

Nectar chemistry mediates the behavior of parasitized bees: consequences for plant fitness

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Abstract. Plants produce an array of secondary metabolites that play important ecological roles as anti-herbivore and anti-pathogen defenses. Many herbivores experience physiological costs when they consume secondary metabolites, yet some also benefit, for example when these chemicals confer resistance to parasites and predators. Secondary metabolites are often present in nectar and pollen, which is paradoxical given that floral rewards are important in the attraction of mutualists rather than deterrence of antagonists. Motivated by studies of interactions among plants, herbivores, and parasites, as well as research showing that secondary metabolites can reduce bee disease, we characterized the occurrence of two iridoid glycosides, aucubin and catalpol, in floral rewards and other tissues of the bee pollinated plant, *Chelone glabra*. We then experimentally investigated effects of nectar iridoid glycoside concentrations on the foraging behavior of bumble bee pollinators naturally afflicted by a parasitoid fly and a protozoan intestinal parasite, and subsequent effects on an estimate of plant reproduction. We found that floral nectar had lower iridoid glycoside concentrations than leaves, pollen, and corollas, and that, compared to those plant parts, the relative ratio of the two primary iridoid glycosides, aucubin and catalpol, was reversed in nectar. Whether bees carried parasitoid fly larvae did not affect their response to nectar chemistry; however, there was a significant interaction between protozoan parasite infection and nectar treatment, with infected bees foraging longer at flowers with high compared to low nectar iridoid glycoside concentrations. Parasitized bees were also more likely to return to inflorescences with high iridoid glycoside nectar. Consequently, flowers in the high iridoid glycoside nectar treatment donated significantly more pollen to conspecific stigmas than did flowers in the low iridoid glycoside treatment, suggesting an increase in male plant fitness. Taken together, these results demonstrate that nectar secondary metabolites can mediate the behavior of pollinators with subsequent benefits for estimates of plant reproduction.

Key words: bees; iridoid glycosides; multispecies interactions; parasitism; parasitoids; pollination; secondary metabolites.

INTRODUCTION

Plants form the base of many terrestrial food webs, and plant traits play important roles in the assembly of ecological communities (Strauss and Irwin 2004). For example, plants commonly defend their leaves and other tissues from herbivores with secondary metabolites (e.g., alkaloids, terpenoids, and phenolics) that are deterrent or poisonous to consumers (Fraenkel 1959). These compounds often structure tritrophic or multispecies interactions, as when herbivores sequester compounds as defenses against their predators and

parasites (Price et al. 1980, Dyer and Bowers 1996). Secondary metabolites are also found in floral nectar and pollen (reviewed in Adler 2000), a surprising occurrence since they are encountered here by plants' mutualist pollinators. On the one hand, nectar secondary metabolites may be an unavoidable, pleiotropic consequence of foliar defense against herbivores (Adler et al. 2012). On the other hand, evidence is mounting that, for some plant species, nectar and pollen secondary metabolites differ in quality and/or quantity from those found in other plant tissues (Cook et al. 2013, Gosselin et al. 2013). A number of adaptive hypotheses have been proposed to explain the existence of secondary metabolites in nectar and, by extension, pollen (Adler 2000). Secondary metabolites might protect nectar from microbes (Sasu et al. 2010) and nectar robbers and thieves (Stephenson 1981, Johnson et al. 2006) or from over-exploitation by pollinators (Heil 2011). Such benefits would be moot, however, if

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pollinators were also deterred from visiting flowers with nectar secondary metabolites, and there are reports of lethal and sublethal effects of floral secondary metabolites on bee consumers (Detzel and Wink 1993, Arnold et al. 2014). Floral secondary metabolites could also be pollinator attractants that affect plant fitness through increased pollination (Adler 2000, Kessler et al. 2008). Given that nectar secondary metabolite concentrations vary within plant populations (Kessler et al. 2012), the degree to which they are avoided or sought after by pollinators may have consequences for plant fitness.

Although most research has focused on nectar secondary metabolites as deterrents (e.g., Adler and Irwin 2005, Kessler et al. 2008), there are a number of mechanisms by which secondary metabolites could function as pollinator attractants. Nectar foragers could learn to associate the odor, taste or color of secondary metabolites with a food reward (Johnson et al. 2006). Secondary metabolites may enhance consumers' memory of floral rewards (Wright et al. 2013), which could increase pollinator fidelity for particular plants. In addition, secondary metabolites might function as rewards themselves if they confer benefits to consumers. For example, consumption of a variety of secondary metabolites in nectar, including alkaloids, terpenoids, and iridoid glycosides, reduces bumble bee infection by a trypanosome gut parasite, *Crithidia bombi* (Manson et al. 2010, Richardson et al. 2015). Given that parasites have been implicated in widespread declines of wild and managed bee populations (Potts et al. 2010), secondary metabolites found in nectar may be important in mediating the effects of bee disease. However, it is unknown whether bees increase their consumption of nectar with secondary metabolites when they are parasitized (Abbott 2014). Such self-medication behavior could increase plant fitness in pollen-limited species, assuming the benefits of increased pollinator visitation and pollination outweigh any costs associated with investment in floral chemistry.

The goal of this study was to examine the degree to which secondary metabolite identities and concentrations differed between nectar and pollen rewards and floral and vegetative tissues, and to ask how nectar secondary metabolites affected interactions among plants, bee pollinators and the parasites of bees. Using *Chelone glabra* L. (Plantaginaceae; hereafter *Chelone*), a bee-pollinated plant that contains iridoid glycosides (Bowers et al. 1993), which are also present in nectar (Richardson et al. 2015), we investigated the following three questions: (1) How do nectar iridoid glycoside concentrations vary within and between *Chelone* populations, and are they correlated with those of other plant parts? (2) Do nectar iridoid glycosides alter the foraging behavior of unparasitized bees relative to those challenged by natural enemies? (3) To what extent do pollinator behavioral responses to nectar iridoid glycosides affect an estimate of plant reproduction? We hypothesized that *Chelone*

nectar iridoid glycoside chemistry would be distinct from that of other plant tissues, reflecting the primary role of nectar in attracting mutualists rather than repelling antagonists. We also predicted that, consistent with Optimal Defense theory, plant tissues whose removal by consumers poses a risk to plant fitness, for example leaves and corollas, would have higher iridoid glycoside concentrations than those of floral attractants such as nectar (McKey 1974). We predicted that the degree to which bee pollinators were deterred from vs. attracted to nectar iridoid glycosides would be dependent on whether or not they were naturally attacked by each of two natural enemies, the gut parasite *Crithidia bombi* and the parasitoid fly *Phyocephala tibialis*. Because iridoid glycosides can reduce bee parasite loads (Richardson et al. 2015), we predicted that bees attacked by natural enemies would preferentially visit *Chelone* flowers with higher iridoid glycoside concentrations whereas uninfected bees would not show such preference or would even be deterred by them (Stephenson 1982). Finally, because *Chelone* pollinators are frequently affected by parasites and parasitoids (L. L. Richardson and R. E. Irwin, unpublished data), we predicted that flowers with higher nectar iridoid glycoside concentrations would receive longer and more frequent visits from parasitized bees, leading to an increase in pollen donation to other plants in the population.

