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LETTER

Ecological context influences pollinator deterrence by alkaloids in floral nectar

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Abstract

Secondary compounds may benefit plants by deterring herbivores, but the presence of these defensive chemicals in floral nectar may also deter beneficial pollinators. This trade-off between sexual reproduction and defense has received minimal study. We determined whether the pollinator-deterring effects of a nectar alkaloid found in the perennial vine *Gelsemium sempervirens* depend on ecological context (i.e. the availability of alternative nectar sources) by monitoring the behavioural response of captive bumblebees (*Bombus impatiens*, an important pollinator of *G. sempervirens* in nature) to nectar alkaloids in several ecologically relevant scenarios. Although alkaloids in floral nectar tended to deter visitation by bumblebees, the magnitude of that effect depended greatly on the availability and nectar properties of alternative flowers. Ecological context should thus be considered when assessing ecological costs of plant defense in terms of pollination services. We consider adaptive strategies that would enable plants to minimize pollinator deterrence because of defensive compounds in flowers.

Keywords

Bumblebee, cost of resistance, ecological costs, gelsemine, *Gelsemium sempervirens*, herbivory, nectar alkaloids, pollination, pollinator behaviour, toxic nectar.

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INTRODUCTION

The interaction between plants and their animal pollinators has been a significant force in the evolution of floral characters. However, plants also simultaneously interact with other types of animal visitors, such as herbivores, which may affect pollination and ultimately influence the evolution of floral characters. Although herbivory can influence pollination through direct damage to reproductive tissues (e.g. pistils and stamens, Leege & Wolfe 2002) or floral characters used to attract pollinators (e.g. corolla characters, Strauss *et al.* 1996; for further review see McCall & Irwin 2006), it can also influence pollination through more subtle, but nonetheless ecologically significant, mechanisms (Agrawal *et al.* 1999; Strauss *et al.* 1999, 2002). For instance, pollination services to plants may be reduced when plant characters used to defend against herbivores are linked to characters used to attract pollinators (Simms & Bucher 1996; Strauss 1997). This integration of attractive and defensive traits presents plants with a potential fitness trade-off between the benefits of pollinator attraction and the costs of reduced herbivore defense. Consequently, many pollination- and herbivory-related traits that have traditionally been considered the result of selection by pollinators or herbivores alone may actually be an evolutionary compromise between the contrasting selection pressures exerted by both plant interactors together (Herrera *et al.* 2002). Despite the important implications of interactions between pollination and herbivory for the ecology and evolution of plant characters, there is little known about how pollinator visitation is influenced by the concurrent presence of plant attractive and defensive traits. Here, we describe a series of controlled experiments that were designed to examine the effects of plant defensive compounds in flowers on the attractiveness of plants to pollinators.

From the pollinator's perspective, the attractiveness of plants is determined primarily by the perceived amount of beneficial compounds such as carbohydrates and amino acids contained in floral nectar (Proctor *et al.* 1996). Paradoxically, floral nectar of some plant species also contains secondary compounds such as phenols and alkaloids (Baker & Baker 1983) that occur in leaves, stems, and roots to defend against attack by herbivores and microorganisms (Berenbaum 1995). Indeed, secondary compounds have been reported in the floral nectar of at least 21 angiosperm families (Adler 2000), indicating that

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this phenomenon is widespread. Although the presence of secondary compounds in floral nectar may provide reproductive benefits to plants in some special cases (see Rhoades & Bergdahl 1981; Adler 2000 for reviews of hypotheses), it is predicted to have detrimental effects if pollinators are deterred from visiting flowers (Strauss *et al.* 1999; Adler & Irwin 2005). Although we are testing adaptive hypotheses in other work, here we implicitly assume that defense chemicals occur in nectar as an unavoidable byproduct of their production in other tissues; we therefore consider the deterrence of pollinators as an *ecological cost of defense* (Strauss *et al.* 1999, 2002).

