may impact pollinators beyond pollination events by shaping pathogen infection, with potential consequences for pollinator fitness. We also found temporal variation in pollen secondary chemistry and effects on pollinator pathogens, highlighting how plant-pollinator-pathogen interactions may change over the course of a single flowering season.

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FIGURE LEGENDS

Fig. 1 Alkaloid concentrations over time in *Nicotiana tabacum* pollen from damaged (red) and undamaged (black) plants. a) Nicotine concentrations with lines indicating the zero-inflated compound Poisson generalized linear model estimate of the mean (dotted), linear model estimate (solid) and the 95% confidence intervals based on linear model estimate (dashed). b) Anabasine concentrations with lines depicting the estimate of the mean for damaged (red) and undamaged (black) plants. Parameter uncertainty for anabasine could not be calculated given current statistical packages in R; all model estimates for nicotine and anabasine are provided in Tables S3 and S4

Fig. 2 *Crithidia* cell counts in *B. impatiens* workers fed pollen from herbivore-damaged and undamaged plants from 3 collection periods (early, mid and late). Means and 95% confidence intervals

Fig 1 Nicotine and anabasine concentrations

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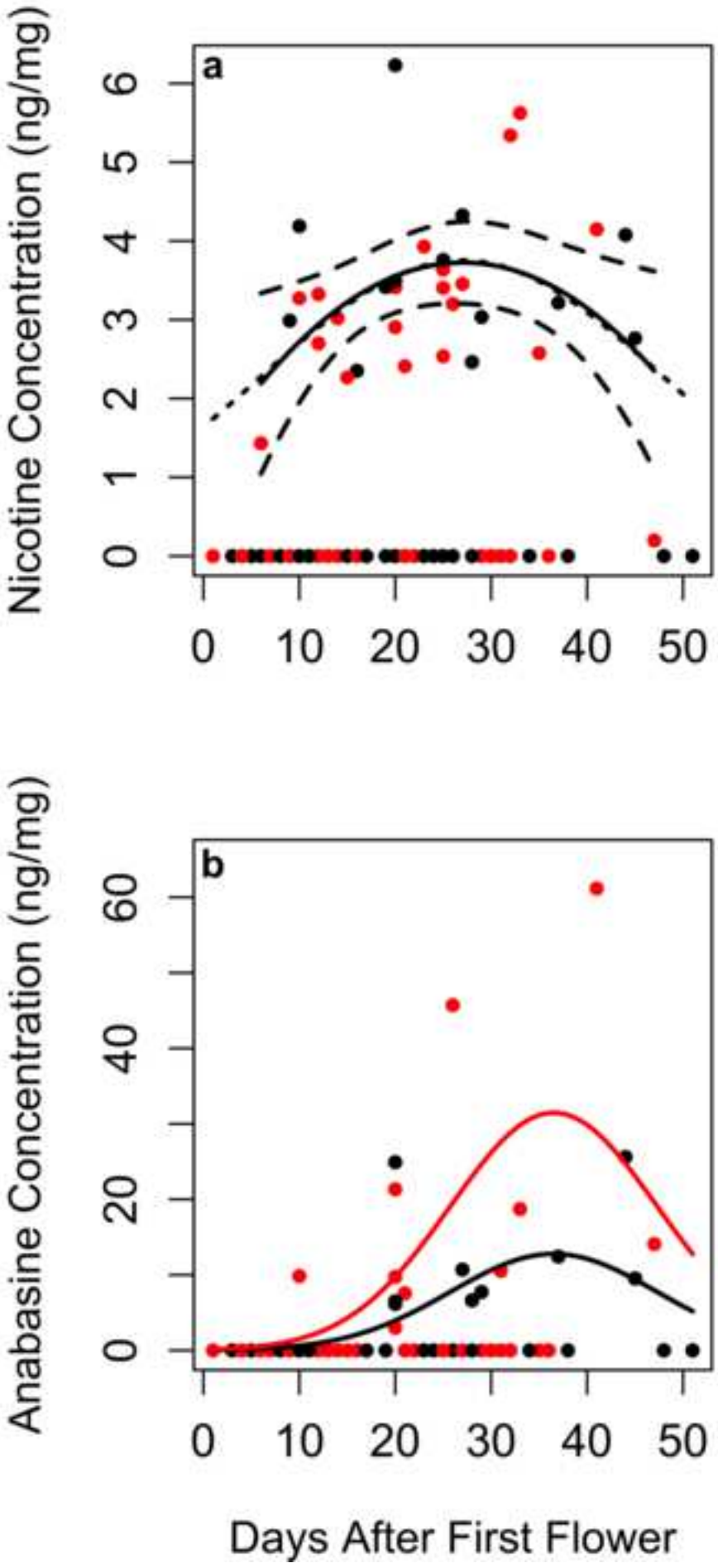

