CORRELATIONS AMONG TRAITS ASSOCIATED WITH HERBIVORE RESISTANCE AND POLLINATION: IMPLICATIONS FOR POLLINATION AND NECTAR ROBBING IN A DISTYLOUS PLANT[1]

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Plants interact simultaneously with a diversity of visitors, including herbivores and pollinators. Correlations among traits associated with herbivory and pollination may constrain the degree to which plants can evolve in response to any one interactor. Using the distylous plant, *Gelsemium sempervirens*, we tested the hypothesis that traits typically associated with pollination (distyly) and herbivore resistance (secondary compounds) were phenotypically correlated and examined how these traits influenced plant interactions with floral visitors. The flowers of *G. sempervirens* are visited by pollinators and a nectar robber, and the leaves and flowers express gelsemine, an alkaloid that is deterrent and sometimes toxic to visitors. Using an observational approach across five populations, we found the thrum floral morph (short-styled) expressed more leaf gelsemine than the pin morph (long-styled). Leaf gelsemine concentrations were positively correlated with flower gelsemine; however, there were no correlations between gelsemine and other floral morphological traits. Trait expression influenced pollination more so than robbing. Thrums received two times less pollen than pins. Moreover, across both morphs, pollen receipt was lower in plants that expressed higher levels of leaf gelsemine in two sites. These results imply that traits associated with pollination and herbivore resistance may not be independent.

Key words: alkaloids; distyly; flower size; *Gelsemium sempervirens*; herbivory; nectar production; nectar robbing; pollination.

Plants interact simultaneously with a variety of visitors, including antagonists such as herbivores, as well as mutualists such as pollinators. Classically, studies of plant defense against antagonists and attraction of mutualists have occurred independently. However, traits such as secondary compounds, thought to function primarily to deter antagonists, may directly or indirectly affect the attraction of floral visitors and subsequent pollination, and vice versa (Strauss and Armbruster, 1997; Herrera, 2000; Adler and Bronstein, 2004). This relationship between defensive and attractive traits may occur through a number of mechanisms (Strauss and Irwin, 2004). For example, defensive and attractive traits may be correlated, either due to pleiotropy or tight linkage. In this case, genes responsible for traits associated with herbivore resistance may influence (or be tightly linked to) floral attractive traits (Fineblum and Rausher, 1997; Irwin et al., 2003). In addition, traits that affect herbivore resistance may alter pollination (Armbruster, 1997; Adler, 2000) and vice versa (Brody, 1992; Galen and Cuba, 2001; Gómez, 2003). Because of the complex associations among traits and species interactions, the evolution of characters associated with pollination may be difficult to predict without some knowledge of how they relate to herbivore resistance traits. Nonetheless, we know surprisingly lit-tle about correlations among traits associated with pollination and herbivore resistance, how they vary spatially, and the consequences of such trait associations for species interactions.

Distylous plant species provide an ideal opportunity to assess the associations among traits related to pollination and antagonistic interactions (Olesen, 1979; Contreras and Ornelas, 1999; Leege and Wolfe, 2002; Ornelas et al., 2004). Populations of distylous plants contain individuals that have flowers that produce either long styles and short filaments ("pin" or L-morph) or short styles and long filaments ("thrum" or S-morph) that are typically self-incompatible. Distyly has traditionally been viewed as an adaptive trait to promote disassortative pollen transfer between anthers and stigmas of floral morphs (reviewed in Ganders, 1979; Barrett, 1990). However, floral morphs of distylous plants often vary systematically in a number of other floral characters related to pollination (Ganders, 1979), including flower and anther color (Wolfe, 2001), pollen and stigma size and morphology (Ornduff, 1970a), flower size (Pailler and Thompson, 1997), and the timing or amount of nectar reward (Arroyo and Dafni, 1995; Contreras and Ornelas, 1999). Such differences in floral characters between distylous (and tristylous) morphs may promote differential pollinator visitation or efficiency, pollen donation and receipt, and male and female plant reproduction (Wolfe and Barrett, 1987, 1989; Nishihiro et al., 2000). Interestingly, distylous morphs have also been shown to vary in the amount and pattern of damage received by herbivores, florivores, and seed predators (Olesen, 1979; Contreras and Ornelas, 1999; Leege and Wolfe, 2002). For example, pin morphs of *Palicourea padifolia* (Rubiaceae) experience higher leaf herbivory than thrum morphs (Ornelas et al., 2004). Although distylous morphs appear to be differentially attractive to pollinators and susceptible to antagonists, no studies to our knowledge report