Because the foraging decisions of pollinators are contingent on current floral conditions and past floral experiences, the ecological cost of defense to plants with secondary compounds in floral nectar likely depends on the ecological context in which pollinators interact with flowers. For example, ecological costs may be reduced if secondary compounds are present in nectar for short periods or increased if pollinators have the option of visiting alternate plants with no secondary compounds in floral nectar. How these costs vary with ecological context has not been rigorously examined, presumably because of the difficulty in manipulating floral environments and tracking the behaviour of individual pollinators under natural conditions.

Based on the pollination ecology of *Gelsemium sempervirens* (L.), we devised laboratory choice experiments in which we used one of its major floral visitors and pollinators, the bumblebee *Bombus impatiens* Cresson (Ornduff 1970; Manson, unpublished data), as a model system to investigate how ecological context influences the effects of secondary compounds in floral nectar on pollinator choice behaviour and foraging proficiency (flowers visited per minute and flower-handling time). *Gelsemium sempervirens* is an obligate outcrosser and secretes the commercially available alkaloid gelsemine in floral nectar. Previous work has shown that bumblebees (*Bombus bimaculatus*, which is closely related to *B. impatiens*) spend less time on *G. sempervirens* flowers and visit fewer flowers per plant when gelsemine concentrations are increased in nectar (Adler & Irwin 2005, 2006), suggesting that nectar gelsemine imposes an ecological cost on plants by altering visitation by bumblebees. We determined whether the behavioural response of bumblebees to gelsemine-rich floral nectar, and thus the ecological cost of defense to plants, depends on ecological context by monitoring the choice behaviour, foraging rate and flower-handling time of freely foraging bees on artificial floral arrays that simulated the following ecologically relevant scenarios: (i) *G. sempervirens* co-occurring and co-flowering with an equally rewarding plant species without alkaloids in floral nectar, (ii) *G. sempervirens* co-occurring and co-flowering with less rewarding plant species without alkaloids in floral nectar, and (iii) a population of *G. sempervirens* in which plants have either a low or high level of gelsemine in floral nectar. By comparing the behaviour of bees foraging under these conditions, we were not only able to assess ecological costs of gelsemine in floral nectar to *G. sempervirens* in terms of potential bumblebee pollination services, but provide new perspectives on the adaptive significance of traits commonly observed in plant species with secondary compounds in floral nectar.

METHODS

Bees and flowers

Colonies of *Bombus impatiens* Cresson, each with 30–50 workers, were obtained from Biobest Canada (Leamington, ON, Canada). Nest boxes were connected to a 2.2 × 2.2 × 2.4 m flight cage by a gated tube so that we could control the number of bees entering the flight cage. Prior to experiments, colonies were allowed to collect 30% w/w sugar solution from feeders located in the center of the flight cage. Colonies were supplied with pollen *ad libitum*. Workers that made regular foraging trips between the colony and feeders were individually marked with coloured liquid paper.

Artificial flowers were constructed by removing the lids from 30 yellow and 30 blue 1.5 mL microcentrifuge tubes and adding 4.2 cm circles of blue and yellow construction paper respectively around the mouth of the tubes. Yellow flowers resembled the tubular yellow flowers of *Gelsemium sempervirens*. A blue–yellow colour dimorphism was used to make it easier for bees to discriminate flowers based on gelsemine and sucrose content of nectar. To access the test solution (hereafter referred to as 'nectar'), bees had to land on the surface of the paper corolla and crawl to the bottom of the tube, much as they do in real *Gelsemium* flowers. Flowers were presented to bees by embedding them upright in a 1.26 × 0.79 × 0.032 m styrofoam board covered in green paper. Flowers were positioned in a 67.5 cm by 56.0 cm grid so that bees had an equidistant choice of each flower type upon departing any flower on the array (with the exception of the two outer columns). Our artificial array was designed in this manner so that we could create realistic floral environments for bees while controlling for the availability, distribution and nectar properties of flowers.

