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NECTAR SECONDARY COMPOUNDS AFFECT SELF-POLLEN TRANSFER: IMPLICATIONS FOR FEMALE AND MALE REPRODUCTION

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Abstract. Pollen movement within and among plants affects inbreeding, plant fitness, and the spatial scale of genetic differentiation. Although a number of studies have assessed how plant and floral traits influence pollen movement via changes in pollinator behavior, few have explored how nectar chemical composition affects pollen transfer. As many as 55% of plants produce secondary compounds in their nectar, which is surprising given that nectar is typically thought to attract pollinators. We tested the hypothesis that nectar with secondary compounds may benefit plants by encouraging pollinators to leave plants after visiting only a few flowers, thus reducing self-pollen transfer. We used *Gelsemium sempervirens*, a plant whose nectar contains the alkaloid gelsemine, which has been shown to be a deterrent to foraging bee pollinators. We found that high nectar alkaloids reduced the total and proportion of self-pollen received by one-half and one-third, respectively. However, nectar alkaloids did not affect female reproduction when we removed the potential for self-pollination (by emasculating all flowers on plants). We then tested the assumption that self-pollen in combination with outcrossed pollen depresses seed set. We found that plants were weakly self-compatible, but self-pollen with outcrossed pollen did not reduce seed set relative to solely outcrossed flowers. Finally, an exponential model of pollen carryover suggests that high nectar alkaloids could benefit plants via increased pollen export (an estimate of male function), but only when pollinators were efficient and abundant and plants had large floral displays. Results suggest that high nectar alkaloids may benefit plants via increased pollen export under a restricted set of ecological conditions, but in general, the costs of high nectar alkaloids in reducing pollination balanced or outweighed the benefits of reducing self-pollen transfer for estimates of female and male reproduction.

Key words: *distyly; geitonogamy; Gelsemium sempervirens; nectar alkaloids; nectar quality; nectar secondary compounds; outcrossed vs. selfed; pollination; toxic nectar.*

INTRODUCTION

Traits that promote outbreeding and reduce the frequency of inbreeding are widespread in plants and animals. The same traits, such as self-incompatibility, separate sexes, and the spatial and temporal separation of male and female sex parts within individuals, often increase the incidence of outbreeding while reducing the frequency of inbreeding (Thornhill 1993). However, this need not always be the case; some traits increase the potential for both outbreeding and inbreeding. For example, floral traits that increase pollinator visitation and subsequent pollen movement in hermaphroditic flowers may also prolong pollinator visits on individual flowers or plants, potentially increasing self-pollen transfer and inbreeding (Dudash 1991, de Jong et al. 1992b, Harder and Barrett 1995). Even in plants with genetic self-incompatibility, self-pollen transfer may reduce female reproduction by clogging stigma surfaces,

interfering with pollen-tube growth in the style, usurping ovules, and increasing fruit abortion, and reduce male reproduction by reducing pollen available for export to other plants (reviewed in Snow et al. 1996, Barrett 2002). Thus, pollen source (selfed vs. outcrossed) can affect female and male reproduction, and plants may experience selection for traits via female and male fitness that lure pollinators while at the same time persuading pollinators to move quickly to a different plant (de Jong et al. 1993). Studies have tested how floral characters, herbivores, and nectar robbers influence within- and among-plant pollen transfer and plant reproduction via changes in pollinator behavior (e.g., Geber 1985, Irwin 2003, Mitchell et al. 2004, Ishii and Harder 2006, Steets et al. 2006). However, we know little about how the quality (or chemical composition beyond sugars) of nectar influences patterns of pollen movement and reproduction, which is unexpected considering that nectar is conventionally viewed as a trait of fundamental importance in pollination. The goal of this study was to test how nectar chemical components, in particular secondary compounds, influenced patterns of self-pollen transfer and subsequent reproduction.

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Nectar with secondary compounds is common among flowering plants, occurring in at least 21 different plant families (reviewed in Baker 1977, Adler 2000). The presence of secondary compounds in nectar seems paradoxical given that nectar is typically considered a trait to entice pollinators. A number of adaptive hypotheses have been proposed to explain the existence of nectar with secondary compounds (reviewed in Adler 2000). Many of the hypotheses fall under the category of deterring floral visitors that reduce plant fitness, such as nectar robbers or thieves (Stephenson 1981), generalist or inefficient pollinators (Johnson et al. 2006), or microorganisms that spoil nectar (Carter and Thornburg 2004). Alternatively, nectar secondary compounds may simply reflect a pleiotropic consequence of plant defense against herbivores in other tissues (as in Ehrlén and Eriksson 1993). However, a largely unexplored influence of nectar secondary compounds is their effects on pollen-movement patterns within and among plants via changes in pollinator behavior. If high levels of nectar secondary compounds cause pollinators to probe fewer available flowers per plant and spend less time per flower, there may be benefits for the plant in terms of reduced self-pollen transfer. For example, bee pollinators probe fewer flowers and spend less time per flower on plants with high vs. low concentrations of nectar alkaloids in *Gelsemium sempervirens* (Adler and Irwin 2005), and nectar alkaloids decrease time spent per flower by pollinators of wild tobacco, *Nicotiana attenuata* (Kessler and Baldwin 2006). In many hermaphroditic plants, increased within-plant pollinator movement and time spent per flower are positively correlated with within-plant and within-flower pollen transfer, affecting subsequent female and male reproduction (reviewed in Snow et al. 1996). Thus, plants with low levels of nectar secondary compounds may benefit from increased per-flower visitation but may also suffer fitness costs associated with increased self-pollination compared to plants with high nectar secondary compounds.

