PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Nectar alkaloids decrease pollination and female reproduction in a native plant

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Abstract The evolution of floral traits may be shaped by a community of floral visitors that affect plant fitness, including pollinators and floral antagonists. The role of nectar in attracting pollinators has been extensively studied, but its effects on floral antagonists are less understood. Furthermore, the composition of non-sugar nectar components, such as secondary compounds, may affect plant reproduction via changes in both pollinator and floral antagonist behavior. We manipulated the nectar alkaloid gelsemine in wild plants of the native perennial vine *Gelsemium sempervirens*. We crossed nectar gelsemine manipulations with a hand-pollination treatment, allowing us to determine the effect of both the trait and the interaction on plant female reproduction. We measured pollen deposition, pollen removal, and nectar robbing to assess whether gelsemine altered the behavior of mutualists and antagonists. High nectar gelsemine reduced conspecific pollen receipt by nearly half and also reduced the proportion of conspecific pollen grains received, but had no effect on nectar robbing. Although high nectar gelsemine reduced pollen removal, an estimate of male reproduction, by one-third, this effect was not statistically significant. Fruit set was limited by pollen receipt. However, this effect varied across sites such that the sites that were most pollen-limited were also the sites where nectar alkaloids had the least effect on pollen receipt, resulting in no significant effect of nectar alkaloids on fruit set. Finally, high nectar gelsemine significantly reduced seed weight; however, this effect was mediated by a mechanism other than pollen limitation. Taken together, our work suggests that nectar alkaloids are more costly than beneficial in our system, and that relatively small-scale spatial variation in trait effects and interactions could determine the selective impacts of traits such as nectar composition.

Keywords Floral evolution · Gelsemine · Nectar robbing · Pollination · Toxic nectar

Introduction

Pollinators have historically been viewed as the driving agent shaping the evolution of floral traits (Darwin 1877; Lloyd and Barrett 1996 translation of Sprengel 1793). In many systems, pollinators are clearly strong agents of selection on both floral display and rewards (e.g., Fishman and Willis 2008; Bolstad et al. 2010; Parachnowitsch and Kessler 2010; Sletvold et al. 2010). However, a wide range of antagonists can also interact with plants at the floral interface. These antagonists include nectar robbers and thieves that consume nectar without pollinating (Irwin et al. 2001), pathogens that sterilize or kill plants via floral infection (Roy 1996; Alexander 2010; Sasu et al. 2010a), florivores that consume flower petals and reproductive structures (McCall and Irwin 2006), seed predators that use flowers as oviposition sites for offspring (Brody and Zimmerman 1995), and insects that have nectar-feeding adults and herbivorous larvae (De Moraes et al. 2001; Adler and Bronstein 2004). Thus, floral evolution may represent a compromise between the benefits and costs of attracting mutualists versus a wide range of antagonists (Strauss and Whittall 2006; Adler 2007).

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Nectar mediates many of the mutualistic and antagonistic interactions that occur at flowers. Nectar is composed primarily of sugars, and traits such as sugar concentration and composition have been the primary focus of research examining how nectar mediates floral interactions (Nicolson 2007). However, minor nectar constituents can have major effects on species interactions and plant fitness. For example, antimicrobial properties of *Cucurbita texana* nectar may help plants resist infection by a fatal pathogen transmitted by pollen-feeding beetles (Sasu et al. 2010b). In addition, floral nicotine in *Nicotiana attenuata* reduced nectar robbing, florivory and per-visit nectar consumption by pollinators, increasing both male and female plant reproduction (Kessler et al. 2008). In spite of current recognition that nectar is made up of numerous components that mediate a wide range of interactions (Nicolson et al. 2007) and the existence of many hypotheses for the adaptive value of such components (Adler 2000), surprisingly few studies have examined how these components affect plant reproduction in natural settings. Because plant traits can have variable or unintuitive effects on reproduction via complex multispecies interactions (e.g., Lankau and Strauss 2008), evaluating the impact of traits on plant reproduction in natural environments is critical to assessing their ecological and evolutionary significance.