The principal alkaloid found in the nectar of *Gelsemium sempervirens* is gelsemine (Irwin & Adler 2006), with natural concentrations ranging across populations from 5.8 to 246.1 ng μL^{-1} (Adler & Irwin 2005). Sucrose concentrations in *G. sempervirens* nectar reportedly range from 11% to 62% under natural conditions (Leege & Wolfe 2002; Adler & Irwin 2005; Manson, personal observation). For our behavioral assays, nectar containing both gelsemine and sucrose was created by adding gelsemine hydrochloride

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Table 1 Descriptions of the reward conditions used in each behavioural assay

	Nectar condition		
Assay	Gelsemine-poor	Gelsemine-rich	Ecological significance
1A	30% w/w sucrose	30% w/w sucrose with 50 ng μL^{-1} gelsemine	50 ng μL^{-1} is in the middle range of natural concentrations of gelsemine in *G. sempervirens* nectar (Adler & Irwin 2005)
1B	50% sucrose	50% sucrose with 50 ng μL^{-1} gelsemine	Simulates high nectar concentrations found in the field (Adler & Irwin 2005)
1C	30% sucrose	30% sucrose with 5 ng μL^{-1} gelsemine	5 ng μL^{-1} is the lowest concentration of gelsemine found in *G. sempervirens* nectar (Adler & Irwin 2005)
2	30% sucrose	50% sucrose with 50 ng μL^{-1} gelsemine	Presents bees with a trade-off between palatability and economic gain
3	30% sucrose with 50 ng μL^{-1} gelsemine	30% sucrose with 125 ng μL^{-1} gelsemine	Simulates a possible induced chemical response to herbivory that increases nectar alkaloid concentrations or natural variation in nectar alkaloid concentrations

Sucrose and gelsemine concentrations used in each assay were selected based on their ecological significance. Concerning Assay 3, note that gelsemine production by *G. sempervirens* has yet to be characterized as either induced or constitutive.

(ChromaDex, Santa Ana, CA, USA) to aqueous sucrose solutions (either 30% or 50% w/w sucrose) until all gelsemine was dissolved (gelsemine concentrations were 0, 5, 50 and 125 ng μL^{-1} sucrose solution; Table 1). Solutions were refrigerated at 4 °C when not in use and replaced every 3–5 days. For brevity, we refer to the sucrose concentration of nectar as either S30 or S50 and the gelsemine concentration of nectar as either G0, G5, G50, or G125.

Experimental procedure

We determined the behavioural response of bumblebee foragers to gelsemine in nectar under the three ecological scenarios described above. Gelsemine and sucrose concentrations used in each assay were selected based on values reported for *G. sempervirens* under natural conditions (Table 1).

Marked bees were trained by allowing them to forage freely on an array of each flower type (i.e. each nectar condition) in succession for three foraging trips This procedure ensured that bees had experienced the nectar condition associated with each flower colour prior to testing. The flower colour associated with each nectar condition was randomized among bees to control for the possibility that floral preference was influenced directly by colour. Immediately following training, bees were individually presented with a mixed array containing 30 flowers of each type, and we videotaped at least 80 flower visits for later analysis. Flowers were filled with 3 μL of nectar and refilled quickly after being drained by bees. Flowers were replaced between bees. After testing, bees were freeze-killed, and body size was estimated by measuring the length of the radial cell on the right forewing (Harder 1982).

Data analysis

For each assay, we determined whether bees overall had a preference for flowers with lower levels of gelsemine on the mixed array (i.e. visitation frequency was non-random with respect to alkaloid level) by using a two-tailed one-sample *t*-test to compare the mean proportion of visits to flowers with the lower concentration of nectar gelsemine to the proportion of visits expected given the abundance of both flower types on the mixed array (0.5 in all cases). Proportions were arcsin-transformed so that they conformed more closely to a normal distribution. We then examined how the flower-choice behaviour of individual bees changed as they gained foraging experience on the mixed array by dividing the first 80 flower visits for each bee tested into four blocks of 20 consecutive visits. For each block, we determined whether individuals had a preference for one of the available flower types by using a G-test of independence (Sokal & Rohlf 1995) to compare the observed frequency of visits to low gelsemine flowers to the frequency of visits expected given random flower selection (10 visits). An observed visit frequency of 15 or greater indicated that the bee had a preference for low gelsemine flowers whereas a visit frequency of five or less indicated a preference for high gelsemine flowers. We then tested for changes in flower-choice behaviour of bees over time by using a repeated-measures ANOVA to compare visit frequency to low gelsemine flowers among the four blocks, followed by Tukey's multiple comparison test.