We used experimental and modeling approaches to test how nectar secondary compounds influenced patterns of self-pollen transfer and reproduction in *Gelsemium sempervirens* (Loganiaceae; hereafter *Gelsemium*). *Gelsemium* is distylous, a trait thought to promote disassortative pollen movement (Darwin 1877). However, the trait does not eliminate self-pollen transfer in this (Ornduff 1979) or other heterostylous plants (reviewed in Ganders 1979, Barrett 1990). *Gelsemium* nectar contains the alkaloid gelsemine, which can reduce pollinator visitation (Adler and Irwin 2005). In a previous study, however, we found that nectar alkaloids did not affect female reproduction (Adler and Irwin 2005). We hypothesized that this lack of effect was due to a trade-off between the benefits of increased per-flower visitation and the costs of increased self-pollen transfer. This hypothesis assumes that plants are pollen limited for seed set, that high nectar alkaloids reduce

within-plant self-pollen transfer, that self-pollen alone or in combination with outcrossed pollen depresses seed set relative to solely outcrossed flowers, and that selfing can result in early-acting inbreeding depression measured in terms of fruits and seeds. Moreover, although nectar alkaloids can reduce pollen donation, a component of male reproduction, under experimental conditions (Adler and Irwin 2005), the range of ecological conditions under which nectar alkaloids may have positive, neutral, or negative effects on estimates of male reproduction remains largely unexplored. Measuring the consequences of nectar alkaloids on female and male reproduction is important; even if *Gelsemium* populations are near carrying capacity, plants that produce or sire more seeds may have a higher frequency of progeny in the next generation, and soft selection can occur if allele frequencies change within populations of constant size. Here, we experimentally tested the degree to which high concentrations of nectar alkaloids affected self-pollen transfer and female reproduction, and we tested the assumption that self-pollen in combination with outcrossed pollen depresses seed set. We also used an exponential decay model of pollen transfer to explore under what conditions high nectar alkaloids might benefit pollen export (an estimate of male reproduction; Stanton et al. 1992) or, alternatively, if the benefits of multiple-flower pollinator visitation for pollen export for plants with low nectar alkaloids always outweigh the costs of within-plant self-pollination.

Specifically, we asked four questions. (1) Do nectar secondary compounds influence within-plant self-pollen transfer? (2) How do nectar secondary compounds influence female reproduction in the absence of self-pollen transfer? (3) Does self-pollen in combination with outcrossed pollen depress fruit and seed set? (4) What are the consequences of nectar alkaloids and self-pollen transfer on pollen export?

METHODS

Study system

Gelsemium sempervirens is a native perennial vine in the southeastern United States, occurring in disturbed forest edges and open pine forests (Ornduff 1970; see Plate 1). *Gelsemium* is distylous; each plant produces flowers with either long styles and short filaments (pins) or short styles and long filaments (thrums). Fruit and seed set are highest when pollination occurs between morphs; however, low fruit and seed set do occur with pollination within the same morph or plant (average of 0.9 seeds per fruit; Ornduff 1970), making the plants largely intramorph and intraplant incompatible (Pasarella 2007). *Gelsemium* blooms from March through April in Athens, Georgia, USA, where our field sites were located. Individual plants produce up to several hundred yellow, tubular flowers. On wild-growing plants at peak bloom, the size of the floral display can be as high as 286 open flowers on plants within pine forests and even higher for plants growing at the top of the

forest canopy, suggesting the potential for self-pollen transfer. Individual flowers have a nectar production rate of $1.1 \pm 0.1 \mu\text{L}$ (mean \pm SE) per 48 h (range 0–6.2 $\mu\text{L}/48$ h) with $48.2\% \pm 1.4\%$ sugar concentration (range 23–62%; Irwin and Adler 2006). Flowers last for three to five days. Fruits mature in October.

The most common pollinating floral visitors to *Gelsemium* at our study sites are *Bombus bimaculatus* (bumble bees, Apidae), *Apis mellifera* (honey bees, Apidae), *Osmia lignaria* (blue orchard bees, Megachilidae), and *Habropoda laboriosa* (blueberry bees, Apidae). *Bombus*, *Osmia*, and *Habropoda* transfer significantly more pollen per visit than *Apis* (Adler and Irwin 2006). Flowers are also visited by *Xylocopa virginica* (carpenter bees, Apidae) that make slits near the corolla base through which they rob nectar. On rare occasions, *Xylocopa* also visit flowers legitimately, transferring less pollen per visit than *Apis* (Adler and Irwin 2006).

Gelsemium leaves, corolla, and nectar contain the alkaloid gelsemine (Adler and Irwin 2005, Irwin and Adler 2006). Gelsemine is toxic to mammals (Kingsbury 1964), can cause bee poisoning (Eckert 1946; but see Elliott et al. 2008), and has been implicated in honey bee developmental abnormalities (Burnside and Vansell 1936). Nectar gelsemine concentrations in wild-growing *Gelsemium* range from 5.8 ng/ μL to 246.1 ng/ μL (Adler and Irwin 2005). Nectar gelsemine has no direct effect on fruit and seed set in hand pollinated flowers (Adler and Irwin 2005). In field arrays, pollinators do not learn to avoid flowers with high gelsemine; they only alter their behavior upon foraging, by reducing the percentage of flowers probed per plant and time spent per flower (Adler and Irwin 2005). The degree to which plants with low nectar alkaloids may benefit from increased per-flower visitation, but also suffer from increased self-pollen transfer, is unknown.

Question 1: do nectar secondary compounds influence within-plant self-pollen transfer?