METHODS

Study system

Chelone glabra is native to northeastern North America, occurring in a variety of wetland types, including calcareous fens, open marshes, and anthropogenic wetlands. In northern Vermont, USA where field work for this study was conducted, *Chelone* blooms from late July to early September. Plants produce numerous stems, each terminating in a racemose inflorescence bearing 2–12 flowers, of which 2.05 ± 0.05 flowers (mean \pm SE) are open at any one time (L. L. Richardson and R. E. Irwin, unpublished data; Fig. 1). *Chelone* flowers have a sympetalous white to rose-colored corolla with a partially restricted entrance that foragers must pry apart before entering. Individual flowers last 3.00 ± 0.12 d (mean \pm SE) and are protandrous, dispensing pollen the first day they are open but gradually elongating the style such that stigmas do not contact pollinators until the second day (L. L. Richardson and R. E. Irwin, unpublished data). Flowers have little or no detectable scent. Nectar production begins when flowers open in male phase and continues until corollas senesce. The average amount of nectar available to bee foragers ("standing crop") is 1.69 ± 0.17 μ L, and sugar concentration is $34.38\% \pm 1.17\%$ sucrose equivalents (L. L. Richardson and R. E. Irwin, unpublished data).

Chelone is pollinated by nectar- and pollen-collecting bees, especially *Bombus impatiens* and *B. vagans*



FIG. 1. A turtlehead (*Chelone glabra*) flower visited by a bumble bee (*Bombus* species). Flowers open sequentially on racemes, and approximately two are open each day.

(Cooperrider 1967, Heinrich 1975, Williams et al. 2014), which sonicate flowers to shake pollen from the anthers (Buchmann 1983, Heinrich 2004). *Chelone* is self-compatible but requires pollinator visitation to transfer pollen, even within flowers or plants, to set seed, and seed production is pollen limited in some populations (L. L. Richardson and R. E. Irwin, *unpublished data*; Cooperrider 1967).

Chelone glabra produces at least two iridoid glycosides, aucubin and catalpol, in foliar tissue (Bowers et al. 1993). Bowers et al. (1993) reported that catalpol was present in all leaf samples, and aucubin was present at lower concentrations and absent from some samples. Iridoid glycosides can be deterrent to generalist and non-adapted specialist herbivores (Bowers and Puttick 1988, Bernays and Chapman 2001, Pankoke et al. 2010); however, *Chelone* is also host to specialized herbivores that consume leaf and corolla tissue, some of which sequester aucubin and catalpol as defense against their natural enemies (Bowers and Puttick 1986, Bowers et al. 1992). Bees are known to avoid some secondary metabolites (Detzel and Wink 1993, Adler and Irwin 2005), so we reasoned that if *Chelone* nectar contained iridoid glycosides, then bees could potentially make foraging decisions based on their relative concentrations.

The bumble bee pollinators of *Chelone* are commonly host to natural enemies (Gillespie and Adler 2012),

of which we consider two here. *Crithidia bombi* Lipa & Triggiani (Trypanosomatida; hereafter *Crithidia*) is a protozoan intestinal parasite spread via contact with feces in the nest and at flowers (Schmid-Hempel 1998, Cisarovsky and Schmid-Hempel 2014). Infection rates of *Bombus* species by *Crithidia* in northeastern North American can be as high as 80% (Gillespie 2010; L. L. Richardson and R. E. Irwin, *unpublished data*). *Crithidia* has a range of negative effects on its host, including reduced overwintering survival, colony founding success and reproduction (Schmid-Hempel 1998). Although *Crithidia* infection reduces the ability of bees to use floral cues and learn while foraging (Gegear et al. 2005), bees can learn to avoid flowers contaminated with *Crithidia* (Fouks and Lattorff 2011). Consumption of the iridoid glycoside catalpol reduces *Crithidia* infection in *B. impatiens* (Richardson et al. 2015). While foraging, bumble bees may also be attacked by *Physocephala tibialis* Say (Conopidae; hereafter *Physocephala*), parasitoid flies that land on hosts in flight, inserting an egg into the abdomen (Malfi et al. 2014). Parasitism by these flies can be common, with up to 80% of bees affected (L. L. Richardson and R. E. Irwin, *unpublished data*; Gillespie 2010, Malfi and Roulston 2014). *Physocephala* parasitism causes sensory impairment (Müller and Schmid-Hempel 1993), changes in floral preference and fidelity (Schmid-Hempel and Stauffer 1998) and a reduction in foraging efficiency (Schmid-Hempel and Müller 1991) in the 10–12 d before host death (Müller 1994, Malfi et al. 2014). It is not known whether *Physocephala* is affected by bee diet chemistry, but given that haemolymph is the site of secondary metabolite sequestration for many herbivores, including Hymenoptera (Boevé and Schaffner 2003), *Physocephala* larvae may be exposed to iridoid glycosides as they develop in bees' haemolymph.

Question 1: How do nectar iridoid glycoside concentrations vary within and between *Chelone* populations, and are they correlated with those of other plant parts?

