Journal of Chemical Ecology (2020) 46:978–986
https://doi.org/10.1007/s10886-020-01213-2

Herbivory and Time Since Flowering Shape Floral Rewards and Pollinator-Pathogen Interactions

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Received: 5 May 2020 / Revised: 7 August 2020 / Accepted: 24 August 2020 / Published online: 2 September 2020

Abstract

Herbivory can induce chemical changes throughout plant tissues including flowers, which could affect pollinator-pathogen interactions. Pollen is highly defended compared to nectar, but no study has examined whether herbivory affects pollen chemistry. We assessed the effects of leaf herbivory on nectar and pollen alkaloids in *Nicotiana tabacum*, and how herbivory-induced changes in nectar and pollen affect pollinator-pathogen interactions. We damaged leaves of *Nicotiana tabacum* using the specialist herbivore *Manduca sexta* and compared nicotine and anabasine concentrations in nectar and pollen. We then pooled nectar and pollen by collection periods (within and after one month of flowering), fed them in separate experiments to bumble bees (*Bombus impatiens*) infected with the gut pathogen *Crithidia bombi,* and assessed infections after seven days. We did not detect 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 days since flowering. Bees fed pollen from damaged plants had *Crithidia* counts 15 times higher than bees fed pollen from undamaged plants, but only when pollen was collected after one month of flowering, indicating that both damage and time since flowering affected interaction outcomes. Within undamaged treatments, bees fed late-collected pollen had *Crithidia* counts 10 times lower than bees fed early-collected pollen, also indicating the importance of time since flowering. Our results emphasize the role of herbivores in shaping pollen chemistry, with consequences for interactions between pollinators and their pathogens.

Keywords *Bombus impatiens* · *Crithidia bombi* · Floral chemistry · Multitrophic interactions · Pollinators

Introduction

Plant defenses modify species interactions, and induced defenses in response to herbivory can mediate interactions over three or even four trophic levels (Harvey et al. 2003; Soler et al. 2005). Induced plant defenses typically are studied in the context of effects on herbivores and their natural enemies; however, they also have the potential to affect pollinators and their natural enemies. Herbivory on vegetative tissues often induces changes to volatile organic compound (VOC) emissions and increases secondary compounds in floral tissues and rewards (reviewed in Lucas-Barbosa 2016; Moreira et al. 2019; Rusman et al. 2019), and many studies have examined how such changes affect pollinator visitation. In general, floral and leaf herbivory deter pollinators (Moreira et al. 2019) although root herbivory sometimes increases flower visitation (Barber et al. 2011; Moreira et al. 2019; Poveda et al. 2003, 2005) and effects on pollinator behavior may depend on the pollinator species (Hoffmeister et al. 2016; Rusman et al. 2018).

The impacts of herbivory on plant-pollinator interactions could continue past the immediacy of the pollinator visit, if herbivore-induced changes to floral rewards affect pollinator health (Jacobsen and Raguso 2018). Herbivory can alter the value of pollen and nectar through decreased quantity

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10886-020-01213-2) contains supplementary material, which is available to authorized users.

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(Krupnick et al. 1999; Lehtilä and Strauss 1999; Chauta et al. 2017), quality and increased secondary compound content (Adler et al. 2006; Halpern et al. 2010; Kaczorowski et al. 2014), although in some cases herbivory can increase nectar sugar concentration (Bruinsma et al. 2014; Chauta et al. 2017). Because pollinators rely on pollen and nectar to meet their nutritional requirements (Heinrich 1979; Roulston and Cane 2000), changes in the nutritional value of floral rewards could have effects on pollinator health. Thus, herbivory could influence interactions between pollinators and their natural enemies, but to our knowledge this relationship has never been assessed.

Pollinators, like other herbivores, ingest secondary compounds in their diet (Irwin et al. 2014), which could have direct negative effects on pollinator performance and indirect positive or negative effects on pollinator-pathogen interactions. Secondary compounds in floral rewards can be directly harmful to pollinators (Detzel and Wink 1993; Tiedeken et al. 2016). For example, Detzel and Wink (1993) found that 17 out of 29 secondary compounds found in honey were lethal to honey bees at concentrations of 0.6% and under, and noted that alkaloids were particularly toxic. Secondary compounds, such as the alkaloid D-lupanine, can also reduce bumble bee microcolony fitness (Arnold et al. 2014). Pollinators may respond to harmful compounds in their diet via processes such as detoxification (du Rand et al. 2015; Mao et al. 2013). These responses can lead to tradeoffs that hinder pathogen resistance, such as energetic stress or reduced immunocompetence (Roger et al. 2017). For example, the alkaloid anabasine can magnify the negative effects of pathogens on bumble bee and microcolony fitness (Palmer-Young et al. 2017), suggesting possible tradeoffs between compound detoxification and immune function or pathogen tolerance.

Secondary compounds could also benefit pollinators by reducing pathogen infection via increasing host resistance or being directly harmful to the pathogens (Stevenson et al. 2017; Koch et al. 2019). Several nectar secondary compounds, including anabasine and nicotine, reduce pathogen infections in bumble bees (Baracchi et al. 2015; Koch et al. 2019; Manson et al. 2010; Palmer-Young et al. 2017; Richardson et al. 2015). For example, Richardson et al. (2015) tested the effects of eight nectar secondary compounds on infections by *Crithidia bombi*, a common gut endoparasite, in the bumble bee *Bombus impatiens* and found that half of those compounds reduced infections. However, these effects can be context-dependent (Palmer-Young et al. 2016; Thorburn et al. 2015) and secondary compounds have mostly been tested at the concentrations found in nectar, but pollinators could be exposed to higher concentrations via consumption of pollen (Cook et al. 2013; Palmer-Young et al. 2019).