The goal of this study was to test how nectar composition affects plant reproduction through changes in pollination and nectar robbing in natural plant populations. We focused on secondary compounds as one aspect of nectar composition. We manipulated the nectar alkaloid gelsemine in naturally growing plants of *Gelsemium sempervirens* (Loganiaceae). In addition, we manipulated pollination in the same study using a factorial design to evaluate the combined effects of a floral trait (nectar alkaloids) and floral interaction (pollination) on plant female reproduction. This experiment builds on previous work examining the role of nectar alkaloids in *Gelsemium* (Adler and Irwin 2005) by moving from an experimental array to multiple natural populations and by directly manipulating pollination service using hand-pollination. Our factorial design has two advantages. First, manipulating nectar gelsemine experimentally isolates its effect rather than effects of unmeasured, correlated traits. Second, manipulating both nectar gelsemine and pollination allowed us to isolate the effect of the trait on female reproduction via a specific interaction of interest. In addition, by manipulating nectar gelsemine and pollination on naturally growing plants at multiple sites, we could assess how general our observed patterns were across a range of *Gelsemium* populations.

We hypothesized that high nectar alkaloids would confer resistance to nectar robbers. In addition, because we studied *Gelsemium* in wild-growing populations with other co-flowering species, we hypothesized that high nectar alkaloids would increase the ratio of conspecific to heterospecific pollen transfer due to a reduction in pollinator visitation by highly generalized insects (akin to the 'pollinator fidelity hypothesis'; Baker and Baker 1975; Rhoades and Bergdahl 1981; Adler 2000). However, we predicted that any potential benefits of high nectar alkaloids for male and female components of plant reproduction would be moderated by the degree to which nectar alkaloids affected rates of pollinator visitation and the degree to which male and female plant reproduction were pollen limited. Moreover, because of spatial variation in the abundance of pollinators and the strength of pollen limitation (Ashman et al. 2004; Price et al. 2005), we predicted that we would find variation in the magnitude of the responses among sites.

Materials and methods

Study system

Gelsemium sempervirens (Loganiaceae; we refer to this species as *Gelsemium* hereafter for simplicity) is a perennial vine native to the southeastern United States that grows naturally in disturbed forest edges and open pine forests (Ornduff 1970; Phillips 1985). *Gelsemium* blooms from early March into late April, producing up to several hundred yellow, tubular flowers per plant. Each flower has five petal lobes; individual flowers bloom for 3–5 days and produce nectar at a rate of 1.1 µl per 48 h with sugar concentration of approximately 48% (Irwin and Adler 2006). Fruits are dehiscent capsules that mature in the fall.

Gelsemium is self-incompatible and distylous; each plant has either long styles and short anthers ("pin" plants) or short styles and long anthers ("thrum" plants). Plants of each morph are incompatible with others of the same morph (Ornduff 1970). Anthers and stigmas appear to ripen simultaneously within a flower. Pollen grains from the two morphs overlap in size, so that the morph of individual pollen grains cannot be identified (Ornduff 1979).

The most common floral visitors of *Gelsemium* at our study sites in Athens, Georgia, USA, are *Bombus bimaculatus* (bumble bees, Apidae), *Apis mellifera* (honey bees, Apidae), *Osmia lignaria* (blue orchard bees, Megachilidae), *Habropoda laboriosa* (blueberry bees, Apidae), and the nectar robber *Xylocopa virginica* (carpenter bees, Apidae), which make slits near the corolla base and insert their proboscis to rob nectar (Adler and Irwin 2005).

Gelsemium contains the alkaloid gelsemine in leaves, flowers and nectar (Adler and Irwin 2005; Irwin and Adler 2006); this alkaloid is highly toxic to mammals (Kingsbury 1964). *Gelsemium* nectar can cause bee poisoning (Eckert 1946; Hardin and Arena 1969), and young worker honey bees develop abnormally when feeding on *Gelsemium*

nectar (Burnside and Vansell 1936). Moreover, gelsemine has sublethal effects on *B. impatiens* by reducing oocyte width in subordinate bees (Manson and Thomson 2009). Gelsemine deterred bumble bee feeding in a laboratory assay, although the extent of deterrence depended on the availability of alternative resources (Gegear et al. 2007). Nectar gelsemine concentrations pooled across plants within populations range from 5.8 to 246.1 ng/ul (Adler and Irwin 2005).

Field sites

We studied three wild populations (hereafter referred to as sites) of *Gelsemium* in Athens-Clarke County, GA, in March and April of 2003. Site latitude/longitudes are: WH: 33°53′45″N, 83°21′51″W; RD: 33°55′41″N, 83°20′46″W; AB: 33°59′31″N, 83°30′34″W. Sites were separated by greater than 1 km and were unlikely connected by gene flow via pollen or seeds. We chose 23 (12 pin, 11 thrum), 58 (39 pin, 19 thrum), and 62 (41 pin, 21 thrum) plants in the three sites, respectively (143 plants total), comprising all the plants in a site that had at least 3 flowers or buds, were distinguishable as individuals, and whose flowers were accessible from a 1.2-m stepladder.