We manipulated nectar gelsemine and estimated self-pollen transfer using a paired-plant design; each pair involved one floral morph. One member of each pair was randomly assigned to a high nectar gelsemine treatment (hereafter “high alkaloid”) and one to a low nectar gelsemine treatment (hereafter “low alkaloid”). We ensured that pairs had the same number of flowers open by pruning flowers when needed. We used 12 plant pairs (six pin and six thrum). To manipulate nectar alkaloids, flowers of high alkaloid plants received 0.5% gelsemine hydrochloride (Indofine Chemical Company, Hillsborough, New Jersey, USA) in a 40% (mass per volume) sucrose solution. Flowers of low alkaloid plants received the sucrose solution without gelsemine. We added 2 μL of sucrose solution to each open flower. We used a 40% sucrose solution and a 2- μL addition because both are within the range of what natural *Gelsemium* flowers produce in the field (Irwin and Adler 2006). We did not remove naturally produced nectar from flowers

to avoid floral damage, which could alter pollinator behavior. As a result, our treatments should be viewed as supplementation or dilution of naturally occurring nectar gelsemine. Based on average nectar standing crop and nectar gelsemine levels, flowers in the high alkaloid treatment had ~ 3200 ng/ μL of gelsemine, and flowers in the low alkaloid treatment had ~ 12 ng/ μL of gelsemine (for calculations, see Adler and Irwin [2005]). In the high alkaloid treatment, we supplemented gelsemine above natural levels to maximize the difference between the high and low treatments and to ask whether nectar alkaloids could affect self-pollen transfer (Power et al. 1998). A previous field study of pollinator behavior and plant reproduction found that some pollinators altered the proportion of flowers probed and time spent per flower similarly using nectar-alkaloid treatments both within and outside of the natural range of gelsemine concentrations (Adler and Irwin 2005).

Between 4 April and 17 April 2003, each pair of plants was placed individually in an open field near the University of Georgia (Athens, Georgia, USA) greenhouses for 4 h. We placed four unmanipulated potted *Gelsemium* (hereafter border plants) around each treatment plant to help attract pollinating insects; *Gelsemium* were also naturally blooming in the area. The border plants were each 1 m from treatment plants, and the paired treatment plants were 3 m apart. Before placing treatment plants in the field, we added nectar to all open flowers near the nectaries at the corolla base using Eppendorf Repeater Plus pipetters (Brinkmann Instruments, Westbury, New York, USA). We cleaned pipet tips with ethanol between flowers to prevent accidental pollen movement.

To estimate self-pollen transfer, we used powdered fluorescent dyes (JST-300, Radiant Color, Richmond, California, USA) as pollen analogs (Adler and Irwin 2006). For each pair, we randomly assigned the nectar treatments to two different dye colors (green or orange). We dyed all of the anthers from one-half of the open flowers after performing nectar treatments. After 4 h in the field, we collected stigmas from undyed flowers from each pair. The number of selfed and outcrossed dye particles on each stigma were counted under a dissecting microscope. For each plant, we calculated the mean number and proportion of self-dye particles received per flower (hereafter self-dye receipt and proportional self-dye receipt, respectively). Proportional self-dye receipt was calculated as: (self-dye)/(selfed + outcrossed dye). Because we dyed the same number of flowers within pairs, we did not standardize by the number of flowers dyed.

We tested the prediction that high nectar alkaloids reduced self-dye receipt ($\log(x + 1)$ -transformed) and proportional self-dye receipt (arcsine square-root transformed) using paired *t* tests. We excluded one pair from the analysis because no dye particles were deposited on any stigma. Floral morph was not included in the

analysis because it had no effect on either estimate of self-dye receipt ($t_{20} < 0.65$, $P > 0.52$).

Question 2: how do nectar secondary compounds influence female reproduction in the absence of self-pollination?

On 11 March 2003, we planted 120 *Gelsemium* into an open field at the University of Georgia Botanical Gardens (Athens, Georgia, USA). Plants were spaced 1 m apart and buried into the ground in a 3.8-L pot in a 10 × 12 plant array. We randomly separated the plants into treatment and pollen-donor plants (60 each), equally divided between pins and thrums. Of the treatment plants, 30 each were randomly assigned to the high and low alkaloid treatments. Nectar treatments were performed as described previously at the whole-plant level each morning (six days per week) throughout blooming. On treatment plants, we emasculated all of the flowers in elongated-bud phase. Removing anthers did not alter final floral development. By emasculating flowers, we removed the potential for self-pollen movement to test how nectar alkaloids affected female reproduction in the absence of within-flower and within-plant self-pollen transfer. Using pollen donors ensured that emasculated plants had access to outcrossed pollen for seed set. We predicted that low nectar alkaloids would increase plant reproduction when the potential cost of self-pollen transfer was eliminated.

On two days during peak bloom (21 March and 23 March 2003) we observed treatment plants for insect visits midday, during peak insect activity, to confirm that high alkaloid nectar reduced per-flower visitation and time spent per flower relative to low alkaloid nectar (as in Adler and Irwin 2005). We conducted a total of eight person-hours of observations over the two dates, noting the number of visits per plant, the number of flowers probed per visit, the time spent per flower, and the identity of each visitor using hand-held tape recorders. Visitors were tracked between plants until they left the array. We also noted whether the visitor entered the flower legitimately through the floral opening (hereafter referred to as a pollinator; Adler and Irwin 2006) or acted as a nectar robber. We only observed *Xylocopa* robbing nectar. Before each observation period, we counted the number of flowers open on each plant. To estimate floral visitation, we calculated three response variables per plant: (1) total number of insect visits, (2) mean proportion of flowers probed, and (3) mean foraging time per flower (square-root transformed). We calculated separate response variables for pollinating visits (all pollinator species combined) and robbing visits. We calculated response variables across pollinator species because we were most interested in general pollinator response; individual pollinator species responses to high and low nectar alkaloids have been reported previously (Adler and Irwin 2005). The distributions of the residuals for total number of insect visits per plant and mean proportion of flowers probed per plant were nonnormal and could not

be transformed to improve normality. Thus, we used nonparametric van der Waerden tests to assess how nectar treatment and floral morph affected these responses. We used ANOVA to test how nectar treatment, floral morph, and their interaction affected mean foraging time per flower by pollinators and robbers.