Pollen chemical composition has the potential to play a central role in mediating pollinator-pathogen interactions. Pollen is the major source of protein and lipids for many floral visitors, and thus is critical for reproduction and survival (Roulston and Cane 2000). Pollen is also the plant's male gamete, and can be defended with secondary compounds. Optimal defense theory predicts that plant tissues that are more tightly linked to reproduction should be better defended (McKey 1974), and this has been supported by studies in which female reproductive tissue such as fruits and seeds are highly defended (Ohnmeiss and Baldwin 2000; Zangerl and Rutledge 1996). By the same logic, pollen should be highly defended relative to nectar, because pollen viability is directly tied to plant male reproductive success. Consistent with this hypothesis, pollen contains an array of "anti-herbivore" secondary compounds (Adler 2000; Rivest and Forrest 2020; Stevenson et al. 2017), that are often more concentrated (Cook et al. 2013; Palmer-Young et al. 2019) and diverse (Palmer-Young et al. 2019) than compounds in nectar. Hence, the effects of pollen quality on pollinator health may be dictated not only by macronutrients but also by secondary chemistry. However, secondary compound concentrations in pollen may not be consistent over time (see Davis et al. 2019). Instead, they could be shaped by both herbivory and time since flowering (i.e., days since the onset of flowering). Secondary compound concentrations in roots and leaves change in response to herbivory (Kaplan et al. 2008) and ontogeny (Boege and Marquis 2005). Additionally, secondary compound concentrations in vegetative tissues, flowers and nectar can be positively correlated (Adler et al. 2006, 2012; Manson et al. 2012). Thus, we hypothesize that herbivory and time since flowering will affect the concentrations of secondary compounds in pollen and consequently shape multitrophic interactions with pollinator pathogens.

We conducted a study to examine whether herbivory can induce chemical changes in floral rewards and influence interactions between a pollinator and its gut pathogen, and whether time since flowering influences these effects. 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 bumble bee, *Bombus impatiens* Cresson (Apidae). We hypothesized that herbivory would induce higher alkaloid concentrations in floral rewards, and that these changes would reduce pathogen counts in bumble bees.

Materials and Methods

Study System We assessed the effects of herbivory on nectar and pollen secondary compounds and the time course of induction using domesticated tobacco (*Nicotiana tabacum* L.,

Solanaceae), and then assessed the effects of pollen and nectar from damaged tobacco on *Crithidia* infection in *B. impatiens*. Tobacco nectar contains the alkaloids anabasine and nicotine (Adler et al. 2006), which are inducible in nectar following leaf herbivory (Adler et al. 2006; Halpern et al. 2010). Tobacco is often damaged by the tobacco hornworm (*Manduca sexta* L., Sphingidae), a specialist herbivore of Solanaceae (Merrill 1916). Furthermore, nicotine and anabasine reduced infection in the common eastern bumble bee *Bombus impatiens* Cresson (Apidae) by the gut pathogen *Crithida bombi* (Trypanosomatidae; hereafter, *Crithidia*) in some studies (Anthony et al. 2015; Baracchi et al. 2015; Richardson et al. 2015), although not in others (Palmer-Young et al. 2016; Thorburn et al. 2015). *Crithidia* is transmitted fecal-orally on flowers and within the colony (Durrer and Schmid-Hempel 1994) and reduces individual survival, colony founding success, and foraging abilities in bumble bees (Brown et al. 2000; Gegear et al. 2006; Shykoff and Schmid-Hempel 1991). We note that *N. tabacum* is an agricultural species that does not grow in the wild, but produces copious nectar and has been a model system for studying defensive chemistry (e. g., Kaplan et al. 2008). Bumble bees have been observed visiting flowers of some wild *Nicotiana* species (Kaczorowski et al. 2005), many *Nicotiana* species have nicotine and/or anabasine in nectar (Adler et al. 2012), and mechanisms of nectar production and biochemical action seem to be generally conserved across the genus (Silva et al. 2018), making our study ecologically relevant.

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 applied herbivory treatments when the first flower buds appeared on each plant (April 14 until June 23, 2017). Three fifth-instar (occasionally third- or fourth-instar) 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. Plants were treated with herbivores or bagged (controls) on average 7.3 days (range: 2–12 days) prior to the onset of flowering.

Floral Induction: Chemical Analysis As plants began to flower, we collected nectar and pollen as described in Online Resource 1 (see *Nectar and Pollen Collection*). Fifty-three 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 were 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 150 °C for 2 min, then increased by 6 °C/min until it reached 240 °C. 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, remaining samples were pooled by treatment and collection periods in 2019. We are unaware of data demonstrating that herbivory induces changes in pollen chemistry, including the time it takes for induction to occur or relax. We compared pollen and nectar collected within one month of damage to samples collected more than one month after damage. We chose a one-month period to collect sufficient material for bioassays and because we hypothesized induced responses would last for at least one month after damage but might attenuate after that; Adler et al. (2006) showed that induction in nectar occurred in samples pooled for several weeks after damage, and Halpern et al. (2010) showed that induction in nectar may last up to two months (nonsignificant trend). Because anabasine was not detected in any samples collected before May 25, we conducted an additional analysis with three categories ("within one month [before May 25]", "within one month [after May 25]" and "after one month"), but results were largely consistent and so we report the simpler analysis (see Online Resource 1; Figures S1–2 and Tables S1–3, for the more complex analysis and results). All 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. During each bioassay, we included control treatments to determine whether all tobacco pollen and nectar