Experimental design

Plants were assigned randomly to a nectar manipulation of either sucrose solution supplemented with gelsemine ('high alkaloid'), or sucrose solution without gelsemine ('low alkaloid'), crossed with a natural or supplemental pollination treatment for a total of four treatment combinations. Treatments were applied at the whole-plant level throughout the flowering season, from March 21 through April 23, 2003. Approximately equal numbers of pin and thrum morphs were assigned to each treatment at each site. 'High alkaloid' plants received 0.5% gelsemine hydrochloride ('gelsemine' hereafter; Indofine Chemical, Hillsborough, NJ, USA) in a 40% (wt/vol) sucrose solution. Low alkaloid plants received the sucrose solution without alkaloids. We did not remove naturally produced nectar from flowers because removal can damage flowers and ovaries. Thus, our treatments should be considered a supplementation (high alkaloid) or dilution (low alkaloid) of existing nectar gelsemine (see Adler and Irwin 2005 for a calculation of manipulated nectar alkaloid concentration). Although the high alkaloid treatment produced nectar gelsemine concentrations outside the natural range documented for *Gelsemium*, our previous work in experimental arrays found effects of nectar gelsemine on floral interactions both at this high level and at a lower augmentation level within the natural range (Adler and Irwin 2005). Moreover, laboratory preference assays also found that nectar gelsemine concen-

trations within the natural range affected bumble bee behavior (Gegear et al. 2007). We used the high nectar gelsemine concentration in an effort to maximize treatment differences between the high and low alkaloid treatments and to ask whether secondary compounds in nectar could affect pollination and plant reproduction in natural populations (Power et al. 1998). Nectar treatments were performed at the whole-plant level each morning (5 days/week) for the entire period that plants flowered. We added 2 μl of nectar treatments to all open flowers using Eppendorf Repeater Plus pipetters (Brinkmann Instruments, Westbury, NY, USA). Pipette tips were narrow enough to allow nectar to be placed at the corolla base near nectaries, and tips were cleaned with ethanol between each flower to prevent accidental pollen transfer.

We hand-pollinated all open flowers on plants in the supplemental pollination treatment using a mixture of pollen from at least 3 different plants. We used a mixture of thrum pollen for pin plants and vice versa. Pollen was applied with a camel-hair paint-brush washed with ethanol between each pollination, and care was taken to avoid moving self-pollen within plants. Because open flowers last 3–5 days, we treated each plant 2–3 times per week to ensure that every flower was pollinated. Plants in the natural pollination treatment were untreated, and all plants were exposed to natural pollination.

Effects of nectar alkaloids on pollinator and nectar robber visitation

Conducting pollinator and nectar-robber observations was not practical in these natural populations because flowers were high in trees and obscured by leaves, and insect visits to flowers were relatively infrequent (Adler and Irwin, personal observation). Instead, we used stigma pollen receipt as an index of pollinator visitation. Higher pollinator visitation to *Gelsemium* in experimental arrays increases pollen deposition on unemasculated pin and thrum flowers ($n = 57$ plants, $r = 0.32$, $P = 0.015$; Irwin and Adler, unpublished data), suggesting that pollen receipt is an appropriate proxy for pollinator visitation. Flowers were emasculated just prior to opening to ensure that stigmas did not receive self-pollen, and thus pollen on these stigmas was brought via pollinators from other flowers. We emasculated one-third of all open flowers, up to four flowers per plant. Stigmas were collected after the corolla fell off due to the expanding ovary; collecting stigmas at this stage is unlikely to affect fruit or seed set, as has been found in other plant species (e.g., Waser and Price 1991). Each stigma was mounted in basic fuchsin dye (Kearns and Inouye 1993) on a glass slide. Pollen grains were counted under a compound microscope (Nikon Eclipse E400, Melville, NY, USA) and classified as conspecific or heterospecific based on a pollen

library we created from local flowering plants. We used the mean number of conspecific pollen grains received per stigma per plant as a measure of pollination that could benefit plant female reproduction, and total pollen grains as a proxy for all pollinator visitation. The mean proportion of conspecific pollen received per stigma per plant was considered a measure of pollen quality since heterospecific pollen will not produce viable seeds, and in other systems heterospecific pollen deposition has been shown to reduce reproduction by clogging stigmas (Fishman and Wyatt 1999). Moreover, a reduction in the mean proportion of conspecific pollen received can indicate increased visits by pollinators that show little host-species fidelity. Only plants in the natural pollination treatment with at least three flowers were used to measure pollen receipt, and stigmas sometimes dried out and were lost before collection, resulting in a total of 38 plants for this measure (25 low alkaloid and 13 high alkaloid).