Because previous work has suggested that nectar alkaloids, including gelsimine, may affect plant fitness by altering the behaviour of pollinators on flowers rather than through pollinator deterrence (Strauss *et al.* 1999; Adler & Irwin

2005), we also examined the effect of gelsemine on bee foraging proficiency. Here, we assess foraging proficiency by measuring foraging rate (number of flowers visited per minute) and flower-handling time (total time in seconds that the bee spends on a flower), which are two components of bumblebee behaviour that may affect how they collect and deposit pollen and thus provide another measure of how nectar gelsemine may influence plant reproductive success though behavioral alterations to bees. We used a generalized linear model with radial cell length as a covariate (Proc Genmod; SAS Institute 1999) to compare foraging rates and flower-handling times of individuals that showed a preference (i.e. visitation frequency was significantly biased in favour of the flower type) for S30G0 flowers ($n = 20$) and those that showed a preference for S30G50 flowers ($n = 10$). Measures of foraging proficiency were calculated based on 10 consecutive flower visits taken randomly between visits 50 and 70 and results are reported as likelihood ratio statistics (*G*; SAS Institute 1999). All foraging proficiency measures were log-transformed to meet the assumptions of normality and equal variance.

RESULTS

Flower preference

Bumblebee choice behaviour was significantly influenced by the level of gelsemine in the nectar of available flower types. Although bees as a group readily collected floral nectar containing gelsemine on monotypic arrays during training, they had a strong preference for nectar with equal sucrose rewards but no gelsemine (Assays 1A-C) or lower levels of gelsemine (Assay 3 – S30G50 vs. S30G125) on mixed arrays (Table 2). Bees showed no overall nectar preference when the sucrose concentration of nectar with alkaloids was increased relative to an alkaloid-free nectar alternative (Assay 2 – S30G0 vs. S50G50; Table 2). In fact, there was a significant decrease in the proportion of visits to flowers with no nectar gelsmine between Assays 1A and 2 ($t = 6.37$, d.f. $= 16$, $P < 0.001$), indicating that an increase in sucrose concentration of floral nectar containing gelsemine relative to alkaloid-free nectar alternatives increased its attractiveness to bees.

Table 2 Results of one-sample *t*-test for Assays 1–3

Assay	Mean proportion of visits to low gelsemine flowers	*t*-value	d.f.	*P*-value
1a	0.86 ± 0.04	7.932	12	< 0.0001
1b	0.76 ± 0.09	2.54	10	0.029
1c	0.84 ± 0.07	4.89	8	0.0012
2	0.50 ± 0.10	−0.149	10	0.88
3	0.82 ± 0.04	7.04	8	0.0001

For each assay, the mean proportion of visits to the flower type with the lower level of nectar alkaloids on the mixed array was compared with the proportion of visits to flowers with lower nectar alkaloids expected given the abundance of both flower types on the mixed array (0.5 in all cases). Means are given ± SE.