Once plants ceased blooming, we dug them up on 23 April, transplanted them into 18.9-L pots, and moved them into a screen house for fruit maturation. In October, we counted all of the expanded fruits and the number of seeds per fruit. We also weighed all of the seeds to the nearest 0.0001 g. For each plant, we calculated four measures of female reproduction: (1) proportion fruit set (number of seed-bearing fruits divided by total number of flowers), (2) mean seeds per fruit, (3) total seeds per plant, and (4) mean seed mass. To test how nectar-alkaloid treatment in the absence of self-pollination affected these intercorrelated measures of female reproduction, we used a MANOVA with nectar-alkaloid treatment, floral morph, and their interaction as factors, and proportion fruit set (arcsine square-root transformed), mean seeds per fruit, total seeds per plant ($\log(x + 1)$ -transformed), and mean seed mass as response variables. A significant MANOVA was followed by univariate ANOVAs for each response variable (Scheiner 1993). We excluded two plants because they did not flower during the study; both were thrums, one each assigned to low and high alkaloid treatments.

Question 3: does self-pollen in combination with outcrossed pollen depress fruit and seed set?

We pollinated flowers by hand in the greenhouse to explore the consequences of selfed and outcrossed pollen mixtures on fruit and seed set. We used *Gelsemium* from three wild-collected populations in Athens, Georgia, USA; cuttings were collected and propagated in the greenhouse. We propagated a second series of cuttings from these plants to remove any environmental effects. We used 20 plants, each of a different genotype (9 pins and 11 thrums). On most plants, we enclosed five mature flower buds of similar size in bags made of bridal veil ~48 h prior to flower opening. In some cases, plants did not have enough buds of similar phenology at the same time; in these cases, we bagged additional buds on alternate plants of the appropriate morph. Bags were used to avoid unintentional pollen transfer. Upon opening, flowers were randomly assigned to receive one of five treatments: (1) self-pollen (from another flower on the same plant), (2) outcrossed pollen (a mixture from four unrelated donors of the opposite morph), (3) 1:1 self:outcrossed pollen mixture (a mixture of equal numbers of anthers from the focal plant and from four unrelated donors of the opposite morph), (4) pollen chase (self-pollen and then outcrossed pollen three hours later), and (5) bag control (left unpollinated to test for accidental pollination within

TABLE 1. Parameter values for *Gelsemium sempervirens* used in an exponential decay model of pollen transfer in order to understand how nectar alkaloids affect pollen export.

Parameter	Definition	Value	Source
B	number of pollen grains in virgin flowers	68 677 pollen grains per flower	Ornduff (1979)
k_1	fraction of pollen on the visitor that deposits per flower	0.28	Adler and Irwin (2006)
k_2	fraction of pollen removed from anthers per visit	not measured; values ranged from 0.001 to 0.65	de Jong et al. (1992)
X	mean number of pollinator visits to a plant	2–20 visits per plant	Adler and Irwin (2005)
F	number of open flowers	1–40 open flowers	Irwin and Adler (2006); R. E. Irwin and L. S. Adler (<i>unpublished data</i>)
f	number of flowers probed per plant visit	varied as a function of nectar alkaloid treatment	median values in this study†

Note: Parameters and their values are discussed in Appendix A.

† See Results: How do nectar secondary compounds influence female reproduction in the absence of self-pollination?

bags). We used camel-hair paintbrushes to coat receptive stigmas with pollen. In the outcrossed treatment, we used pollen from four unrelated donors to avoid any potential incompatibilities with single donors. The pollen donors were different plants than the treatment plants. In the pollen chase treatment, we applied outcrossed pollen three hours after self-pollen because we have observed visits to individual flowers approximately every three hours (Adler and Irwin 2005).

We counted the number of expanded seeds and total ovules per fruit under a dissecting microscope. For each fruit, we calculated proportion seed set as (number of seeds)/(total number of ovules). We calculated proportion seed set (rather than seed number) to take into account any difference in ovule numbers among fruits, although results were similar for proportion seed set and seed number (R. E. Irwin and L. S. Adler, *unpublished data*). Flowers in the bag control treatment did not make seed-bearing fruits, suggesting that bags were successful at deterring pollinators; thus, this treatment was excluded from further analysis. To test how pollination treatment and floral morph affected probability of fruit set, we used a multi-way contingency table. We assessed how pollination treatment altered proportion seed set for expanded fruits using ANOVA with pollination treatment, floral morph, and their interaction as factors. The proportion of flowers that we treated was small compared with the hundreds of flowers these plants produced, reducing the likelihood that there was resource competition among flowers on the same plant in different treatments.

Question 4: what are the consequences of nectar alkaloids and self-pollen transfer on pollen export?

We used a model of pollen carryover described by Klinkhamer et al. (1994) and Iwasa et al. (1995). Briefly, when a pollinator visits a flower, it deposits a fraction of pollen from its body onto the flower stigma (k_1), and it removes a fraction of pollen from the anthers (k_2). Because *Gelsemium* typically have multiple flowers open

per plant (F , hereafter floral display size) and pollinators probe multiple flowers during a plant visit (f) prior to moving to a new plant, some fraction of pollen will be lost to flowers on the same plant and will not be available for export. Although the number of times a *Gelsemium* plant is visited (X) is not a function of nectar alkaloids, the number of flowers probed per visit (f) can be influenced by nectar alkaloids (Adler and Irwin 2005). Decreasing f results in a lower fraction of pollen transferred within the plant and an increased fraction exported to other plants. Pollen export per plant is then the product of the number of pollen grains removed from anthers, the average fraction of pollen exported to other plants instead of being deposited on flowers of the same plant, and the number of flowers open.

We parameterized the model primarily using published and unpublished data specific to *Gelsemium* (Table 1, Appendix A). We used the model to predict how variation in the fraction of pollen removed from anthers (k_2), the number of times plants were visited (X), and the number of flowers probed per visit (f) affected the number of pollen grains exported per plant as a function of floral display size (F) for plants with low and high nectar alkaloids.

RESULTS

Question 1: do nectar secondary compounds influence within-plant self-pollen transfer?