We censused nectar robbing by counting the number of flowers robbed and total flowers open on all plants on March 29, 2003 at the peak of the flowering season. Carpenter bees are the only species that we have observed robbing *Gelsemium* plants, and they leave a distinctive hole at the corolla base. We censused for robbing once at peak flowering for two reasons. First, our prior research in natural *Gelsemium* populations documented that robbing levels were highest at peak flowering (Irwin and Adler 2006). For example, in 2002 (the year prior to this study), peak flowering occurred during our first nectar-robbing census, and robbing levels were over 10% lower at two subsequent robbing censuses (Irwin and Adler 2006). Second, we were most interested in relative differences in susceptibility to robbing among our treatments and not absolute levels of robbing that plants received; thus, censusing robbing at peak levels should capture relative differences.

Effects of nectar alkaloids on plant reproduction

Male reproduction

Previous research suggested that male reproduction, estimated as dye (pollen) donation to other plants, may be more affected by nectar alkaloids than female reproduction (Adler and Irwin 2005). Thus, we measured both male and female plant function in this study. In a field setting with plants at widely spaced intervals, measuring the movement of fluorescent dye particles was not feasible. Instead, we estimated pollen removal as one component of male reproduction (Stanton et al. 1991). We chose pairs of buds at the same developmental stage. Within each pair, one anther was collected from one bud just before opening (prior to dehiscence) using a pair of forceps cleaned with ethanol between flowers. One anther was collected from the other

flower of the pair 48 h after opening and exposure to natural pollinator visitation. We did not use the same flower for both anther collections since removal of the first anther could adversely affect pollinator preference, and thus result in an underestimate of pollen removal. Each anther was placed in an open microcentrifuge tube in a closed cabinet (to prevent dust in samples) for 2 weeks while the anther dehisced. We counted pollen using a hemacytometer by adding 1 ml of ethanol to each anther, sonicating, and counting eight subsamples of 5 µl each per anther. Pollen counts per anther were multiplied by 5 to estimate pollen per flower. We calculated per-flower pollen removal as the difference in pollen between the paired anther exposed to pollinators for 48 h and the anther collected from the bud within each pair. We collected 1–3 pairs of anthers per plant, and used mean per-flower pollen removal per plant in all analyses. In the few cases where the number of pollen grains was higher in 2-day-old compared to unopened flowers from the same plant, pollen removal was defined as zero because it is unlikely that the 2-day-old flowers had pollen removed by pollinators. Due to challenges finding plants with two buds at the same phenological stage, and timing the collection of anthers at multiple sites, we ended with 38 replicates measuring this response (24 low alkaloid, 14 high alkaloid).

Female reproduction

We measured female reproduction on all plants as fruit and seed production. During the flowering season, we counted flower production per plant by marking all newly opened flowers every 2–3 days using a small piece of green tape on the adjacent woody stem. We collected and counted mature fruits from all marked stems in October 2003, counted seeds per fruit, and estimated weight per seed as (total seed weight per fruit)/(total seeds per fruit). We estimated proportion fruit set as the ratio of mature fruits to flowers produced per plant. We used multiple components of female reproduction because they provide insight into how species interactions affect pollination. For example, proportion fruit set can provide insight into visitation by pollinators because *Gelsemium* is self-incompatible and, without pollinator visitation, no fruits can be produced. Seeds per fruit and seed weight can provide insight into pollen quality (such as the proportion of conspecific pollen receipt) and the ability of plants to take advantage of pollen deposition. Flowers whose stigmas were removed to estimate pollen deposition were not included in fruit set measures.

Statistical analysis

All data were analyzed using SAS 9.2. To test whether nectar alkaloids affected pollinator visitation, estimated as

Gelsemium and total pollen receipt and the proportion of conspecific pollen grains on stigmas, we used ANCOVAs with nectar treatment, floral morph (pin/thrum), site, and all interactions as fixed effects and floral display (daily average number of open flowers) as a covariate, since display could influence visitation independent of treatment effects. Robbing levels were very low in the year of this study (see "Results"), and so the effects of nectar treatment, site, floral morph, and total open flowers on the number of robbed flowers was analyzed using generalized linear models (PROC GENMOD) with a Poisson distribution, verifying that the dispersion factor was approximately 1. To assess whether nectar alkaloids affected male reproduction, estimated as the number of pollen grains removed from the anthers, we used the same ANCOVA model as for pollen deposition. Finally, to test whether nectar alkaloids and hand-pollination treatments affected female reproduction per plant, measured as fruit set (total fruits/total flowers), mean seeds per fruit, and mean weight per seed, we analyzed response variables using ANOVAs with nectar treatment, pollination treatment, floral morphology, and site as fixed main effects and including all two- and three-way interaction terms; the four-way interaction term was never significant and was eliminated from the models.