Plant collections

To characterize patterns of iridoid glycoside occurrence and concentration, in August 2012 we collected nectar, pollen, corollas, and leaves from approximately 15 plants at each of four *Chelone* populations in northern Vermont (Appendix S1: Table S1). Populations were separated by >4 km and all populations had >500 flowering *Chelone* stems. We used bags made of bridal veil to exclude insect visitors from newly opened flowers for 1–2 d. We removed pollen by sonicating anthers with an electric toothbrush, then extracted nectar with 5- μ L microcapillary tubes. To collect enough material for chemical analysis, we made nectar and pollen collections from up to five flowers for each individual plant. After removing nectar and pollen, we collected five leaves from the same plant as well as the corolla and attached

androecium (depleted of pollen), which we hereafter refer to as corolla tissue. We did not collect the gynoecium. For samples where we had a large enough volume, we measured nectar sucrose-equivalent sugars, expressed as percent Brix, with a refractometer (National Industrial Supply, Temecula, California, USA). Nectar was frozen after collection, and all corolla, leaf, and pollen samples were air dried for 1 week. All samples were thereafter stored at -13°C until sample preparation.

Sample preparation and gas chromatography

Leaves and corollas were finely ground with a coffee mill, which was cleaned with 70% ethanol after each sample. Leaves, corollas, and pollen were dried to a constant mass at 50°C before preparing approximately 50 mg samples (approximately 10 mg for pollen) for analysis. Nectar samples of 10–25 μL were prepared for analysis by adding 1 mL methanol, vortexing, and allowing liquid to evaporate. To extract iridoid glycosides from samples, we followed methods of Gardner and Stermitz (1988) and Bowers et al. (1993). Briefly, samples were extracted overnight in methanol and plant solids were removed by filtration and the eluate evaporated to dryness. We then added 1.0 mL of the internal standard phenyl- β -D-glucopyranoside (PBG, 0.500 mg/mL; Sigma Aldrich, St. Louis, Missouri, USA) and partitioned the sample with water and diethyl ether to remove hydrophobic compounds. The ether layer was removed and the water layer (containing the iridoid glycosides) evaporated. This residue was suspended in 1.0 mL methanol, and a 100- μL aliquot was removed for analysis. The methanol was evaporated and the remaining residue was derivatized using Tri-Syl-Z (Thermo Fisher, Waltham, Massachusetts, USA) in pyridine and heating to 70°C for 20 min before injection into a gas chromatograph (GC).

To quantify iridoid glycosides, we used an Agilent 7890A GC equipped with a DB-1 column (30 m, 0.320 mm, 0.25 μm particle size), using flame-ionization detection (Agilent Technologies, Santa Clara, California, USA). The temperature program used an initial temperature of 200°C held for 1 min, followed by a 3-min increase to 260°C , which was held for 8 min, followed by a 3-min increase to a final temperature of 320°C , held for 10 min. Mass of aucubin and catalpol in *Chelone* samples was quantified using the manufacturer's ChemStation B-03-01 software, based on calibration with pure compounds.

Statistical analyses

Except where otherwise noted, we used JMP 11.2 software (SAS Institute 2014) for data analyses. We used sample dry mass to calculate the percent dry mass and parts per million (ppm) concentration of each iridoid glycoside compound in samples. For nectar,

we estimated sample dry mass based on initial volume and sugar concentration. We report mean percent dry mass of each compound, but to improve data normality, for other statistical analyses we log-transformed concentrations, first adding 1 to values in order to retain ecologically relevant zero values for some samples. We used Spearman's ρ rank correlations to test whether aucubin, catalpol, and total iridoid glycoside (i.e., the sum of these two compounds) concentrations were correlated within and between plant tissues and rewards. We combined data across all four populations for the correlation analyses; examining correlations within populations yielded qualitatively similar results (data not shown). To test how aucubin and catalpol concentrations varied among plant parts, individuals, and populations, we used a linear mixed model with iridoid glycoside compound (aucubin vs. catalpol), plant part (corolla, leaf, nectar, and pollen) and their interaction as fixed effects and plant population and plant individual nested within population as random effects. In this analysis, we also included fully factorial interactions between plant population, plant part, and iridoid glycoside compound as random effects. For nectar iridoid glycoside concentrations, we used Tukey's honest significant difference (HSD) tests to compare aucubin and catalpol across the four sites.

Question 2: Do nectar iridoid glycosides alter the foraging behavior of unparasitized bees relative to those challenged by natural enemies?

Finding iridoid glycosides in the nectar of *Chelone* (see *Results*), we studied the effects of these compounds on bee foraging behavior using arrays of inflorescences bearing flowers with manipulated nectar iridoid glycoside concentrations. Experiments were conducted at two field sites between 08:00 and 17:00 in July–August 2013. We cut stems with inflorescences bearing pairs of open flowers to 75 cm and placed them in florist's water picks in 6×4 arrays with picks 25 cm apart, matching common plant spacing in natural *Chelone* populations. The arrays were surrounded by unmanipulated, wild-growing *Chelone*. For each trial, we randomly assigned half of the inflorescences to each of two nectar addition treatments, which we applied to both flowers of an inflorescence with a repeating micropipettor (Eppendorf, Hamburg, Germany). We prepared 30% sucrose solutions and added pure aucubin and catalpol (Sigma Aldrich, St. Louis, Missouri, USA) at concentrations similar to means measured at one plant population, Morse Farm (aucubin 1961 ppm; catalpol 455 ppm; see *Results*). Three treatments were applied to flowers over the course of the experiment: (1) 2 μL 30% sucrose solution without aucubin or catalpol addition; (2) 2 μL 30% sucrose solution with mean aucubin and catalpol concentration; and (3) 2 μL 30% sucrose solution with aucubin and catalpol concentrations +2 SD above the mean. Because of *Chelone's* floral morphology,

we could not remove existing nectar without damaging flowers; thus, our treatments represent alterations of existing floral nectar volume and chemistry. Given an average nectar standing crop of $1.69 \pm 0.17 \mu\text{L}$ (mean \pm SE), our treatments resulted in aucubin and catalpol concentrations approximately -1 , 0 , and $+1$ SD, respectively, from mean concentrations of the reference Morse Farm population. We hereafter refer to these additions as low, mean, and high iridoid glycoside treatments and, in each trial, we paired either high and low or high and mean iridoid glycoside treatments. The $2\text{-}\mu\text{L}$ nectar additions resulted in nectar standing crops above population means but within the natural range of variation we observed and the 30% sucrose solutions approximated mean *Chelone* nectar sugar concentration. This approach of diluting and augmenting nectar secondary metabolites has been used successfully in other systems to understand how nectar compounds affect pollinator behavior and pollination (e.g., Adler and Irwin 2005).