could affect parasite counts relative to a more standard diet. In the pollen bioassay, the control diet was the wildflower pollen mix and in the nectar bioassay, the control diet was a 17% sucrose solution, which was the same sugar concentration as the tobacco nectar. We also chemically analyzed one subsample from each pooled pollen treatment but did not detect alkaloids. Because a high proportion of pollen samples did not contain alkaloids originally, pollen with detectable levels of alkaloids may have been diluted by pollen with alkaloid concentrations too low to detect in our pooled sample (See *Statistical Analyses* in Methods and *Floral Induction* in Results; Fig. 1). Alternatively, it is possible that after two years, although the samples were stored at −20 °C, the alkaloids originally present at low concentrations (see Results) had decomposed to below our detection threshold. Nonetheless, some macronutrients remain stable over long periods of time (P.C. Stevenson, personal observation), and changes in macronutrient concentrations could mediate interactions between pollinators and their pathogens.

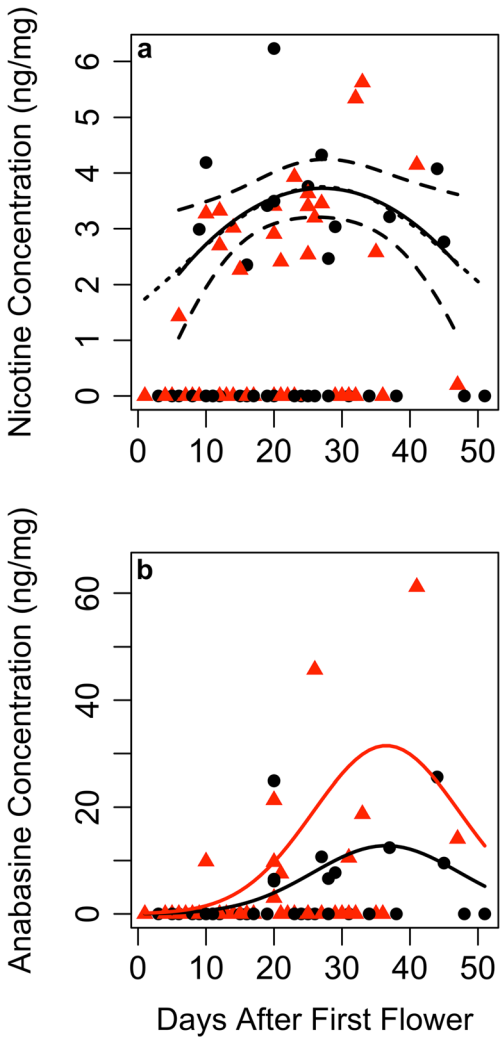

Fig. 1 Alkaloid concentrations over time in *Nicotiana tabacum* pollen from damaged (red triangles) and undamaged (black circles) plants. **a** Nicotine concentrations with lines indicating the zero-inflated compound Poisson generalized linear model estimate of the mean (dotted), linear model estimate (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 triangles) and undamaged (black circles) plants. Parameter uncertainty for anabasine could not be calculated with the current statistical packages in R; all model estimates for nicotine and anabasine are provided in Tables S4–5

Bee-Pathogen Bioassays: Experimental Procedure In 2019, we tested effects of the pooled pollen and nectar on *Crithidia* infection. Nectar and pollen bioassays were conducted separately but identically, apart from the manipulation of pollen or nectar and the dates of inoculation. The pollen bioassays were conducted with 84 *B. impatiens* workers inoculated on February 14 and 15 (9–22 bees per treatment combination; sample sizes in Online Resource 1, Table S1). The nectar bioassays were conducted with 175 *B. impatiens* workers inoculated from April 26 to May 6 (19–40 bees per treatment combination; total of 5 inoculation dates; sample sizes in Online Resource 1, Table S2).

Workers were taken from four commercial colonies (Biobest, Leamington, ON, Canada) for each bioassay; a subsample of five workers per commercial colony was screened for *Crithidia* before experiments began and weekly thereafter to confirm colonies were *Crithidia*-free. After a 2-h starvation period, workers were inoculated with 10 μL of inoculum; the infection inoculum was made from a mix of sucrose, Ringer's solution and infected bee gut contents. The inoculum contained 25% sucrose and 6000 cells of a lab-reared *Crithidia* 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 seven days. In the pollen bioassay, bees were fed ~ 0.5 mg of their pollen treatment in 16 × 19 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 1.5 mL 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 h. In the nectar bioassay, we did not need to wet the pollen because wildflower pollen retained its moisture over 48 h. During the seven days, workers were kept at 27 °C in darkness in 16-oz individual containers. Bees were then dissected to assess infection levels as 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 h to allow the tissues to settle. We micro-pipetted 10 μL of the supernatant onto a hemocytometer and counted the number of live *Crithidia* cells in a 0.02 μL volume. We also measured the radial cell length

from the right forewing (hereafter referred to as "body size" for simplicity), which serves as a proxy for bee size (Nooten and Rehan 2020) 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-h consumption period. On one occasion we assessed consumption at 24 h; 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). We also recorded deaths daily to assess whether diet treatments affected bee mortality.