Results

Floral visitation

Nectar alkaloids reduced *Gelsemium* pollen receipt by nearly half (Fig. 1a; $F_{1,25} = 4.02$; $P = 0.056$), suggesting that pollinators either visited flowers with high nectar alkaloids less frequently or deposited less pollen per visit. Site, floral morph, all interactions, and display as a covariate had no significant effect on *Gelsemium* pollen receipt (all $F < 2.0$, $P > 0.15$). Total pollen receipt was less than half in thrum compared to pin plants (mean ± SE, thrum: 174.3 + 39.7 grains; pin: 393.1 + 60.6 grains; $F_{1,25} = 4.82$; $P = 0.038$) and varied by site ($F_{1,25} = 3.62$; $P = 0.042$), but there was no main effect of nectar alkaloids. However, there was a marginally significant three-way interaction between nectar treatment, morph and site ($F_{2,25} = 3.36$; $P = 0.051$), indicating that the effect of nectar alkaloids on total pollen varied with site-morph combinations. The proportion of conspecific pollen grains on stigmas was reduced by nectar alkaloids ($F_{1,25} = 19.77$; $P = 0.0002$), although this effect varied with site (nectar treatment × site interaction: $F_{2,25} = 5.48$; $P = 0.011$; Fig. 1b). The proportion of conspecific pollen on each floral morph also varied by site (morph × site interaction: $F_{2,25} = 3.74$; $P = 0.038$), such that pin plants received more conspecific pollen than thrum plants at RD and WH, but equivalent ratios at AB. There
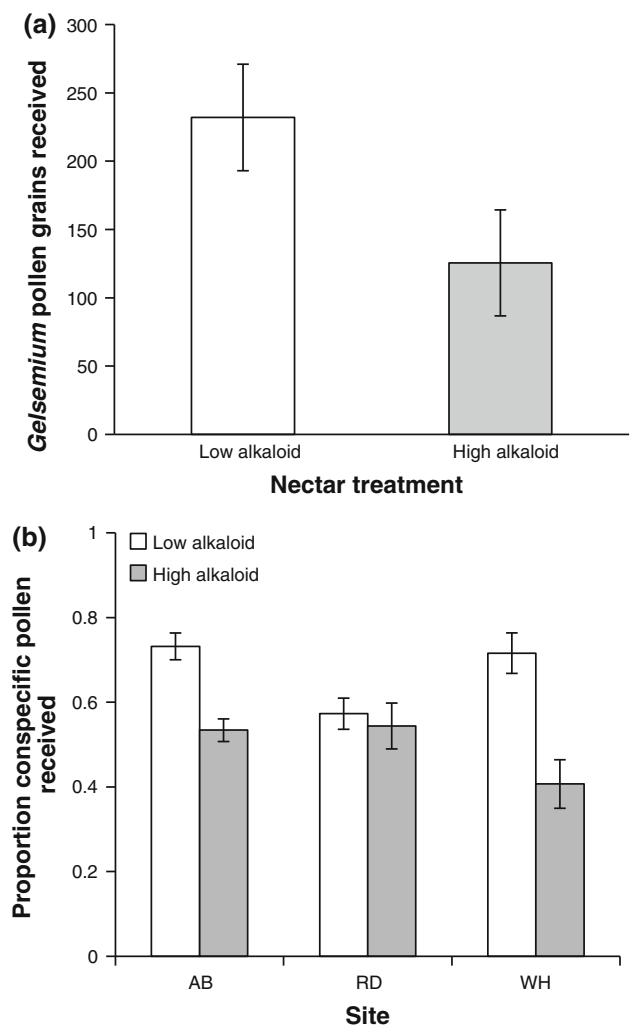

Fig. 1 **a** Effect of nectar treatment on *Gelsemium* pollen receipt to stigmas of emasculated flowers ($n = 43$ for low alkaloid and 31 for high alkaloid plants). **b** Interaction between nectar treatment and site on the proportion of conspecific pollen grains received; the main effect of nectar treatment was also significant ($F_{1,25} = 19.8$, $P = 0.0002$; $n = 6–22$ plants per treatment/site combination). *Bars* means ± SE

were no other significant main effects or interactions ($F < 2.2$, $P > 0.14$ for all).