At the individual level, there was a considerable amount of variation in flower-choice behaviour of bees on the mixed array over time (Fig. 1). With the exception of Assay 2, most individuals had a strong preference for flowers with nectar containing sucrose only or low levels of gelsemine in the final visit block (percentage of bees with a preference for flowers with no or low nectar gelsemine in the final visit block was: 92.3% (Assay 1A), 63.6% (Assay 1B), 77.8% (Assay 1C), 36.4% (Assay 2), and 77.8% (Assay 3). Interestingly, a small percentage of bees showed a preference for flowers with higher nectar gelsemine concentrations in the final visit block [7.7% (Assay 1A), 9.1% (Assay 1B), 11.1% (Assay 1C), 45.5% (Assay 2)]. The mean proportion of visits to the flower type on the mixed array with the lower nectar gelsemine concentration differed significantly among visit blocks for Assay 1A ($F_{3,12} = 12.32$, $P < 0.0001$), 1B ($F_{3,10} = 7.09$, $P = 0.001$), 1C ($F_{3,8} = 6.98$, $P = 0.0015$, and 3 ($F_{3.8} = 6.61$, $P = 0.0021$), but not for Assay 2 ($F_{3,10} = 0.542$, $P = 0.657$). Pairwise comparisons showed that the proportion of flower visits to low gelsemine flowers significantly increased between blocks 1 and 3 and blocks 1 and 4 for Assays 1A-C and Assay 3 and also between blocks 2 and 3 and blocks 2 and 4 for Assay 1A, indicating that bees tended to sample both flower types on the mixed array prior to developing a preference for the flower type with the lower level of nectar gelsemine.

Foraging proficiency

Visitation to gelsemine-rich flowers had no significant effect on the foraging rate or mean handling time (Table 3). Bee size, determined from radial cell length, did positively correlate with foraging rate ($G_{1,27} = 5.01$, $P = 0.03$), but did not correlate with mean handling time. There was no interaction between preference and size for any of the three foraging efficiency measures, so the interaction term was removed from the model.

DISCUSSION

Our study supports the hypothesis that defensive compounds in floral nectar impose an ecological cost on plants in the form of reduced pollinator visitation, and demonstrates for the first time that such ecological costs to plants depend heavily on the ecological context in which pollinators make foraging decisions. Bumblebees readily foraged on monotypic arrays of alkaloid-rich flowers