Plants with low nectar alkaloids received over two times more self-dye than plants with high nectar alkaloids ($t_{10} = 2.99$, $P = 0.01$; Fig. 1a). Moreover, the proportion of self-dye received was 30% higher in plants with low vs. high nectar alkaloids ($t_{10} = 2.53$, $P = 0.03$; Fig. 1b).

Question 2: how do nectar secondary compounds influence female reproduction in the absence of self-pollination?

We observed 83 foraging bouts comprising visits to 481 flowers, primarily by *Bombus*, *Habropoda*, *Apis*, and the robber *Xylocopa*. Across all pollinators, we found no

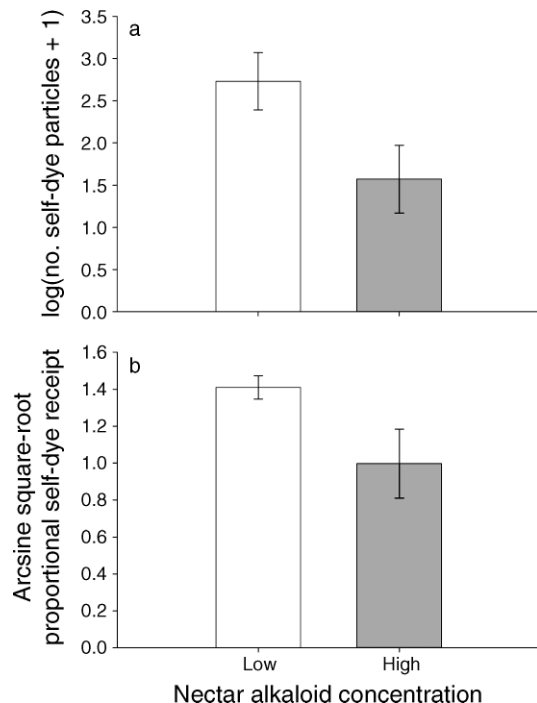


FIG. 1. *Gelsemium sempervirens* with nectar containing low alkaloid concentrations (open bars) (a) receive two times more self-dye and (b) have 30% higher proportional self-dye receipt than plants with nectar containing high alkaloid concentrations (gray bars). Bars are means \pm SE.

effect of nectar alkaloids on the number of times plants were visited ($\chi^2_1 = 0.07$, $P = 0.79$). However, nectar alkaloids influenced behavior once pollinators started probing flowers on a plant. Pollinators probed 52% more flowers per plant on plants with low nectar alkaloids (median, 100% of flowers probed; quartiles, 50–125% of flowers probed) relative to plants with high nectar alkaloids (median, 48% of flowers probed; quartiles, 29–92% of flowers probed; $\chi^2_1 = 4.86$, $P = 0.03$). Moreover, pollinators spent 33% more time per flower on plants with low (7.67 ± 1.15 s [mean \pm SE]) relative to high alkaloids in nectar (5.12 ± 1.41 s; a difference of 2.55 s per flower); this difference was marginally statistically significant ($F_{1,28} = 3.06$, $P = 0.07$). Floral morph had no effect on number of times plants were visited, percentage of flowers probed per visit, or time per flower ($P > 0.18$ in all cases), and we found no interaction between nectar treatment and floral morph for time per flower ($F_{1,28} = 0.71$, $P = 0.41$). Finally, we found no significant effects of nectar alkaloids on nectar-robber behavior (Appendix B).

Even though nectar alkaloids influenced some aspects of pollinator visitation, nectar alkaloids did not affect any measure of female reproduction in the absence of self-pollination (MANOVA, $\lambda = 0.10$, $F_{4,51} = 1.30$, $P = 0.28$; Appendix C), and we found no interaction between nectar alkaloids and floral morph (MANOVA: $\lambda = 0.07$,

$F_{4,51} = 0.89$, $P = 0.48$). Floral morph was the only factor that affected female reproduction (MANOVA, $\lambda = 0.27$, $F_{4,51} = 3.48$, $P = 0.01$). Thrums had 38% higher fruit set ($F_{1,54} = 5.23$, $P = 0.03$) and produced 60% more total seeds per plant than pins ($F_{1,54} = 8.45$, $P = 0.005$). Morph did not affect seed set per fruit ($F_{1,54} = 1.42$, $P = 0.24$) or seed weight ($F_{1,54} = 1.78$, $P = 0.19$).

Question 3: does self-pollen in combination with outcrossed pollen depress fruit and seed set?

Pollination treatment affected probability of fruit set ($\chi^2_3 = 15.29$, $P = 0.002$). Only 21% of flowers made seed-bearing fruits in the self-pollen treatment whereas $\sim 80\%$ of flowers made seed-bearing fruits in the outcrossed, self: outcrossed, and pollen chase treatments. There was no effect of floral morph on probability of fruit set ($\chi^2_1 = 2.19$, $P = 0.14$).

Pollination treatment also affected seed set per fruit ($F_{3,41} = 6.15$, $P = 0.002$). Self-pollinated fruits produced at least 70% fewer seeds per fruit than all other pollination treatments (Fig. 2). However, we found no significant difference in percentage seed set among the outcrossed, self: outcrossed, and pollen chase treatments (Tukey's hsd at $\alpha = 0.05$; Fig. 2). We also found no significant effect of floral morph or a treatment \times morph interaction on percentage seed set ($P > 0.7$ in both cases). These results suggest that solely self-pollinated flowers have lower fruit and seed production, but self-pollen in combination with outcrossed pollen or chased with outcrossed pollen did not depress fruit or seed set.