The number of robbed flowers per plant differed across sites ($\chi^2 = 27.84$, $P < 0.0001$) and increased with the number of open flowers ($\chi^2 = 5.80$, $P = 0.016$), but was not affected by nectar treatment or floral morph ($\chi^2 < 1.7$, $P > 0.18$ for both). However, robbing levels were low in the year of this study; only 18 of our 143 study plants had any robbed flowers.

Male reproduction

Although flowers with high nectar alkaloids had one-third less pollen removed than anthers from low-alkaloid flowers (low alkaloid nectar: 61,691 ± 15,166 grains; high alkaloid
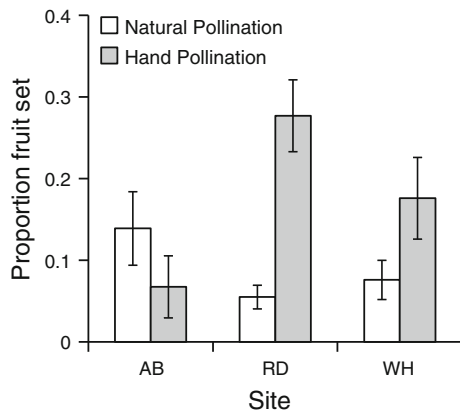
Fig. 2 Interaction between pollination treatment (natural pollination vs. supplemental hand pollination) and site on proportion fruit set, measured as the proportion of total flowers produced per plant that set fruit. The main effect of pollination treatment on fruit set was also significant ($F_{1,109} = 4.18$, $P = 0.04$). *Bars* means ± SE; $n = 11$–29 plants per treatment/site combination

Fig. 3 Interaction between nectar treatment (low vs. high alkaloid nectar) and site on seed weight. The main effect of nectar treatment on seed weight was also significant ($F_{1,49} = 5.92$, $P = 0.019$). *Bars* means ± SE; $n = 4$–22 plants per treatment/site combination

nectar: 39,615 ± 16,807 grains), this effect was not statistically significant ($F_{1,26} = 2.04$; $P = 0.16$). Post-hoc power analysis found that with our variation and effect size, we would need 82 replicates per treatment (compared to our actual 24 low alkaloid and 14 high alkaloid replicates) to detect a significant effect at alpha = 0.05. There were no other significant effects of any factor, covariate or interaction on pollen removal ($F < 3.0$, $P > 0.08$ for all).

Female reproduction

Proportion fruit set was low overall but more than doubled with hand-pollination (hand-pollinated: 0.20 ± 0.030; open pollinated: 0.08 ± 0.015; $F_{1,109} = 4.18$, $P = 0.04$). The effect of supplemental pollination on fruit set varied across sites (pollination treatment × site interaction: $F_{2,109} = 4.08$, $P = 0.02$; Fig. 2), indicating that pollen limitation varied spatially. Although high alkaloid nectar reduced proportion fruit set by 20% (low alkaloid nectar: 0.15 ± 0.025; high alkaloid nectar: 0.12 ± 0.021), this difference was not statistically significant ($F_{1,109} = 0.63$, $P = 0.43$). No other factor or interaction significantly affected proportion fruit set ($F < 2.5$, $P > 0.09$ for all). Moreover, the number of seeds per fruit was not affected by any factor ($F < 3.0$, $P > 0.09$ for all).

High nectar alkaloids significantly reduced seed weight (low alkaloid nectar: 2.24 mg ± 0.104; high alkaloid nectar: 2.14 mg ± 0.150; $F_{1,49} = 5.92$, $P = 0.019$) although this effect was stronger in some sites than others (nectar treatment × site interaction: $F_{2,49} = 3.32$, $P = 0.045$; Fig. 3). Seed weight did not differ overall across floral morphs ($F_{1,49} = 3.14$, $P = 0.08$), but the effect of floral morphology (pin vs. thrum) on seed weight varied across sites
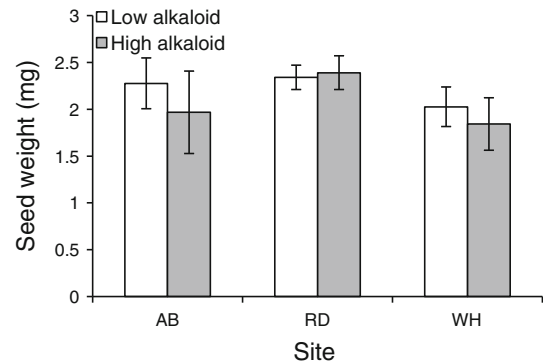
(morph × site interaction: $F_{2,49} = 5.07$, $P = 0.01$). Finally, we found no effect of pollination treatment on seed weight ($F_{1,49} = 0.31$, $P = 0.58$), and there was no pollination × nectar alkaloid treatment interaction ($F_{1,49} = 0.05$, $P = 0.83$).