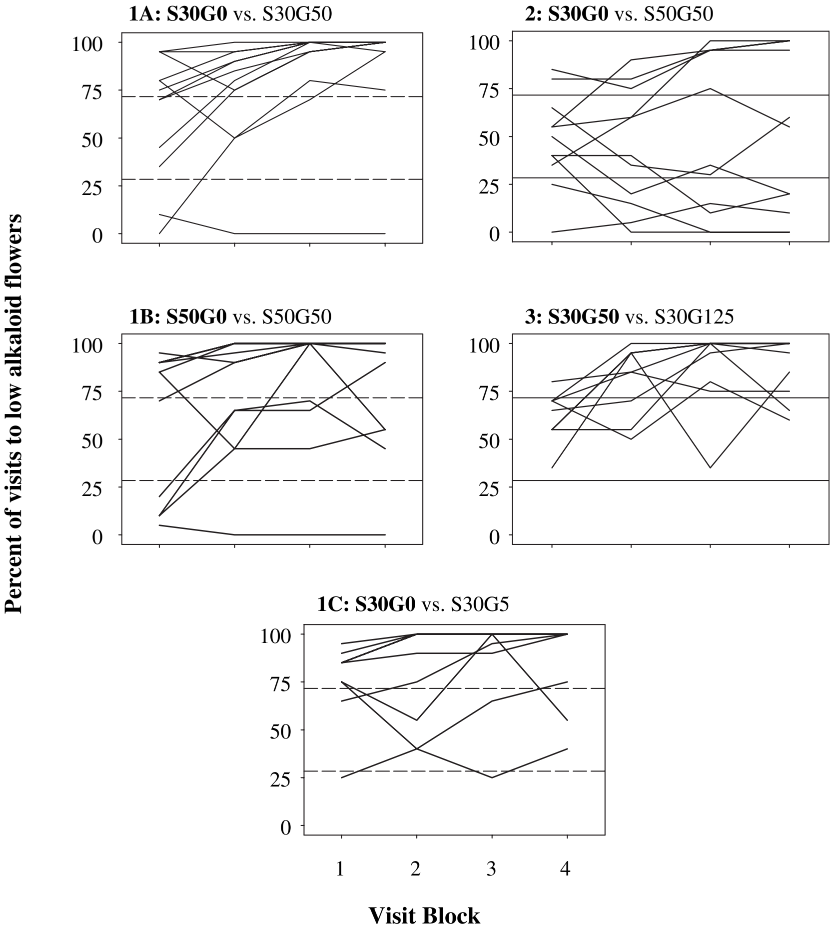

Figure 1 Choice behaviour of individual bees over four consecutive blocks of 20 flower visits. Figure panel numbers correspond to assay numbers from Table 1. Changes are displayed in percent preference for flowers with low or no gelsemine in nectar, as indicated by the treatment in bold text, and each line represents the preference trajectory of a single foraging bee. Dashed reference lines demarcate zones of significance as determined by G-test values, with percent of visits to flowers with low or no gelsemine in nectar above 71.6% indicating that the bee had a preference for flowers with no or low levels of gelsemine in nectar and below 28.4% indicating that the bee had a preference for flowers with gelsemine in nectar.

Table 3 Generalized Linear Model results for bee foraging rate (flowers visited per minute), and flower-handling time (in s)

Foraging proficiency measure	Sucrose only	Sucrose and gelsemine	G	*P*-value
Foraging Rate (flowers/min)	9.38 ± 0.36	8.91 ± 0.29	1.82	0.18
Flower-handling time (s)	4.76 ± 0.6	5.77 ± 0.42	3.2	0.07

Data are from foragers in Assays 1A, 1C and 3 that had a significant preference for either sucrose-only ($n = 20$) or sucrose plus gelsemine ($n = 10$) nectar. All values are mean ± SE and d.f. = 1, 27 for each analysis.

regardless of nectar alkaloid level, but quickly developed a strong aversion to them when flowers with lower levels of nectar alkaloids were made available. These results suggest that nectar alkaloids would only be a significant ecological cost to *Gelsemium sempervirens* plants when they compete for pollination services with alternate plants that have lower levels of nectar alkaloids. Interestingly, *G. sempervirens* flowers very early in the spring (Pascarella, 2007), perhaps because pollinator response to alkaloids in floral nectar was an important selective pressure on flowering time. Nectar quality has been postulated to be a significant factor in the evolution of flowering phenology (e.g. Mosquin 1971; Heinrich 1975; Brody 1997), with plant species of low nectar quality evolving earlier bloom times to escape competition for pollinators with plant species of high nectar quality. Early bloom time may thus be one adaptive mechanism, or 'counteradaptation' (Strauss *et al.* 1999), in plants to mitigate the loss of pollination services due to nectar alkaloids.

Although most bees quickly learned to associate alkaloid concentration with flower-colour cues, and avoided alkaloid-rich flowers when equally rewarding alkaloid-free alternatives were available, the deterrent effect of the alkaloid was offset by higher sugar concentrations. Thus, bees acted as if they were balancing economic gains (sugar collection) against palatability (alkaloid concentration).

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Previous work on feeding behavior in herbivorous insects has shown that carbohydrates can counteract the deterrent effects of many plant secondary compounds, including alkaloids, and do so through a variety of complex physiological response mechanisms (e.g. Dethier 1982; Mitchell & Sutcliffe 1984; Mitchell 1987; e.g. Dethier & Bowdan 1992; Shields & Mitchell 1995; Glendinning *et al.* 2000). Similar mechanisms may mediate the behavioral response of bumblebees, and other insect pollinators, to nectar alkaloids. For instance, the unpleasant taste of the alkaloids may be 'masked' by higher sucrose concentrations (Glendinning 2002), in the same way that a person can make a bitter food like chocolate more palatable by adding sugar. Alternatively, alkaloids may interfere with the ability of sucrose-sensitive receptor cells in the peripheral taste system to detect the correct sucrose concentration of nectar (a process called sensory inhibition; Mitchell & Sutcliffe 1984). In this view, higher sucrose concentrations are required for alkaloid-rich nectar to be perceived as a profitable resource. Regardless of the behavioral mechanism involved, the combined effect of nectar alkaloids and sugars on floral attractiveness has important implications for our understanding of how pollinators assess nectar quality and make adaptive foraging decisions. For example, pollinators performing a "behavioral titration" (Kotler & Blaustein 1995; Webster & Dill 2006) of sucrose and alkaloid uptake would alter predictions on how they should allocate foraging effort to available plant species.