Statistical Analyses All statistical analyses were performed using R ver. 3.3.3 (R Core Team 2019) and all plots were created using the graphics (base), emmeans (Lenth 2019), ggeffects (Lüdecke 2018) and ggplot2 packages (Wickham 2016). For all analyses, we used Akaike information criterion (AIC) and parsimony to select the best model amongst all possible models. We first fitted a global model with all possible factors including interactions between herbivory treatment and collection period. Then we selected the best model by removing interactions first, and then removing factors until simpler models did not yield lower AIC values. When 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 do not calculate AIC and log-likelihood values. Hence, to select the best models we simply removed the least significant term until all remaining factors were significant at $P < 0.05$. Because calculating 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 estimates in Online Resource 1 (Tables S4–5).

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 analyzed (i.e., higher probability of detection due to greater sampling effort) per plant as fixed effects. The top models for nicotine and anabasine 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 (Zhang 2013). We chose a Tweedie distribution because our data were continuous, highly dispersed and included many zeros (Jørgensen 1992). The global models 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 pollen anabasine and nicotine concentrations over time by Davis et al. (2019) detected 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 herbivory 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 using the lme4 package (Bates et al. 2015). The global models for the pollen and nectar bioassays included herbivory treatment, collection period ("within one month" and "after one month") and their interactions as fixed effects, and colony of origin and inoculation date as random effects. The global model for nectar also included body size (estimated by wing radial cell length) as a fixed effect; the pollen bioassay did not include body size due to many missing values for the covariate. The top model for the pollen bioassay included collection period ("within one month" and "after one month") and the interaction between collection period and herbivory treatment as fixed effects. The top model for the nectar bioassay included collection period and body size as fixed effects, and colony of origin and inoculation date as random effects. To adjust for multiple comparisons within each bioassay, we adjusted *p*-values using the false detection rate (FDR) and reported the corrected *p*-values. To assess whether nectar diets affected bee survival, we performed a Cox Proportional Hazards test. We did not analyze mortality for the pollen bioassay due to too few deaths (only 5 of 84 bees).

Data and Code Availability Data and R script are available via Dryad (https://doi.org/10.5061/dryad.bk3j9kd8m

Results

Floral Induction Herbivory did not increase the likelihood of detecting nicotine or anabasine (i.e., presence vs. absence) in tobacco nectar or pollen. Nectar did not have detectable levels of alkaloids. Anabasine was more likely to be present in pollen collected later (z-value = 2.51, $P = 0.01$), but collection period did not significantly affect the likelihood of detecting nicotine in pollen (z-value = 1.76, $P = 0.08$). Herbivory did not affect nicotine concentration in pollen (z-value = −1.26, $P = 0.20$; Fig. 1a), but significantly increased pollen anabasine by about 250% (damaged: 31.45 ng/mg 95% CI [25.37, 37.74], undamaged: 12.77 ng/mg, 95% CI [6.69, 18.85], z-value = 2.29, $P = 0.03$; Fig. 1b). Both nicotine and anabasine concentrations changed through time. Pollen nicotine concentrations initially increased before peaking 27 days after the first flower and then declining (time since flowering: z-value = 2.52, $P = 0.01$, time since flowering (quadratic): z-value = −2.53, $P = 0.01$; Fig. 1a). Similarly, anabasine concentrations in pollen increased until day 37 and then decreased (time since flowering: z-value = 3.07, $P = 0.01$, time since flowering (quadratic): z-value = −2.61, $P = 0.01$; Fig. 1b).

Bee Pathogen Bioassays In the pollen bioassays, herbivory treatment and collection period both affected *Crithidia* counts. There was a significant interaction between herbivory treatment and collection period (z-value = −2.685, $P = 0.01$) such that damage only affected *Crithidia* counts when pollen was collected after one month of flowering. Contrary to expectations, bees fed late-collected pollen from damaged plants had 15 times higher pathogen counts than bees fed late-collected pollen from undamaged plants (damaged plants: 15.00 cells/0.02 μL, 95% CI [5.42, 41.52], undamaged plants: 1.11 cells/0.02 μL, 95% CI [0.31, 3.97]; $P = 0.01$; Fig. 2). Time since flowering also affected *Crithidia* counts within the undamaged treatment. Within undamaged plant diets, bees fed pollen collected within 1 month of flowering had 10 times higher *Crithidia* counts than bees fed pollen collected after 1 month of flowering (within one month: 10.82 cells/0.02 μL, 95% CI [5.25, 22.29], after one month: 1.11 cells/0.02 μL, 95% CI [0.31, 3.97]; $P = 0.01$; Fig. 2), indicating differences in pollen quality across the flowering period. However, in bees fed pollen from damaged plants we found no effect of collection period on *Crithidia* counts (within one month: 10.45 cells/0.02 μL, 95% CI [5.07, 21.55], after one month:15.00 cells/0.02 μL, 95% CI [5.42, 41.52], $P = 0.57$; Fig. 2). *Crithidia* counts did not differ in bees fed the control pollen diet compared to bees fed any tobacco pollen diet (Table S6).