Fig 2 Pollen bioassay

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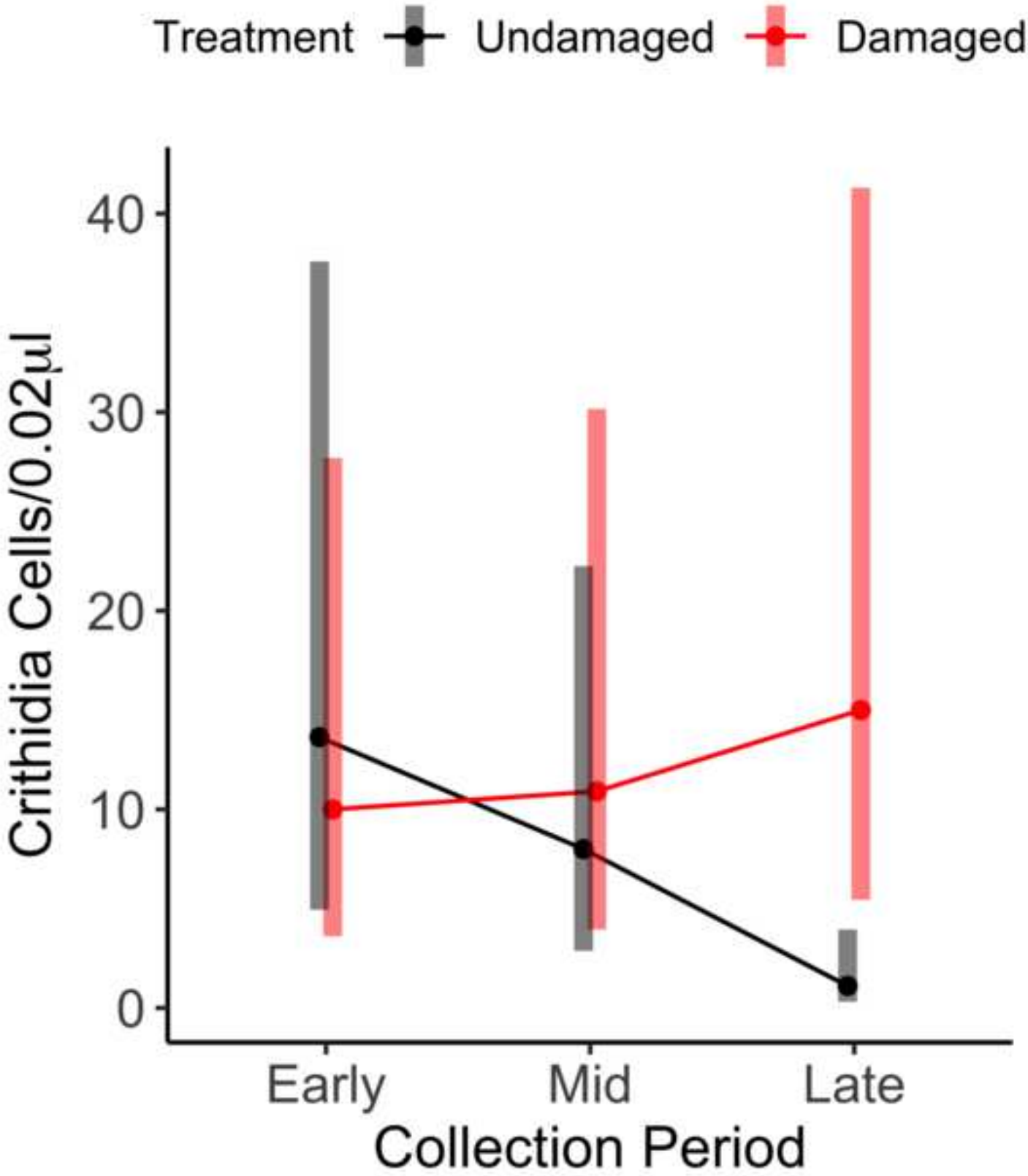

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