Discussion

This is the first study to experimentally manipulate nectar secondary compounds in naturally growing wild plants. Consistent with our previous research (Adler and Irwin 2005), we found costs, including reduced conspecific pollen receipt, reduced proportion of conspecific pollen receipt, and reduced seed weight, but no discernable benefits of nectar containing high alkaloid levels. The 'pollinator fidelity hypothesis' (Baker and Baker 1975; Rhoades and Bergdahl 1981; Adler 2000) predicts that nectar secondary compounds should encourage specialized pollinators and therefore increase the proportion of conspecific pollen grains received. Our results were contrary to this prediction, since high nectar gelsemine reduced the proportion of conspecific pollen grains received. High nectar gelsemine also did not deter nectar robbing, contrary to the nectar robbing hypothesis (Janzen 1977; Baker and Baker 1978; Adler 2000); however, robbing levels were quite low compared to previous years (cf. Irwin and Adler 2006). Contrary to our findings, some studies in other systems suggest that non-sugar nectar components may sometimes benefit plants via changes in species interactions (Stephenson 1982; Johnson et al. 2006; Kessler et al. 2008). For example, nectar can have antimicrobial properties (Thornburg et al. 2003; Carter and Thornburg 2004; Sasu et al. 2010b), but the consequences for plant fitness have not yet been demonstrated. In one of the few studies that has examined fitness effects of nectar secondary compounds, Kessler et al. (2008) showed that transgenic *Nicotiana attenuata* plants

without nicotine had increased floral herbivory, nectar robbing, and pollinator nectar consumption per visit, and reduced male and female reproduction compared to wild-type plants. This annual species grows ephemerally in large populations (Kessler and Baldwin 2004) whereas our study species is a long-lived perennial at relatively low densities. The benefits of pollinator deterrence may therefore depend on the availability of both conspecifics and pollinators; when conspecifics and pollinators are abundant, as with *N. attenuata*, reducing nectar consumption per visit may increase total visitation and conspecific pollen deposition (Kessler et al. 2008), whereas when pollinator visits are relatively rare, traits that deter pollinators may be costly. In addition, eliminating nicotine transgenically provides a phenotype that is consistent (as compared to our nectar additions, which only last until that nectar is consumed), but tests effects of whole-plant nicotine production rather than only nectar or floral tissue. Clearly, work is needed in more systems to determine the conditions under which floral defenses are beneficial or costly to plants due to effects on pollinators and floral antagonists.

Studies that manipulate floral traits and measure effects on interactions are common (e.g., reviewed in Campbell 2009), as are manipulations of pollinator service to determine effects on reproduction (Burd 1994; Ashman et al. 2004; Knight et al. 2005, 2006). However, we are aware of few prior studies that manipulate both a floral trait and floral interaction to disentangle direct effects of the trait on reproduction from interaction-mediated indirect effects. In our study, manipulating both nectar alkaloids and pollination suggests that both are important for plant reproduction, but that nectar alkaloids may have effects on reproduction mediated by factors other than pollination. For example, nectar alkaloids reduced conspecific pollen receipt by nearly half, and pollen receipt limited fruit set. However, although nectar alkaloids reduced fruit set by 20%, this effect was not significant. The lack of effect may be driven by variation across sites in both pollen limitation and trait effects. Pollen limitation of fruit set was high at the RD site, moderate at WH, and there was no limitation at the AB site (Fig. 2). However, high nectar alkaloids reduced the quality of pollen delivered (i.e., the proportion of conspecific pollen grains on stigmas) in the AB and WH sites more than the RD site (Fig. 1). Thus, the sites where nectar alkaloids reduced pollination the most were the sites where pollen was least limiting for reproduction. Future work across multiple sites and years would help determine if the relative importance of pollination across sites is consistent over time (Thompson and Cunningham 2002). If so, there is the potential for trait evolution to vary across relatively small spatial scales. We also found that nectar alkaloids caused a small but significant reduction in seed weight, although our hand-pollination treatment demonstrated that seed weight

was not pollen-limited and there was no nectar alkaloid by pollination treatment interaction. This suggests that nectar alkaloids reduced seed weight via a mechanism other than pollination. One explanation is that nectar alkaloids could be directly toxic to developing ovules, reducing seed quality. Although a previous study found no direct effect of nectar gelsemine (at the same concentration as this study) on flower production, fruit production, fruit set, seeds per fruit or seed set (Adler and Irwin 2005), we did not examine direct effects on seed weight. An alternative possibility is that nectar alkaloids influenced seed weight through some other interaction, such as nectar microbes, that was not considered in this study.