In the nectar bioassay, *Crithidia* counts were not affected by herbivore damage or collection period. However, larger bees had a non-significant tendency to have lower infection levels (z-value = −1.83, $P = 0.07$; Online Resource 1; Figure S3). Larger bees also consumed more nectar (Pearson's correlation: $r = 0.23$, df = 130, $P = 0.01$), but nectar consumption was not affected by herbivory treatment or collection period (Herbivory Treatment: $X^2(2) = 1.94$, $P = 0.38$; Collection Period: $X^2(2) = 1.36$, $P = 0.51$). Bee mortality rates were not affected by nectar treatments (*Wald*-test = 0.71, df = 3, $P = 0.9$). *Crithidia* counts did not differ in bees fed the control nectar diet compared to bees fed any tobacco nectar diet (Table S7).

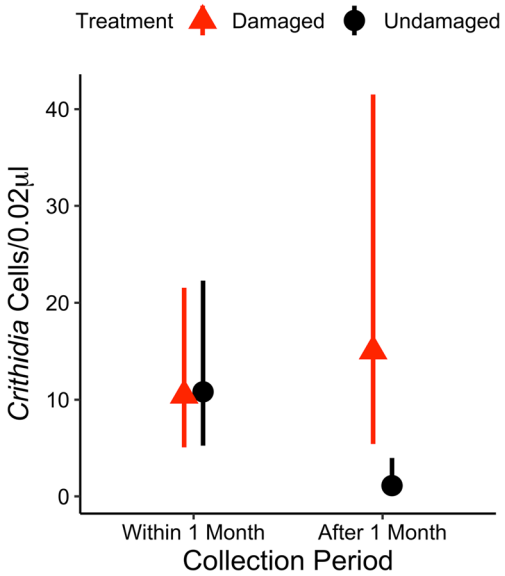

Fig. 2 *Crithidia* cell counts in *B. impatiens* workers fed pollen from herbivore-damaged and undamaged plants from two collection periods (within 1 month of flowering, 1 month after flowering). Means and 95% confidence intervals

Discussion

We provide the first example of multitrophic effects of herbivory on pollinators and their pathogens 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; Moreira et al. 2019; Rusman et al. 2019). Surprisingly, we found that bumble bees that consumed pollen from tobacco plants damaged by herbivores had more *Crithidia* cells than bees that consumed pollen from undamaged plants, but only when the pollen was collected after one month of flowering (Fig. 2). This result does not support our original hypothesis that damage would reduce *Crithidia* infection, but demonstrates that in some contexts consequences of herbivory can extend beyond plant-pollinator interactions to affect higher trophic levels in unexpected ways.

The length of time elapsed between production of the first flower (i.e., time since flowering) and nectar or pollen collection was a surprisingly important mediator of the effects of herbivory on pollinator-pathogen interactions. Bees that consumed pollen from damaged plants had higher *Crithidia* infection intensity than bees that consumed pollen from undamaged plants, but only when the pollen was collected after one month of flowering (Fig. 2). Surprisingly, this effect is largely because bees fed control pollen collected after one month of flowering had considerably lower *Crithidia* cell counts than bees fed control pollen collected early (Fig. 2), suggesting some quality of pollen that reduces infection as plants age. Thus, pathogen infections may be greatly affected by the period within which infected individuals exploit a specific resource that fluctuates in quality. Temporal effects on pollen chemistry are largely unexplored, but temporal variation in secondary compound production in other tissues can have repercussions for plant-animal interactions, such as when herbivores' digestive efficiency and growth rate decrease as plants age (Quintero and Bowers 2018). We found that pollen chemical properties changed over time; concentrations of nicotine and anabasine rose and fell along the progression of the plants' flowering period (Fig. 1), a pattern similar to that found by Davis et al. (2019). However, further laboratory and field studies will be needed to test the hypothesis that temporal variation in floral rewards is widespread and affects pollinator-pathogen dynamics.

Herbivory did not induce higher concentrations of nicotine but did increase anabasine in tobacco pollen (Fig. 1). While anabasine and nicotine share a biosynthetic pathway (Solt et al. 1960), our results suggest differential regulation in floral rewards. Our work is consistent with previous research showing that herbivory induced higher concentrations of some alkaloids in nectar (Adler et al. 2006; Halpern et al. 2010; Kaczorowski et al. 2014), but it is the first to examine induction in pollen. Moreover, we note that we only focused on alkaloids; it is possible that other primary or secondary compounds that we did not consider could also be affected by herbivory. For example, proteins in tobacco nectar have been characterized as part of a nectar redox cycle involved in antimicrobial defense (Thornburg et al. 2003), but we know less about how herbivory affects these proteins or whether they occur in pollen. Understanding how pollen secondary chemistry is regulated is crucial because its role in plant-pollinator interactions is largely unexplored relative to nectar chemistry (Parachnowitsch and Manson 2015; Stevenson 2019). Optimal defense theory predicts that pollen should be more heavily defended than nectar, and several studies have shown that pollen contains higher concentrations (Cook et al. 2013; Davis et al. 2019) and diversity (Palmer-Young et al. 2019) of secondary compounds than nectar. Consistent with this prediction, we detected alkaloids only in pollen, although we note that both nicotine and anabasine are commonly found in tobacco nectar. Because pollinators can quickly assess some pollen qualities (i.e. taste) (Muth et al. 2016; Ruedenauer et al. 2016), pollinators may be sensitive to variation and rapid changes in pollen chemistry. Thus, our result that herbivory induces higher pollen anabasine provides a new potential mechanism for pollinator deterrence in response to herbivory (Kessler and Halitschke 2009; Moreira et al. 2019).