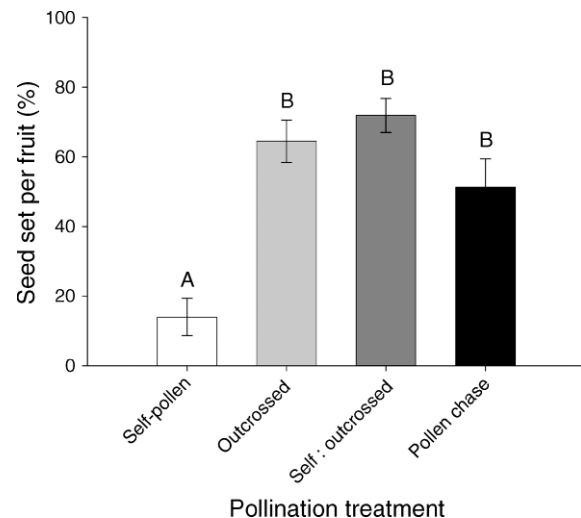


FIG. 2. Pollination treatment significantly affected percentage seed set per fruit. The self: outcrossed treatment refers to stigmas that received a 1:1 mixture of self-pollen and outcrossed pollen. The pollen chase treatment refers to stigmas that received self-pollen and then outcrossed pollen three hours later. Self-pollinated *Gelsemium* had significantly lower percentage seed set per fruit than flowers in all other pollination treatments. Bars are means \pm SE. Different uppercase letters indicate significant differences at $P < 0.05$.

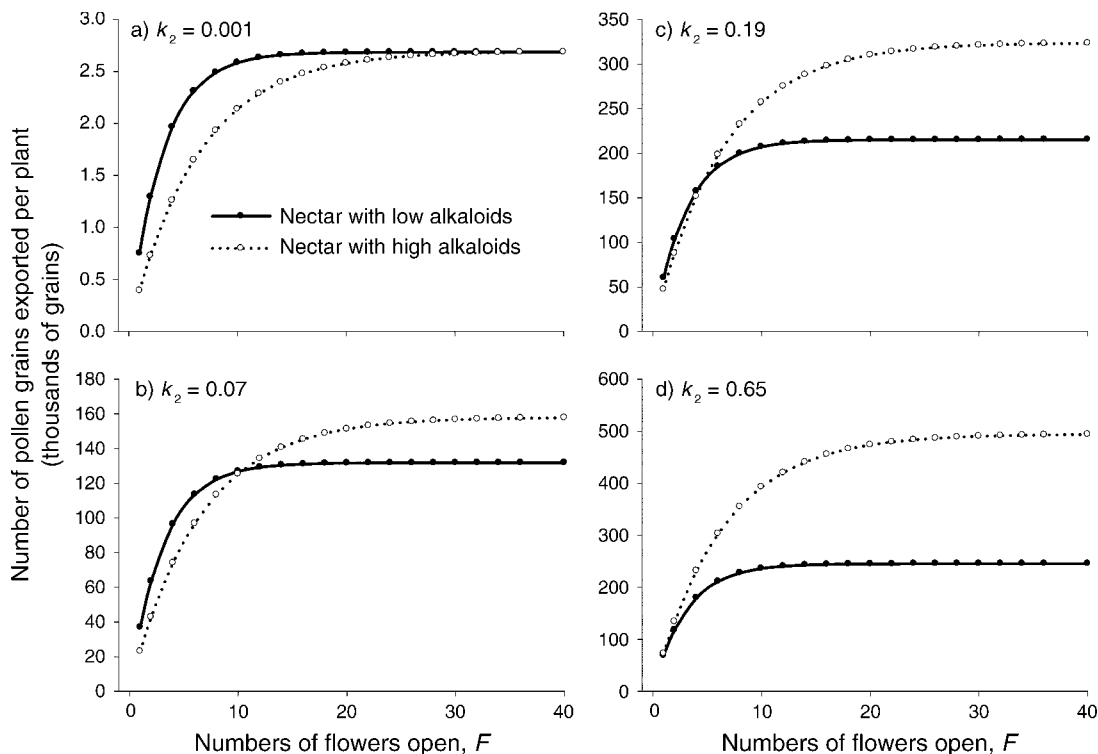


FIG. 3. The relationship between pollen export per plant and number of flowers open (the size of the floral display, F) for plants with nectar containing low vs. high alkaloid concentrations. The panels (a–d) show the fraction of pollen removed from anthers in a single visit (k_2) varying from inefficient (low) to efficient (high) values. The model was parameterized with values as in Table 1, with $X = 11$ pollinator visits/plant.

Question 4: what are the consequences of nectar alkaloids and self-pollen transfer on pollen export?

The model showed that high nectar alkaloids could benefit plants via increased pollen export relative to low nectar alkaloids, but only when pollinators were efficient and abundant and plants had large floral displays (Figs. 3 and 4).

Fraction of pollen removed per visit across a range of floral display sizes.—At high values of k_2 (fraction of pollen removed from anthers per visit), plants with high nectar alkaloids had higher pollen export than plants with low nectar alkaloids across most of the parameter space (Fig. 3c, d). However, at smaller values ($k_2 = 0.07$), high nectar alkaloids only benefited plants with large floral displays (Fig. 3b), and at the smallest value of k_2 that we used ($k_2 = 0.001$) there were no conditions under which high nectar alkaloids benefited plants in the currency of pollen export (Fig. 3a). Thus, when pollinator efficiency at removing pollen from anthers is low and plants have small floral displays, plants should entice pollinators to visit all open flowers because the benefits of multiple-flower visitation outweigh the costs of self-pollen transfer.

Number of pollinator visits to plants across a range of floral display sizes.—When the frequency of pollinator visits (X) was low, low nectar alkaloids were more beneficial for pollen export when plants had smaller

floral displays (Fig. 4a). However, as the number of pollinator visits increased (Figs. 3c and 4b) and as the size of the floral display increased, pollen export was higher for plants with high nectar alkaloids due to the benefit of stimulating pollinators to leave the plant, reducing self-pollen transfer.