A consistent result across our current and previous work is the cost of high alkaloid nectar in reducing pollinator visitation, either measured as observed pollinator visitation rate (Adler and Irwin 2005) or estimated using stigma pollen loads (this study). Thus, high nectar alkaloids in *Gelsemium* are deterrent to pollinators under a variety of field conditions and at a variety of nectar alkaloid levels. However, changes in pollination have different consequences for plant reproduction in each study. Here, nectar gelsemine reduced seed weight, an aspect of female reproduction, and reduced an estimate of male fitness, but not significantly so given our small sample size. By contrast, Adler and Irwin (2005) found significant effects of nectar gelsemine on estimates of male but not female reproduction. This contrast is likely due to differences in pollination between a natural setting in which plants and pollinators were at relatively low density and interspersed with co-flowering species (this study), and an experimental array in a botanical garden with high pollinator densities (previous work). The advantage of our current experiment is that, by manipulating pollination directly, we demonstrated that this interaction was important for plant female reproduction, whereas in our previous study we suspected pollen receipt did not limit female reproduction but could not say so conclusively. For male reproduction, the experimental array provided the advantage of measuring pollen movement to receptive stigmas using fluorescent dye as a pollen analog, allowing a more precise estimate of male reproduction than pollen removal since removed pollen may not reach conspecifics. Although we found a non-significant trend for nectar alkaloids to reduce male reproduction in our field study, measuring pollen movement was not possible in a natural setting due to widely spaced plants.

The differences in the nature of costs that we detected in these two experiments highlight how environmental context can alter results. In natural settings, costs of phenotypic traits may be more readily discerned due to greater competition or lower resources; these resources may be abiotic as well as biotic, such as the abundance of pollinators. For example, genetic correlations or tradeoffs are typically

stronger under more stressful conditions (Agrawal et al. 2010). However, greater environmental variation or logistical constraints may limit the ability to detect certain effects. More controlled studies allow us to discern effects that may go undetected amidst the variability of natural settings, but the high resources and uniformity of samples found in many laboratory, greenhouse, and experimental array studies may reduce or eliminate our ability to detect effects that occur under more natural, low resource conditions. Thus, both approaches may be valuable to discern the ecological and physiological effects of traits on fitness.

Selection by floral antagonists and pollinators on floral traits may be context-dependent, creating a geographic mosaic in which traits are under different selective regimes in different environments (Thompson and Cunningham 2002; Gomez et al. 2009). Although our study was not designed explicitly to address spatial variation in selection on nectar traits, we found that treatment effects often varied across sites. Our other work over greater spatial scales suggests that selection by different floral antagonists, such as nectar robbers in northern Georgia compared to floral herbivores in southern Georgia, may shape local adaptation of floral traits that also affect pollinator preference and plant reproduction (Leege, Irwin and Adler, in preparation). Additionally, the effect of nectar gelsemine on pollinator behavior may depend on the community of co-flowering plants providing nectar resources (Gegear et al. 2007) and the community of pollinators if they respond differently to nectar compounds (Adler and Irwin 2005; Kessler and Baldwin 2007). Thus, the ultimate consequences of nectar defenses on plant reproduction may be dependent both on the communities of animals interacting with flowers and on the co-flowering plant species that modify animal behavior.

In conclusion, we found that plants were pollen-limited for fruit set and that nectar alkaloids reduced conspecific pollen receipt but did not significantly reduce fruit set. The sites with the highest pollen limitation were also the sites where nectar alkaloids had the least effect on pollen receipt, suggesting that relatively small-scale spatial variation in trait effects and interactions could determine the selective impacts of traits. Furthermore, nectar alkaloids reduced seed weight, but our pollination treatment indicated that this effect was mediated by a mechanism other than pollination. This study is consistent with previous work (Adler and Irwin 2005) demonstrating costs rather than benefits of nectar alkaloids, although the nature of the costs (male vs. female reproduction) differed between the studies. These results contrast with recent work in other systems demonstrating benefits of non-sugar nectar components in terms of deterring floral antagonists and increasing pollinator efficiency (Johnson et al. 2006; Kessler et al. 2008; Sasu et al. 2010b), suggesting that the ecological consequences of nectar secondary compounds may depend on how different species respond to such compounds and the fitness consequences of the interactions.

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