For each of 107 individual bees that visited arrays, we recorded bee species, time spent per flower (to the nearest millisecond), number of flowers visited on each inflorescence, and sequence of inflorescences visited, using a FileMaker Go database (Filemaker Pro 12.0; Filemaker, Santa Clara, California, USA; Supplementary file S1) on an Ipad tablet computer (Apple, Cupertino, California, USA). After each bee foraging bout, we refilled visited flowers with experimental solutions, and, to limit the influence of prior bouts, we changed all inflorescences hourly. Environmental variables, including atmospheric pressure, temperature, humidity, and wind speed, affect bee foraging behavior (e.g., Corbet et al. 1993), and to control for these, we paired each time-stamped foraging observation with the nearest quarter-hour meteorological record downloaded from a weather station 0.5–4.5 km from the field sites (www.weatherunderground.com, station KVTMONTP7).

We were able to capture 90% of bees after recording their foraging behavior to measure naturally occurring levels of parasite and parasitoid attack. We placed them in individual vials in a cooler at approximately 4°C . Live bees were kept refrigerated for up to 3 d, then transported to the lab for dissection. After sacrificing each bee, we removed the entire gastrointestinal tract, ground it in $300 \mu\text{L}$ dH_2O , vortexed the sample and allowed this to settle 6–8 h at room temperature. We then placed $10\text{-}\mu\text{L}$ aliquots on a haemocytometer slide and counted live *Crithidia* cells in five $0.004\text{-}\mu\text{L}$ gridded subfields. We summed these counts and calculated the density of cells per sample (cells/mL solution). Because many bees were free of infection, *Crithidia* cell count was strongly right skewed, and we here consider presence vs. absence of the parasite in bees. After removing the intestine, we dissected the abdomen, scoring each bee for the presence of *Physocephala* larvae. We measured the right forewing radial cell of

each bee with a dissecting microscope reticule to estimate body size (Harder 1985).

Statistical analyses

We calculated foraging metrics for bees visiting array flowers, including visit sequence (i.e., where an individual flower visit fell in a bee's total foraging bout in the array), duration of each flower visit, and duration and linear distance of flights between flowers. We log-transformed these variables, as well as wind speed and radial cell length, to satisfy assumptions of normality. We recorded visits by 10 species of bees, but here consider only the two species that together made $>98\%$ of all visits, *Bombus impatiens* and *B. vagans*.

Bee foraging bout length (i.e., total number of flowers visited) varied greatly (range 1 to >100 array flower visits, including revisits to the same flowers), thus we restricted the analyses to the first five flower visits made by each bee; 72% of *B. impatiens* and *B. vagans* observed visited at least five flowers in the array (results were qualitatively similar when we include greater numbers of flower visits). We analyzed the following metrics of bee foraging behavior in the array: proportion of flowers of each treatment visited, effect of treatment on inter-plant movement distance, effect of treatment on treatment of next flower visited, flower visit duration, and probability that bees would visit second flowers on inflorescences after visiting the first.

We used likelihood ratio tests to compare the proportion of visits to each treatment, considering a null hypothesis of equal visitation between treatments. In this analysis, we considered only visits to inflorescences where bees had no information about nectar treatment (e.g., from previous visits to flowers) and analyzed the two experiments (i.e., high vs. low and high vs. mean iridoid concentration) separately. To compare the distance and duration of flight away from flowers as a function of their nectar treatment, we used the Akaike information criterion (AIC) to select a best-fit ANCOVA model, considering in the full model as fixed effects visit sequence, nectar treatment, *Crithidia* and *Physocephala* infection, bee radial cell length, wind speed, atmospheric pressure, and temperature and, as random effects, plant population and bee individual nested within species. Finding that experiment type (i.e., high vs. low and high vs. mean) had no effect on bee foraging behavior (see *Results*), we combined both types of trials in analyses that follow.

We used a generalized linear mixed model (lme4 library for R statistical software; Bates et al. 2014, R Core Team 2014) to analyze flower visit duration and compared AIC scores to select a best-fit model (Bolker et al. 2009). In the full model, we evaluated as fixed effects nectar treatment, *Crithidia* and *Physocephala* presence/absence, radial cell length, air temperature, wind speed, array position of

inflorescence (interior vs. edge), array visitation sequence, duration of flight from last flower visited and experiment (high vs. low iridoids or high vs. mean iridoids); as random effects, we included plant population, individual bee and bee nested within species. To study the effect of parasites on bee foraging behavior, we constrained all candidate models to include interactions between nectar treatment \times *Crithidia* presence and nectar treatment \times *Physocephala* presence. We also constructed a similar generalized linear mixed model to study how nectar chemistry affected bees' decisions to visit second flowers or to move to new inflorescences in the array. Our binary response variable described whether bees visited the second flower on an inflorescence (yes vs. no).

Question 3: To what extent do pollinator behavioral responses to nectar iridoid glycosides affect an estimate of plant reproduction?

In 2013, we studied the impact of nectar iridoid glycosides on an estimate of male plant reproductive success, measured as pollen donation, by offering bees experimentally manipulated flowers. To estimate pollen donation, we used powdered fluorescent dye as a pollen analogue (JST-300; Radiant Color, Richmond, California, USA). Powdered fluorescent dyes have successfully served as pollen analogs in a variety of flowering species (e.g., Waser and Price 1982, Rossum et al. 2011), and have been used to compare pollen (dye) donation among flower or nectar treatments in other studies (Adler and Irwin 2005, Schaeffer and Irwin 2014). We focused on measuring male rather than female components of plant reproduction because male reproduction is often more closely tied to changes in pollinator behavior than female reproduction (Young and Stanton 1990), and measuring male reproduction as pollen (dye) donation allowed us to link changes in pollinator behavior as a function of nectar chemistry and pollinator parasitism to pollen (dye) transfer. Measuring female reproduction was beyond the scope of this study but can be measured in future research. For each replicate, we offered bees experimentally manipulated flowers using the "interview stick" method (Kearns and Inouye 1993). We placed a *Chelone* inflorescence with two open flowers in a water pick wired to a 1-m bamboo pole. We then manipulated nectar chemistry of each flower in one of three ways: (1) addition of 2 μ L of 30% sucrose solution with a high iridoid glycoside concentration; (2) addition of 2 μ L of 30% sucrose solution with a low iridoid glycoside concentration; or (3) a control treatment where we probed flowers with a micropipette tip but did not add any nectar solution. Artificial nectar solutions were mixed at the same concentrations as in Question 2. Following application of nectar treatments, we applied fluorescent dye powder (blue or green) to the anthers of dehiscing flowers using a wooden toothpick. We alternated use

of the blue and green dye colors haphazardly and used each for approximately equal numbers of replicates of each treatment.