Herbivory altered pollen enough to modify interactions between bees and their pathogens, but the mechanism remains unclear. Although we predicted that anabasine or nicotine in floral rewards would mediate multitrophic interactions between herbivores, plants, pollinators and pathogens, we did not find evidence to support this hypothesis. Previous studies have shown 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), although at higher concentrations than typically found in *N. tabacum*. However, we did not detect alkaloids in the nectar or 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 contrary to our prediction, herbivory also increased *Crithidia* counts when pollen was collected after one month of flowering. This suggests that induced anabasine is not the mechanism underlying the effect of herbivory on *Crithidia* counts. Nonetheless, the differences in *Crithidia* levels between bees that consumed control and damaged 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 provides an example of the role that biotic factors may play in shaping pollinator diet. We found that *Crithidia* infections were substantially higher in bees fed pollen from damaged plants than those fed pollen from undamaged plants, but only when pollen was collected after one month of flowering; this could either be because components induced by damage increase pathogen infection, or because components that reduce infection in undamaged plants are degraded by herbivory. 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 could potentially 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 both soil nutrients and mycorrhizal fungi can shape floral reward quality (Davis et al. 2019). Soil fertilizer increased pollen alkaloids in *N. tabacum*, while mycorrhizal association decreased them. Fertilizer and

mycorrhizae treatments affected nectar and pollen quality, which in turn influenced bumble bee-*Crithidia* interactions, but effects did not correlate with alkaloid concentrations. This is very similar to our study, in which herbivory increased pollen anabasine but the effects of pollen diets on *Crithidia* were not mediated by alkaloids. Both these studies highlight the importance of a plant's ecological context in shaping pollinator-pathogen interactions through changes in floral rewards.

In conclusion, we demonstrate that pollen secondary chemistry varies with time since flowering and in response to herbivory. We also demonstrate that herbivore damage may affect pollen quality and under some conditions modify interactions between pollinators and their pathogens, a novel example of multitrophic effects resulting from herbivory. Surprisingly, pollen from undamaged plants increasingly reduced *Crithidia* infection as time since flowering progressed, while in damaged plants this reduction over time did not occur. To understand the mechanisms driving differences in pollen quality due to temporal changes and herbivory, surveys of how broader arrays of nutrients and secondary compounds vary with time and ecological context will be essential. We conclude that biotic factors can impact pollinators beyond pollination events by shaping pathogen infection, with potential consequences for pollinator fitness.

Acknowledgements We thank E. Stone, E. Amponsah, B. Joyce, R. Pasquale, G. Cox, L. Cleary and E. Palmer-Young for help with data collection and thoughtful feedback, the UMass Amherst Quantitative Statistics Group for feedback on statistical analyses, Biobest (Ontario, Canada) for donating bumble bee colonies, and J. van Wyk, R. Malfi, M. Hanusch and three anonymous reviewers for providing constructive comments on the manuscript. This work was funded by the PGAV Destinations Pollinator Research grant, the IMSD/NEAGEP First-Year Pre-Doctoral Fellowship (NIH 25 GM099649), the Lotta M. Crabtree Fellowship, the National Science Foundation Graduate Research Fellowship (NSF 1451512; 1938059), and the National Science Foundation and Austrian Science Fund GROW Program (NSF 1938059; FWF GRW 7-B) to LAA, the Torrey Plant Biology Fellowship to JKD and NIH 1 R01 GM1220 62-01 to LSA.

Author Contributions The study design was devised by LAA and LSA. LAA and JKD carried out the experiments. PCS undertook the chemical analysis. LAA conducted the statistical analyses and wrote the manuscript. All authors contributed critically to the manuscript and approved the final manuscript.

References

Adler LS (2000) The ecological significance of toxic nectar. Oikos 91: 409–420

Adler LS, Wink M, Distl M, Lentz AJ (2006) Leaf herbivory and nutrients increase nectar alkaloids. Ecol Lett 9:960–967

Adler LS, Seifert MG, Wink M, Morse GE (2012) Reliance on pollinators predicts defensive chemistry across tobacco species. Ecol Lett 15: 1140–1148

Anthony WE, Palmer-Young EC, Leonard AS, Irwin RE, Adler LS (2015) Testing dose-dependent effects of the nectar alkaloid anabasine on trypanosome parasite loads in adult bumble bees. PLoS One 10:e0142496

Arnold SEJ, Peralta Idrovo ME, Lomas Arias LJ, Belmain SR, Stevenson PC (2014) Herbivore defence compounds occur in pollen and reduce bumblebee colony fitness. J Chem Ecol 40:878–881

Baracchi D, Brown MJF, Chittka L (2015) Behavioural evidence for self-medication in bumblebees? F1000 Research 4:73

Barber NA, Adler LS, Bernardo HL (2011) Effects of above- and below-ground herbivory on growth, pollination, and reproduction in cucumber. Oecologia 165:377–386

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using {lme4}. J Stat Softw 67:1–48

Boege K, Marquis RJ (2005) Facing herbivory as you grow up: the ontogeny of resistance in plants. Trends Ecol Evol 20:441–448

Brown M, Loosli R, Schmid-Hempel P (2000) Condition-dependent expression of virulence in a trypanosome infecting bumblebees. Oikos 91:421–427

Bruinsma M, Lucas-Barbosa D, ten Broeke CJM, van Dam NM, van Beek TA, Dicke M, van Loon JJA (2014) Folivory affects composition of nectar, floral odor and modifies pollinator behavior. J Chem Ecol 40:39–49