From the plant's perspective, the mitigating effect of higher sucrose concentrations on bumblebee deterrence by alkaloids indicates that pollination services to plants with secondary compounds can be increased by increasing the relative caloric content of floral nectar. At present, there is little information on the relationship between secondary compounds and caloric content of nectar because past studies have either held caloric content constant (Stephenson 1982; Masters 1991; Hagler & Buchmann 1993; Landolt & Lenczewski 1993; Singaravelan *et al.* 2005) or not compared caloric content and secondary compound concentration among plants available to pollinators (Stephenson 1981; Adler & Irwin 2005). We predict that floral nectar containing secondary compounds will have a higher caloric content (concentration and possibly volume) than floral nectar containing lower levels of such compounds. This hypothesis is akin to the "nutrient/toxin titration" model proposed for the presence of toxins in fruit (Cipollini & Levey 1997).

Our results indicate that ecological costs of alkaloids in floral nectar to *G. sempervirens* are due to a reduction in the quantity (number of individuals visiting plants) and not the quality (individual behaviour on flowers) of floral visitation by pollinators. Bees that collected gelsemine-rich nectar spent the same amount of time on flowers and visited the same number of flowers per minute as bees that collected gelsemine-free nectar, indicating that there would be no reproductive cost to *G. sempervirens* in terms of a reduction in the ability of pollinators to remove and deposit pollen. In contrast to our results, Adler & Irwin (2005) found that increasing gelsemine concentrations in flowers of natural *G. sempervirens* populations had no effect on initial attraction of pollinators to plants, but reduced the amount of time that they spent per flower and the number of flowers visited per plant. One likely explanation for the discrepancy between our results and those of Adler and Irwin is that bees in our study visited flowers with different levels of gelsemine in sucrose rewards for extended periods of time and were thus able to learn the association between floral cue (colour in our case) and nectar properties. Indeed, many bees in our study entered and quickly departed from flowers with gelsemine-rich nectar while learning to associate flower colour with reward condition (see Fig. 1), suggesting that ecological costs to plants increase with pollinator foraging experience. This point underscores the importance of identifying and tracking individual pollinators over ecologically relevant time periods in field studies aimed at determining the effects of pollinator behaviour on plant fitness.

Plasticity in the behavioural response of pollinators to secondary compounds in floral nectar has important implications for the evolution of plant defenses against herbivory. Optimal defense theory (ODT) predicts that constitutive defenses in valuable tissues should be more advantageous to plants than induced defenses because such tissues are protected prior to damage by herbivores (Rhoades 1979; McCall & Karban 2006). Our study suggests that predictions of ODT for plants with defense compounds in flowers need to incorporate potential trade-offs between benefits of herbivore resistance and costs of pollinator deterrence. Based on the behavioural response of bumblebees to nectar alkaloids observed in our study, we expect constitutive defense in floral tissues and nectar (i.e. reproductive traits) of out-crossed plants to be favoured only when pollinators have few other floral resources available or caloric rewards compensate for reduced nectar palatability. In contrast, we expect induced defenses in flowers to be advantageous when there is strong competition for pollinators, since floral attractiveness would be reduced for short time periods subsequent to damage by herbivores. Moreover, induced defenses would reduce the floral attractiveness of a small subset of plants in the population (assuming that levels of herbivory are low), thereby decreasing the likelihood that pollinators will learn to discriminate against all plants with a floral signal similar to that of the defended plant (i.e. other plants in the population). Thus, inducible defenses can benefit plants under many ecological conditions by allowing them to mount a strong defense against herbivores while minimizing ecological costs because of pollinator deterrence. Although there is growing evidence for induced defenses in flowers (e.g. Adler *et al.* 2006; McCall & Karban 2006; McCall 2006), the costs and benefits of induced vs. constitutive defense strategies has not been considered in terms of pollinator deterrence. In future field experiments, we plan to determine how plant defense strategies affect pollination services, and thus plant fitness, in different ecological contexts.

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