We positioned experimental inflorescences among unmanipulated *Chelone* plants and bees appeared to forage normally on them. After a bee visited one flower of the treated inflorescence, we allowed it to visit three successive unmanipulated recipient flowers, after which we collected the bee and excised stigmas of the recipient flowers with forceps. The number of bees visiting each treatment was 16, 16, and 11, respectively. We were not able to reliably follow bees beyond the third flower they visited; however, research on other plant species has shown that >50% of pollen carryover by bumble bees is to the first three flowers (Thomson and Plowright 1980). We stained stigmas with basic fuchsin dye (Kearns and Inouye 1993), and used a dissecting microscope to count the number of dye particles present at 20 \times magnification. Bees were stored at approximately 4°C for up to 3 d before dissection to assess parasite load and body size (as described for Question 2).

Statistical analyses

We log-transformed right-skewed dye particle counts to satisfy assumptions of normality, first adding one to all replicates in order to retain observations of stigmas with no dye deposition. We excluded all visits to recipient flowers we assessed to be in male phase, given the assumption that stigmas of these flowers were not yet receptive. We used a repeated-measures ANOVA to test the effect of nectar treatment on pollen (dye) donation, evaluating as fixed effects nectar treatment, *Crithidia* presence, bee species (*B. impatiens* vs. *B. vagans*), radial cell length and, as a random effect, individual bee nested within bee species. The repeated term was flower visit sequence (first, second, or third). We could not evaluate effects of *Physocephala* on dye transport due to the small number of affected bees in this sample ($N = 5$ of 43 bees). We predicted that flowers with higher iridoid glycoside concentrations in nectar would donate significantly more dye, and we thus specified *a priori* linear contrasts between pairs of high and low iridoid glycoside treatments and low iridoid glycoside and control treatments to each of the three successively visited flowers.

RESULTS

Question 1: How do nectar iridoid glycoside concentrations vary within and between Chelone populations, and are they correlated with those of other plant parts?

We found aucubin and catalpol in 94% and 98% of all plant samples, respectively. Only a few nectar and pollen samples showed no iridoid glycosides and

Table 1. Spearman's rank correlation coefficients (ρ) of aucubin and catalpol concentrations in *Chelone glabra* from plants collected at four sites.

Plant part	Aucubin				Catalpol		
	Nectar	Pollen	Leaf	Corolla	Nectar	Pollen	Leaf
Aucubin							
Nectar							
Pollen	0.058						
Leaf	-0.053	0.044					
Corolla	0.001	-0.427	0.656***				
Catalpol							
Nectar	0.006	0.016	0.069	0.202			
Pollen	-0.016	-0.560**	-0.136	0.375	-0.191		
Leaf	0.275	0.198	0.751***	0.318	0.151	-0.262	
Corolla	-0.024	0.224	0.534**	0.419*	0.295	0.253	0.483**

Note: Values in boldface type indicate significant correlations between plant-part–chemical-compound combinations.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

it may have been that the samples were so small that iridoid glycoside content was below our analytical threshold. No other iridoid glycosides present in our library of known compounds were detected in the samples; however, in many nectar samples we noted the presence of an unknown compound eluting at approximately 20.4 min that could represent an undescribed iridoid glycoside.

The iridoid glycoside profile of nectar was distinctive from that of other plant tissues, with nectar concentrations of aucubin and catalpol not correlated with those of either compound in pollen, corollas, or leaves (Spearman's $|\rho| < 0.295$, $P > 0.13$; Table 1). Aucubin and catalpol concentrations were positively correlated in corolla and leaf tissue, and negatively correlated in pollen. Aucubin and catalpol concentrations were each positively correlated between leaves and corollas. Furthermore, corolla catalpol was positively correlated with leaf aucubin. All other individual iridoid-glycoside–plant-part combinations were not statistically associated with one another (Table 1). Total iridoid glycoside concentration (i.e., aucubin and catalpol together) was positively correlated between leaves and corollas ($\rho = 0.556$; $P = 0.002$), but not correlated among other plant part combinations, including those with nectar ($|\rho| < 0.265$; $P > 0.12$).

Iridoid glycoside concentration varied among plant parts over two orders of magnitude (corolla > leaf > nectar > pollen; $F_{3,9,2} = 30.8$, $P < 0.0001$; Table 2). Across all *Chelone* plant parts sampled, we found significantly higher concentrations of catalpol than aucubin ($F_{1,3,0} = 50.16$, $P = 0.006$), and there was a significant interaction between iridoid glycoside (aucubin vs. catalpol) and plant part driven by nectar, in which there was 1.24 times more aucubin than catalpol ($F_{3,8,7} = 14.0$, $P < 0.001$; Table 2). Tukey HSD tests showed that there were no significant differences among populations in either nectar aucubin or nectar catalpol concentrations ($P > 0.94$).

Question 2: Do nectar iridoid glycosides alter the foraging behavior of unparasitized bees relative to those challenged by natural enemies?

Nectar treatment had no effect on the proportion of flowers visited in either of the experiments (high vs. low or high vs. mean iridoid nectar treatments; $\chi^2 < 2.18$, $P > 0.14$), suggesting that bees did not alter their foraging behavior prior to tasting the nectar treatments of flowers. Once bees chose to visit flowers, however, we observed differences in foraging behavior, including per-flower visit duration and probability of visiting a second flower on inflorescences. The model that best fit per-flower visit duration included nectar treatment, *Crithidia* and *Physocephala* presence, and interactions between nectar treatment and parasites as fixed effects, flower visit sequence within the array, duration of flight from the last flower visited, and wind speed as covariates, and individual bee as a random effect. We found no overall effect of nectar treatment on the duration of bee visits to flowers ($\chi^2_6 = 8.10$, $N = 154$, $P = 0.23$, Fig. 2a). However,

Table 2. Aucubin and catalpol as a percentage of dry mass (mean \pm SE) in *Chelone glabra* across four plant parts sampled at four sites in 2012.