Chauta A, Whitehead S, Amaya-Marquez M, Poveda K (2017) Leaf herbivory imposes fitness costs mediated by hummingbird and insect pollinators. PLoS One 12:e0188408

Cook D, Manson JS, Gardner DR, Welch KD, Irwin RE (2013) Norditerpene alkaloid concentrations in tissues and floral rewards of larkspurs and impacts on pollinators. Biochem Syst Ecol 48:123–131

Davis JK, Aguirre LA, Barber NA, Stevenson PC, Adler LS (2019) From plant fungi to bee parasites: Mycorrhizae and soil nutrients shape floral chemistry and bee pathogens. Ecology:e02801

Detzel A, Wink M (1993) Attraction, deterrence or intoxication of bees (*Apis mellifera*) by plant allelochemicals. Chemoecology 4:8–18

du Rand EE, Smit S, Beukes M, Apostolides Z, Pirk CWW, Nicolson SW (2015) Detoxification mechanisms of honey bees (*Apis mellifera*) resulting in tolerance of dietary nicotine. Sci Rep 5:11779

Durrer S, Schmid-Hempel P (1994) Shared use of flowers leads to horizontal pathogen transmission. Proc R Soc Lond B 258:299–302

Gegear RJ, Otterstatter MC, Thomson JD (2006) Bumble-bee foragers infected by a gut parasite have an impaired ability to utilize floral information. Proc R Soc Lond B 273:1073–1078

Halpern SL, Adler LS, Wink M (2010) Leaf herbivory and drought stress affect floral attractive and defensive traits in *Nicotiana quadrivalvis*. Oecologia 163:961–971

Harvey JA, van Dam NM, Gols R (2003) Interactions over four trophic levels: Foodplant quality affects development of a hyperparasitoid as mediated through a herbivore and its primary parasitoid. J Anim Ecol 72:520–531

Heinrich B (1979) Bumblebee economics. Harvard University Press, Cambridge

Hoffmeister M, Wittkoepper N, Junker RR (2016) Herbivore-induced changes in flower scent and morphology affect the structure of flower-visitor networks but not plant reproduction. Oikos 125: 1241–1249

Irwin RE, Cook D, Richardson LL, Manson JS, Gardner DR (2014) Secondary compounds in floral rewards of toxic rangeland plants: Impacts on pollinators. J Agric Food Chem 62:7335–7344

Jacobsen DJ, Raguso RA (2018) Lingering effects of herbivory and plant defenses on pollinators. Curr Biol 28:R1164–R1169

Jørgensen B (1992) Exponential dispersion models and extensions: A review. Int Stat Rev 60:5–20

Kaczorowski RL, Gardener MC, Holtsford TP (2005) Nectar traits in *Nicotiana* section *Alatae* (Solanaceae) in relation to floral traits, pollinators, and mating system. Am J Bot 92:1270–1283

Kaczorowski RL, Koplovich A, Sporer F, Wink M, Markman S (2014) Immediate effects of nectar robbing by Palestine sunbirds (*Nectarinia osea*) on nectar alkaloid concentrations in tree tobacco (*Nicotiana glauca*). J Chem Ecol 40:325–330

Kaplan I, Halitschke R, Kessler A, Sardanelli S, Denno RF (2008) Constitutive and induced defenses to herbivory in above- and belowground plant tissues. Ecology 89:392–406

Kessler A, Halitschke R (2009) Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: Predictions and case study. Funct Ecol 23:901–912

Koch H, Woodward J, Langat MK, Brown MJF, Stevenson PC (2019) Flagellum removal by a nectar metabolite inhibits infectivity of a bumblebee parasite. Curr Biol 29:3494–3500.e5

Krupnick GA, Weis AE, Campbell DR (1999) The consequences of floral herbivory for pollinator service to *Isomeris arborea*. Ecology 80: 125–134

Lehtilä K, Strauss SY (1999) Effects of foliar herbivory on male and female reproductive traits of wild radish, *Raphanus raphanistrum*. Ecology 80:116–124

Lenth R (2019) Emmeans: estimated marginal means, aka least-squares means. R package version 1(4):1

Lucas-Barbosa D (2016) Integrating studies on plant-pollinator and plant-herbivore interactions. Trends Plant Sci 21:125–133

Lüdecke D (2018) Ggeffects: tidy data frames of marginal effects from regression models. R package version 0.12.0

Manson JS, Otterstatter MC, Thomson JD (2010) Consumption of a nectar alkaloid reduces pathogen load in bumble bees. Oecologia 162:81–89

Manson JS, Rasmann S, Halitschke R, Thomson JD, Agrawal AA (2012) Cardenolides in nectar may be more than a consequence of allocation to other plant parts: A phylogenetic study of *Asclepias*. Funct Ecol 26:1100–1110

Mao W, Schuler MA, Berenbaum MR (2013) Honey constituents up-regulate detoxification and immunity genes in the western honey bee *Apis mellifera*. Proc Natl Acad Sci U S A 110:8842–8846

McKey D (1974) Adaptive patterns in alkaloid physiology. Am Nat 108: 305–320

Merrill GB (1916) Report of the tobacco insect investigations. In: 4th report board commissioners Porto Rico, from 1st July 1914 to 30th June 1915. San Juan, pp 50-52

Moreira X, Castagneyrol B, Abdala-Roberts L, Traveset A (2019) A meta-analysis of herbivore effects on plant attractiveness to pollinators. Ecology 100:UNSP e02707