DISCUSSION

Understanding how the traits of organisms influence mating patterns is important for individual fitness, the spatial scale of genetic variation, sex-allocation theory, and life history evolution (reviewed in Thornhill 1993). Here, we used experiments and a model to understand the degree to which nectar secondary compounds affected estimates of female and male reproduction via changes in pollinator behavior and self-pollen transfer. Although we predicted that nectar secondary compounds might benefit reproduction by reducing self-pollination, we found only partial support for this prediction. Our modeling results suggest that under some conditions high nectar alkaloids might benefit plants through increased pollen export (a component of male reproduction). However, in the experiments and in many conditions in the model, the costs of high nectar alkaloids in reducing pollination had either no effect or a negative effect on estimates of female and male

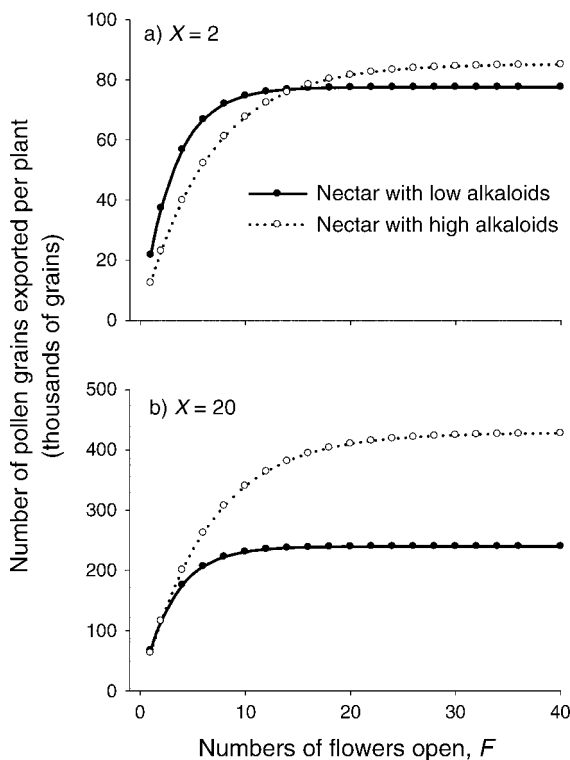


FIG. 4. The relationship between pollen export per plant and number of flowers open (the size of the floral display) for plants with low vs. high alkaloid nectar concentrations and (a) low ($X = 2$ visits/plant) and (b) high ($X = 20$ visits/plant) pollinator visitation rates. The model was parameterized with values as in Table 1, with $k_2 = 0.19$.

reproduction compared to the benefits of reducing self-pollen transfer.

Nectar alkaloids reduce self-pollen transfer

Our results demonstrate that nectar alkaloids can reduce within-plant self-pollen transfer. This result was not merely a function of plants with low nectar alkaloids receiving more total dye; low nectar alkaloids increased the proportion as well as total self-dye receipt. Changes in pollinator behavior are likely responsible for reduced self-dye transfer in plants with high nectar alkaloids. High nectar alkaloids reduced the percentage of flowers probed by $\sim 50\%$ across all pollinators. To our knowledge, few studies have tested how the chemical composition of nectar (beyond sugars) influences self-pollen transfer; however, a wealth of studies has examined how nectar quantity affects self-pollen transfer (e.g., Johnson and Nilsson 1999, Biernaskie et al. 2002, Smithson 2002). For example, one hypothesis for the absence of floral nectar in many orchid species is that it reduces self-pollen transfer by encouraging pollinators to leave plants after visiting few flowers (Dressler 1981). Given that 55% or more of plant species contain secondary compounds in their nectar, the effects of

these nectar compounds on pollinator behavior and self-pollen transfer warrant more attention.

Nectar alkaloids, self-pollen, and female reproduction

Because high nectar alkaloids reduced self-pollen transfer and per-flower visitation, we predicted that removing the possibility of self-pollination would increase female fitness to a greater extent in plants with low compared to high nectar alkaloids. With this prediction, we assumed that self-pollen depresses seed set, that increased per-flower visitation increases pollen deposition, and that plants are pollen limited for seed set. However, when we emasculated flowers, we found no benefit of low relative to high alkaloid nectar on female reproduction, even though pollinators visited 50% more flowers and spent marginally more time per flower on plants with low compared to high alkaloid nectar. This result appears to disprove our speculation from a previous study, in which nectar alkaloids did not affect female reproduction in *Gelsemium* (Adler and Irwin 2005), that the benefits of increased per-flower visitation and time per flower might be balanced by the costs of increased self-pollen transfer. Any such costs were removed in emasculated flowers. An alternate hypothesis that may explain the lack of effect on female fecundity is that plants may not have been pollen limited for seed set in the array. Therefore, increased per-flower pollinator visitation to plants with low vs. high nectar alkaloids may not have translated into differences in seed set. Supplemental hand pollination experiments in arrays of *Gelsemium* are needed to test this hypothesis further.

Three methodological considerations should be addressed when interpreting our results. First, one concern about using an emasculation treatment to remove the effects of self-pollen transfer is that anthers often attract pollinators to flowers (especially bees foraging for pollen) and may affect the morphological fit between flowers and pollinators. Nonetheless, emasculation treatments have been used in previous studies to assess levels of self-pollen transfer and reproduction in bird- and insect-pollinated species (e.g., Barrett and Glover 1985, Irwin 2003). To assess how emasculation might have altered insect pollinator behavior, we recorded pollinator visitation to emasculated and pollen-donor plants. We found no effect of emasculation on number of plant visits ($\chi^2_1 = 0.29$, $P = 0.59$), proportion of flowers probed per plant ($\chi^2_1 = 0.02$, $P = 0.89$), or time spent per flower ($F_{1,56} = 0.003$, $P = 0.96$), and we did not observe any striking difference in how pollinators handled flowers. These results suggest that within the context of our field array, emasculation did not strongly affect visitation behavior.

A second methodological consideration is that we manipulated nectar alkaloids and thus did not consider physiological costs of nectar alkaloid production on reproduction. Whether secondary compounds in nectar confer physiological costs to plants is unknown to our



PLATE 1. (Left) Floral display of *Gelsemium sempervirens* and (right) a pollinator visiting *G. sempervirens*. Photo credits: (left) Greg Crutsinger, (right) L. Adler.

knowledge, although physiological costs of secondary compounds in other tissue types are common (Strauss et al. 2002).