Plant part	N	Dry mass (%)		
		Aucubin	Catalpol	Total iridoid glycosides
Corolla	23	0.26 \pm 0.07	8.36 \pm 0.39	8.62 \pm 0.44
Leaf	31	0.63 \pm 0.06	4.18 \pm 0.34	4.81 \pm 0.38
Nectar	20	0.33 \pm 0.07	0.07 \pm 0.42	0.40 \pm 0.48
Pollen	25	0.01 \pm 0.07	0.36 \pm 0.38	0.38 \pm 0.43

Notes: Data are from ANOVA of untransformed dry mass values. Percent dry mass could not be computed for some nectar samples because sugar concentration (and thus total dry mass) could not be measured.

we did find a significant interaction between nectar treatment and *Crithidia* infection, with visits by infected bees to high iridoid glycoside treatment flowers >3 times longer than those to low iridoid glycoside treatment flowers (difference of 5.9 s; $\chi^2_2 = 6.68$, $N = 154$, $P = 0.04$; Fig. 2a). Moreover, across nectar treatments, visit duration of bees naturally infected with *Crithidia* was 17.2% shorter (difference of 2.9 s) than that of uninfected bees ($\chi^2_3 = 7.81$, $N = 154$, $P = 0.05$). *Physocephala* infection did not affect visit duration, and there was no interaction between nectar treatment and *Physocephala* infection ($\chi^2 < 1.49$, $P > 0.68$ in all cases; Fig. 2b). Covariates explained some of the variation in per-flower visit duration; in particular, wind speed was negatively associated with visit duration ($\chi^2_3 = 55.35$, $N = 154$, $P < 0.0001$) as was duration of flight from the previous flower ($\chi^2_3 = 29.58$, $N = 154$, $P < 0.0001$).

The model that best explained the probability that bees made second flower visits to inflorescences included nectar treatment, *Crithidia* and *Physocephala* infection, and nectar treatment \times *Crithidia* and nectar treatment \times *Physocephala* interactions as main effects, position of the inflorescence in the array (edge vs. interior) as a covariate, and individual bee as a random effect. Similar to visit duration, the probability of bees visiting second flowers on plants was affected by an interaction between nectar treatment and *Crithidia* infection ($\chi^2_2 = 6.13$, $N = 199$, $P = 0.047$). Notably, bees infected with *Crithidia* were nearly twice as likely as

bees without infection to make second visits to inflorescences with high iridoid glycoside nectar treatment (Fig. 2c). We found no significant main effects of nectar treatment ($\chi^2_6 = 11.37$, $N = 199$, $P = 0.08$) or *Crithidia* infection ($\chi^2_3 = 6.17$, $N = 199$, $P = 0.10$) on probability of second visits. Moreover, *Physocephala* infection and the nectar treatment \times *Physocephala* interaction were not statistically significant ($\chi^2 < 1.35$, $P > 0.56$ in all cases; Fig. 2d).

Nectar iridoid glycosides did not affect patterns of bee foraging behavior once bees departed from flowers. The best-fit models comparing flight distance and duration away from flowers included nectar treatment, *Crithidia* and *Physocephala* infection, and interactions as fixed effects, wind speed and bee size as covariates, and individual bee nested within species as a random effect. We found no significant effect of nectar treatment or any other factors for either the high vs. low ($F < 0.42$, $P > 0.52$) or high vs. mean ($F < 3.29$, $P > 0.07$) array trials. There were no significant effects on flight duration in the high vs. low comparison ($F < 1.80$, $P > 0.20$), but for the high vs. mean comparison, we found that bees infected with *Crithidia* took more than twice as long as uninfected bees to travel between flowers (difference of 4.8 s; $F_{1,8.17} = 15.40$, $P = 0.004$). In addition, flight duration between flowers was significantly positively associated with wind speed ($F_{1,76.71} = 4.56$, $P = 0.04$); other effects in the model were not significant ($F < 3.49$, $P > 0.08$).

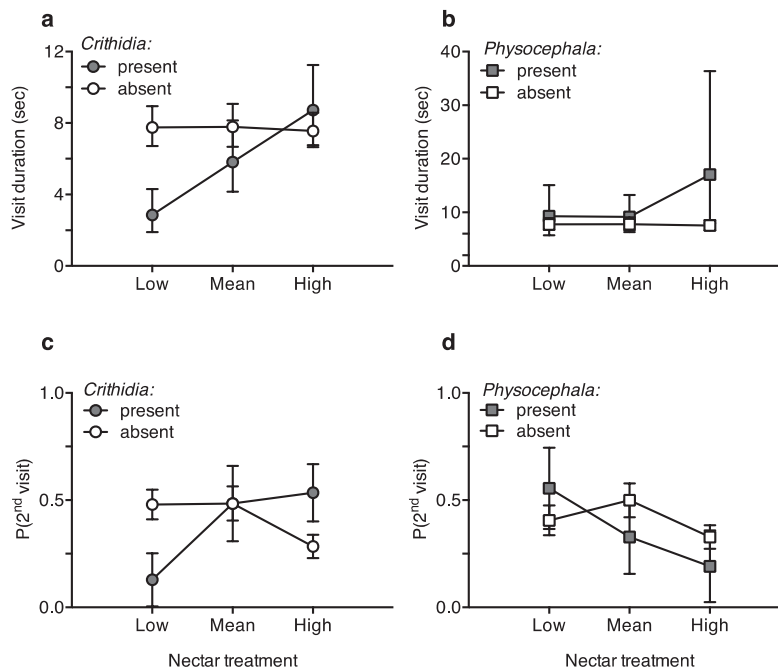


FIG. 2. Effects of nectar chemistry and bee parasites on (a, b) duration of bee visits to *Chelone glabra* flowers and (c, d) probability of second visits by bees to inflorescences. Values shown are least square means \pm SE from generalized linear mixed models (a, b) and probabilities from generalized linear mixed models (c, d).