Muth F, Francis JS, Leonard AS (2016) Bees use the taste of pollen to determine which flowers to visit. Biol Lett 12:20160356

Nooten SS, Rehan SM (2020) Historical changes in bumble bee body size and range shift of declining species. Biodivers Conserv 29:451–467

Ohnmeiss TE, Baldwin IT (2000) Optimal defense theory predicts the ontogeny of an induced nicotine defense. Ecology 81:1765–1783

Palmer-Young EC, Sadd BM, Stevenson PC, Irwin RE, Adler LS (2016) Bumble bee parasite strains vary in resistance to phytochemicals. Sci Rep 6:37087

Palmer-Young EC, Hogeboom A, Kaye AJ, Donnelly D, Andicoechea J, Connon SJ, Weston I, Skyrm K, Irwin RE, Adler LS (2017) Context-dependent medicinal effects of anabasine and infection-dependent toxicity in bumble bees. PLoS One 12:e0183729

Palmer-Young EC, Farrell IW, Adler LS, Milano NJ, Egan PA, Junker RR, Irwin RE, Stevenson PC (2019) Chemistry of floral rewards: Intra-and interspecific variability of nectar and pollen secondary metabolites across taxa. Ecol Monogr 89:e01335

Parachnowitsch AL, Manson JS (2015) The chemical ecology of plant-pollinator interactions: Recent advances and future directions. Curr Opin Insect Sci 8:41–46

Poveda K, Steffan-Dewenter I, Scheu S, Tscharntke T (2003) Effects of below- and above-ground herbivores on plant growth, flower visitation and seed set. Oecologia 135:601–605

Poveda K, Steffan-Dewenter I, Scheu S, Tscharntke T (2005) Effects of decomposers and herbivores on plant performance and aboveground plant-insect interactions. Oikos 108:503–510

Quintero C, Bowers MD (2018) Plant and herbivore ontogeny interact to shape the preference, performance and chemical defense of a specialist herbivore. Oecologia 187:401–412

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Richardson LL, Adler LS, Leonard AS, Andicoechea J, Regan KH, Anthony WE, Manson JS, Irwin RE (2015) Secondary metabolites in floral nectar reduce parasite infections in bumblebees. Proc R Soc Lond B 282:20142471

Rivest S, Forrest JRK (2020) Defence compounds in pollen: Why do they occur and how do they affect the ecology and evolution of bees? New Phytol 225:1053–1064

Roger N, Michez D, Wattiez R, Sheridan C, Vanderplanck M (2017) Diet effects on bumblebee health. J Insect Physiol 96:128–133

Roulston TH, Cane JH (2000) Pollen nutritional content and digestibility for animals. Plant Syst Evol 222:187–209

Ruedenauer FA, Spaethe J, Leonhardt SD (2016) Hungry for quality-individual bumblebees forage flexibly to collect high-quality pollen. Behav Ecol Sociobiol 70:1209–1217

Rusman Q, Lucas-Barbosa D, Poelman EH (2018) Dealing with mutualists and antagonists: Specificity of plant-mediated interactions between herbivores and flower visitors, and consequences for plant fitness. Funct Ecol 32:1022–1035

Rusman Q, Lucas-Barbosa D, Poelman EH, Dicke M (2019) Ecology of plastic flowers. Trends Plant Sci 24:725–740

Shykoff JA, Schmid-Hempel P (1991) Incidence and effects of 4 parasites in natural-populations of bumble bees in Switzerland. Apidologie 22:117–125

Silva FA, Guirgis A, Thornburg R (2018) Nectar analysis throughout the genus *Nicotiana* suggests conserved mechanisms of nectar production and biochemical action. Front Plant Sci 9:1100

Soler R, Bezemer TM, der Putten V, Wim H, Vet LEM, Harvey JA (2005) Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. J Anim Ecol 74:1121–1130

Solt ML, Dawson RF, Christman DR (1960) Biosynthesis of anabasine and of nicotine by excised root cultures of *Nicotiana glauca*. Plant Physiol 35:887–894

Stevenson PC (2019) For antagonists and mutualists: The paradox of insect toxic secondary metabolites in nectar and pollen. Phytochem Rev

Stevenson PC, Nicolson SW, Wright GA (2017) Plant secondary metabolites in nectar: Impacts on pollinators and ecological functions. Funct Ecol 31:65–75

Thorburn LP, Adler LS, Irwin RE, Palmer-Young EC (2015) Variable effects of nicotine, anabasine, and their interactions on parasitized bumble bees. F1000 Research 4:880

Thornburg RW, Carter C, Powell A, Mittler R, Rizhsky L, Horner HT (2003) A major function of the tobacco floral nectary is defense against microbial attack. Plant Syst Evol 238:211–218

Tiedeken EJ, Egan PA, Stevenson PC, Wright GA, Brown MJF, Power EF, Farrell I, Matthews SM, Stout JC (2016) Nectar chemistry modulates the impact of an invasive plant on native pollinators. Funct Ecol 30:885–893

Wickham H (2016) ggplot2: elegant graphics for data analysis. R package version 3(2):1

Zangerl AR, Rutledge CE (1996) The probability of attack and patterns of constitutive and induced defense: A test of optimal defense theory. Am Nat 147:599–608

Zhang Y (2013) Likelihood-based and bayesian methods for tweedie compound poisson linear mixed models. R package version 0.7–8