A final methodological consideration is that our high alkaloid treatment had gelsemine concentrations above the natural range (Adler and Irwin 2005) and used a constant 40% sucrose solution. Gelsemine dose-response studies in the lab using artificial flowers suggest that *Bombus impatiens* may alter their foraging behavior depending on gelsemine concentration (Gegear et al. 2007). Thus, one could argue that we biased our study towards finding a benefit of low nectar alkaloids on female reproduction in the absence of self-pollen transfer. However, we found no effect of nectar alkaloids on female reproduction in emasculated plants, suggesting that this potential bias did not strongly influence our results. Moreover, the ratio of sucrose to gelsemine affects pollinator behavior. A higher concentration of sucrose solution can reduce the deterrent effects of nectar gelsemine on pollinator visitation (Gegear et al. 2007). Dose-response studies in the field using a gradient of sugar and gelsemine concentrations in nectar are needed to assess their effects on pollinator behavior, within-plant and among-plant pollen transfer, and reproduction.

The effects of self-pollen on female reproduction

We initially assumed that self-pollen in combination with outcrossed pollen would reduce *Gelsemium* female reproduction. However, we found no evidence that a 50:50 mixture of self- to outcrossed pollen depressed fruit or seed set relative to completely outcrossed pollen. Moreover, even the application of outcrossed pollen three hours after self-pollen did not reduce reproduction compared to the outcrossing treatment. At $\alpha = 0.05$, we would have needed at least 76 replicates per treatment to

find a significant difference in proportion seed set. We cannot rule out the possibility, however, that waiting a longer period of time to provide outcrossed after self-pollen would affect seed set, as seen in other species (e.g., Gibbs et al. 2004).

Why didn't we find a negative effect of self-pollen in combination with outcrossed pollen on seed set? It is unlikely that the lack of effects was due to localized placement of pollen on stigmas, given that we fully mixed pollen mixtures. The negative effects of self-pollen may only be evident when plants receive high amounts of pollen, providing the opportunity for pollen competition (de Jong et al. 1992b), or when there is long-distance signaling between self-pollen (or self-pollen tubes) and carpel tissue that induces the maternal tissue to abort unfertilized ovules (Sage et al. 2006). Because we hand pollinated flowers with large amounts of pollen, it seems likely that pollen loads were large enough to create the opportunity for competition among selfed and outcrossed pollen, if it exists. Alternatively, if there is no interference between selfed and outcrossed pollen, we may have provided ample amounts of outcrossed pollen to ensure seed set. Thus, one possible explanation is that self-pollen may not significantly interfere with outcrossed pollen at the stigma, style, or ovule-penetration stages. Detailed hand pollination and histochemical staining studies are required in *Gelsemium* to test this hypothesis further.

Nectar alkaloids, self-pollen, and pollen export

Nectar alkaloids and self-pollen transfer could affect male as well as female reproduction. Pollen transferred within a plant cannot be donated to other plants, potentially reducing male reproduction if pollen removal and donation are positively correlated with male siring (Stanton et al. 1992). Using a pollen-carryover model,

we explored whether parameter space existed where high alkaloid nectar could benefit plants via increased pollen export. We found higher estimates of pollen export for plants with high alkaloid nectar when the fraction of pollen removed from anthers per visit was high, when pollinator visitation rate was high, and when a plant had a large floral display. How realistic are these conditions for *Gelsemium*?

Fraction of pollen removed per visit (k_2).—Based on our knowledge of the natural history of *Gelsemium*, we suspect that k_2 is low for this species relative to other plants (de Jong et al. 1992a). The amount of naturally occurring *Gelsemium* pollen on the bodies of bees is low compared to the amount of pollen produced per flower (see Adler and Irwin 2006). Moreover, Ornduff (1979) found large amounts of pollen left in the anthers of open flowers (up to 74%), suggesting either low visitation rate and/or low efficiency at picking up pollen.

Pollinator visitation rate (X).— X can vary widely as a function of biotic and abiotic factors (e.g., Price et al. 2005). For *Gelsemium*, pollinator visitation likely varies depending on the weather, insect abundance, and the abundance of other co-flowering plants (Gegeer et al. 2007; R. E. Irwin and L. S. Adler, *personal observations*), among other factors. During peak *Gelsemium* flowering on warm days, we observed 15 foraging bouts per plant per day, suggesting that values of $X = 20$ visits may be possible. Thus, in some cases, visitation rate may be high enough to confer an advantage to plants with high nectar alkaloids for pollen export (Fig. 4).

Floral display size (F).—If we assume that the fraction of pollen removed from anthers is low (e.g., $k_2 = 0.07$; Fig. 3b), then pollen export would be greater on plants with high compared to low nectar alkaloids only when $F > 10$ flowers open. The size of the floral display of naturally growing *Gelsemium* in pine forests can range from one to 286 open flowers, and *Gelsemium* growing in the forest canopy can have thousands of flowers open at once (R. E. Irwin and L. S. Adler, *personal observations*). For plants with these large floral displays, nectar secondary compounds could benefit plants by encouraging pollinators to leave after visiting few flowers. Taken together, the natural history of *Gelsemium* suggests that ecological conditions exist under which high nectar alkaloids might benefit plants through pollen export by reducing within-plant pollen transfer and increasing pollen export relative to plants with low nectar alkaloids. However, how often these ecological conditions exist and how they influence realized male function remain to be tested.

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APPENDIX A

Exponential decay model of pollen transfer: model assumptions and parameter estimates (*Ecological Archives* E089-126-A1).

APPENDIX B

The effect of nectar alkaloids on nectar-robber visitation (*Ecological Archives* E089-126-A2).

APPENDIX C

Female plant reproduction in the absence of self-pollen transfer for plants with low and high nectar alkaloid concentrations (*Ecological Archives* E089-126-A3).