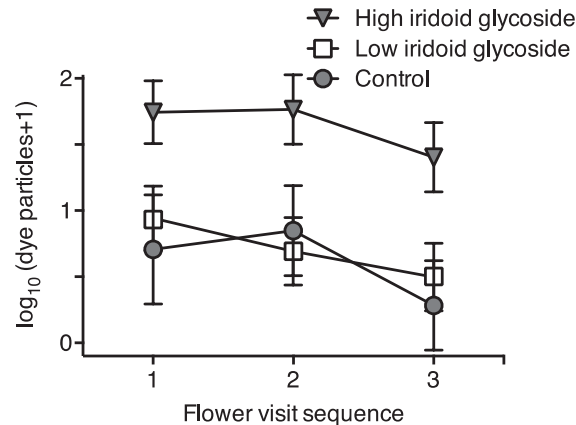


FIG. 3. Effects of experimentally manipulated high and low nectar iridoid glycoside concentrations and unmanipulated controls on the donation of pollen (dye) to stigmas of unmanipulated flowers. Values shown are least square means \pm SE from repeated-measures ANOVA.

Question 3: To what extent do pollinator behavioral responses to nectar iridoid glycosides affect an estimate of plant reproduction?

The best-fit model explaining pollen (dye) donation included nectar treatment, *Crithidia* infection, the *Crithidia* \times nectar treatment interaction, and bee species as fixed effects, and individual bee nested within species as a random effect. Flowers with a high concentration of iridoid glycosides in nectar donated 2.3 times more dye particles than those with low iridoid glycoside-nectar and 2.7 times as many dye particles as control flowers ($F_{2,27,21} = 5.85$, $P = 0.008$; Fig. 3). However, there was no effect of *Crithidia* infection, the *Crithidia* \times nectar treatment interaction, or bee species on dye deposition ($F < 0.81$, $P > 0.37$). Dye donation declined significantly from first to third flower visited ($F_{2,40,14} = 4.49$, $P = 0.02$; Fig. 3). Linear contrasts demonstrated that mean dye donation was different between high and low iridoid glycoside treatments at each of the three successively visited flowers (first flower, $t_{3,43,7} = 2.37$, $P = 0.02$; second flower, $t_{3,43,7} = 2.93$, $P = 0.0005$; third flower, $t_{3,43,7} = 2.48$, $P = 0.02$). By contrast, there were no significant differences in dye donation between low iridoid glycoside and control flowers for any of the three successively visited flowers (all $t_{3,44,8} < 0.51$, $P > 0.61$; Fig. 3).

DISCUSSION

The role of plant secondary metabolites as defenses against herbivores is well documented (Rosenthal and Berenbaum 1992), but their frequent occurrence in pollinator rewards, including floral nectar, is less well understood (Adler 2000). We provide evidence that *Chelone glabra*, a plant known to have foliage containing iridoid glycosides (Bowers et al. 1992, 1993), also expresses those compounds in floral parts where

they alter pollinator behavior and increase an estimate of male plant fitness. We show that, in this case, bee pollinators' interactions with the trypanosome parasite *Crithidia*, but not the parasitoid fly *Physocephala*, influence their response to experimentally manipulated nectar chemistry. Nectar secondary metabolites can thus mediate both pollination mutualisms and pollinator–natural-enemy interactions, acting as pollinator attractants that increase an estimate of plant reproductive success (shown here) while reducing bee disease loads (Richardson et al. 2015). Moreover, our observation of uninfected bees having reduced probability of visiting a second flower on stems with high nectar iridoid glycosides accords with previous studies that have demonstrated pollinator deterrence by nectar secondary metabolites (Detzel and Wink 1993, Kessler et al. 2008, Adler and Irwin 2012, Manson et al. 2013). Taken together, these results suggest that pollinators may be able to use nectar chemistry to self-medicate in the wild with implications for plant reproductive success.

Relatively few studies have compared the identities and concentrations of secondary metabolites in nectar to other foliar and floral tissues (reviewed in Irwin et al. 2014). We found that the iridoid glycosides aucubin and catalpol occur throughout *Chelone* vegetative and floral parts, and, similar to reports for other plants, concentrations were lower in nectar than leaves (Cook et al. 2013, Gosselin et al. 2013). This is the third plant species, to our knowledge, in which iridoid glycosides have been reported in floral nectar (Stephenson 1982, Lohaus and Schwerdtfeger 2014). Our study is novel in showing that the relative concentration of the two iridoid glycosides, aucubin and catalpol, varied between nectar and other plant parts: in nectar, aucubin was the main iridoid glycoside, while catalpol predominated in other *Chelone* parts. Moreover, in contrast to allocation patterns we observed in corollas,

leaves, and pollen, nectar aucubin and catalpol concentrations were not correlated with concentrations of either compound in other plant tissues. In particular, it is notable that plants with higher investment in foliar defense did not also have higher nectar iridoid glycoside concentrations. These patterns are interesting given that catalpol is reportedly more of a deterrent to consumers than aucubin is (Bowers and Puttick 1986). While the sites of iridoid glycoside biosynthesis in *Chelone* are unknown, the patterns of chemical expression described here suggest that plants may regulate expression of secondary metabolites in different tissues, and that the presence of these compounds in floral nectar is not a pleiotropic consequence of foliar defense. It is important to note that, although both compounds are products of the same biosynthetic pathway in *Chelone*, aucubin is a chemical precursor of catalpol (Damtoft 1994, Konno et al. 1999). Understanding the relative importance of these two compounds in floral rewards will require a careful examination of each compound's production by plants and impact on pollinator behavior and performance.

Nectar secondary metabolites are known to both attract and repel consumers, and their effects are often dose dependent (Detzel and Wink 1993). Previous studies that manipulated nectar chemistry have shown that the compounds deter pollinator visitation, at least at the highest concentrations (Adler and Irwin 2005, 2012, Galen et al. 2011, Manson et al. 2013). Our results are novel in that we find a dose-dependent effect of nectar secondary metabolites on bee foraging behavior that is mediated by bee parasites: we found no response to iridoid glycosides among uninfected bees, but a reduced consumption of low and increased consumption of high iridoid glycoside nectar by infected bees. The mechanisms underlying such context-dependent behavioral plasticity are not known. One hypothesis that warrants examination is that, following parasitism, bees experience an increase in taste perception of catalpol, as has been demonstrated for a lepidopteran foliar herbivore whose diet includes iridoid glycosides (Bernays and Singer 2005).

Previous research has demonstrated that insect herbivores can self medicate, changing their foraging behavior when parasitized and resulting in an increased consumption of secondary metabolites (Singer et al. 2009, Abbott 2014). Combined with the discovery that consumption of catalpol can lower *Crithidia* infection of *B. impatiens* (Richardson et al. 2015), our results suggest the interesting possibility that bumble bees may self medicate with iridoid glycosides when challenged by natural enemies, increasing visitation to flowers with high iridoid glycosides in nectar relative to uninfected bees. This potential self-medication behavior is not unique to bumble bees. For example, honey bees (*Apis mellifera* L.) increase their collection of plant resins (propolis) with antibiotic function in response to nest pathogens (Simone-Finstrom and Spivak 2012), and have been shown to increase consumption of nectar

secondary metabolites with antibiotic properties when parasitized (Gherman et al. 2014). Our foraging experiment suggests that parasitized bumble bees return to the nest with greater quantities of nectar iridoid glycosides than uninfected conspecifics, ingestion of which should lower their *Crithidia* parasite load (Richardson et al. 2015). Bumble bees are social insects and workers make foraging decisions based both on individual and colony needs; we could thus have underestimated the importance of parasites in shaping bee foraging behavior, since many individuals from infected colonies show no sign of infection themselves (Otterstatter and Thomson 2007). Our experimental design did not allow us to assess whether colony characteristics such as degree of parasitism affect individual bee foraging behavior, but this possibility warrants further research.

In contrast to the effects of *Crithidia* on the foraging behavior of its host, we found no effect of *Physocephala* attack on bee response to nectar chemistry. There are several possible explanations for this result. First, *Crithidia* inhabits the bee gut, where it is likely in direct contact with secondary metabolites consumed by bees, whereas *Physocephala* larvae develop in the haemolymph of the bee abdomen, where their exposure to these compounds could be lower. Second, ingestion of iridoid glycosides can weaken consumers' ability to fight off parasitoid attack, specifically by impairing the melanization response (Smilanich et al. 2009), so bees attacked by parasitoids might suffer greater negative effects of parasitism when they forage at flowers with higher concentrations of iridoid glycosides. Third, attack by *Physocephala* causes changes in the foraging behavior of bumble bees (Müller and Schmid-Hempel 1993), which could impair their ability to respond to dietary cues such as nectar chemistry.

Changes in the foraging behavior of parasitized bees as a function of nectar chemistry, such as those that we documented, are likely to increase plant reproductive success. In other systems, per-flower pollinator visit duration is positively correlated with male as well as female estimates of plant fitness (Mitchell and Waser 1992, Ivey et al. 2003, Sahli and Conner 2007). And, visitation to greater numbers of flowers on an inflorescence may increase seed set via both outcrossing (Krannitz and Maun 1991) and selfing (Robertson and MacNair 1995, Karron et al. 2003). Here we document an increase in one estimate of male plant reproduction, donation of pollen (dye) to conspecifics, by bees visiting flowers with higher iridoid glycosides in nectar. However, in this experiment, we did not find an interaction between nectar treatment and parasite status that would have been consistent with the results of the array foraging experiment (Question 2). Future research that manipulates both bee parasitism and nectar chemistry and measures pollen (dye) transfer will yield additional mechanistic insight. Moreover, although we only measured male plant reproduction in this study, male and female function often show correlated responses to

changes in pollinator behavior (reviewed in Schaeffer et al. 2013), and *Chelone* is pollen limited for female plant reproduction in some sites and years (L. L. Richardson and R. E. Irwin, *unpublished data*). Thus, we predict that there would be a correlated response in female fitness, measured as seed set. However, because *Chelone* is self compatible, we cannot rule out the possibility that increased within-plant pollinator visitation could increase inbreeding via selfing (Dejong et al. 1993). Future research would benefit from measuring not only estimates of male, but also female, reproduction.

Four caveats are important to the interpretation of this study. First, we measured natural rates of parasite infection and parasitoid attack of individual bumble bee foragers, but were not able to manipulate individual- or colony-level disease in the field setting of our experiments. The correlation we report between individual bee parasite infection and response to nectar chemistry suggests that bees make foraging decisions based on concentration of medicinal chemicals in nectar, but more rigorous manipulative experiments will be necessary to fully understand the patterns we observed. Second, *Crithidia* is known to impair bee foraging behavior and learning (Müller and Schmid-Hempel 1993, Gegeer et al. 2005). Thus, it is possible that the patterns we observed in pollinator foraging behavior were caused by bee impairment and not dietary plasticity. Our observations that bees infected with *Crithidia* made shorter per-flower visits yet took longer to fly between flowers could be evidence of such impairment; however, bee responses to nectar manipulations, no matter what the underlying mechanism, would still translate into changes in pollination. Third, it is important to recognize that bees, including those in our field experiments, commonly collect *Chelone* pollen and nectar simultaneously. They could therefore be making foraging decisions based on taste and odor cues from both resources (Dobson et al. 1996), but we only manipulated nectar in this study. We found that nectar and pollen iridoid concentrations were not correlated, meaning that if bees evaluate chemistry of both rewards upon landing at flowers, they must evaluate each independently. Finally, we did not account for the influence of plant genetic diversity or environmental factors on variation in nectar iridoid glycosides; yet, such factors often shape plant chemistry. For example, plant genetic diversity can exert trait-mediated interaction modifications on tritrophic systems involving herbivores and their natural enemies (Abdala-Roberts and Mooney 2014). Foliar herbivores can also exert top-down pressure on plant trait expression, as when their feeding causes increases in nectar secondary metabolite concentrations (Adler et al. 2006). In order to understand how selective pressures shape tritrophic interactions mediated by *Chelone* chemistry, it will be necessary to experiment with plants of known genetic background in a context where herbivory can be controlled.

Secondary metabolites are critical in structuring tri-trophic interactions among plants, herbivores, and

their parasites (Price et al. 1980). We extend these concepts to the floral interface, demonstrating that nectar secondary metabolites structure multispecies interactions among plants, pollinators, and their natural enemies, with important consequences for outcomes of pollination mutualisms. We find evidence that secondary metabolites could be a “reward” exchanged between plants and their pollinators for the service of pollen transfer. Considering the widespread occurrence of secondary metabolites in nectar of pollinator-dependent angiosperms (Baker 1977), these results suggest that nectar chemistry should be considered by studies evaluating the relative benefits of pollination mutualisms to both plants and pollinators. Moreover, given widespread declines of bees caused in part by parasites (Potts et al. 2010, Cameron et al. 2011), nectar secondary metabolites could play important roles in maintaining bee health.

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