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the strength of the relationships among floral morph, floral attractive characters, and herbivore resistance traits (i.e., secondary compounds), representing a gap in our understanding of the significance of distyly within the context of multispecies plant-animal interactions.

If all floral visits to distylous morphs were from equally efficient pollinators, then interpreting how attractive and resistance traits alter floral visitor behavior and pollination would be relatively straightforward. However, some floral visitors do not act as pollinators. Nectar robbers take nectar through holes bitten in flowers, often without providing pollination service (Inouye, 1980). Nectar robbing is extremely common among flowering plants; almost all flowering species with tubular corollas or nectar spurs undergo some form of floral larceny (Irwin and Maloof, 2002). Moreover, nectar robbing occurs in some distylous species (e.g., Contreras and Ornelas, 1999; Borges et al., 2003). Nectar robbing has a range of fitness consequences for plants, from positive to negative, through both direct and indirect mechanisms (Maloof and Inouye, 2000; Irwin et al., 2001). The addition of nectar robbers to a distylous plant system that includes pollinators and herbivores introduces another layer of complexity to our understanding of the ecological and evolutionary causes and consequences of trait expression. Floral traits thought to function primarily in relation to pollination may attract non-pollinating visits by nectar robbers (Lara and Ornelas, 2001). Additionally, chemical traits thought to function primarily to deter herbivores may also be expressed in floral tissue (Euler and Baldwin, 1996; Strauss et al., 2004), which could deter nectar robbers. Moreover, in a variety of tropical plant species, corolla tube damage by robbers may release secondary compounds from petal tissue into nectar, which could serve to protect the nectar from floral larceny (Guerrant and Fielder, 1981). The benefits and costs of such traits depend on the effects of nectar robbing on plant reproduction, the magnitude of their ecological costs (such as deterring pollinators) and allocation costs, and the degree to which such traits are correlated or tightly linked to other traits associated with species interactions and plant fitness.

As a first step toward understanding the ecological and evolutionary outcomes of complex trait associations and species interactions, we examined the degree to which traits typically associated with pollination (distyly and floral traits) and herbivore resistance (secondary compounds) were phenotypically correlated in a distylous plant, *Gelsemium sempervirens* (L.) (Loganiaceae; Carolina Jessamine). We also assessed how such trait correlations varied among floral morphs and populations and how the traits were related to levels of pollination and nectar robbing. Spatial variation in the strength of correlations and species interactions associated with particular traits could lead to differentiation of floral and defensive traits among populations, if the correlations have a genetic basis and species interactions affect plant fitness.

Gelsemium sempervirens has already been the focus of study on the effects of floral morphology on flower susceptibility to herbivory. Leege and Wolfe (2002) found that thrum flowers experienced higher herbivore damage to anthers and pin flowers experienced higher damage to stigmas, a result likely driven by increased susceptibility in more exposed floral structures. This result suggests that traits important in promoting disassortative pollen movement, such as distyly, may also alter floral susceptibility to herbivory. The work presented here builds on the existing study by Leege and Wolfe (2002)

by explicitly integrating the measurement of resistance traits (leaf and corolla secondary compounds) with floral traits, and relating how these traits influence floral visitation. Using an observational study in the field, we asked: Do floral and resistance characters and the magnitude of correlations among characters (1) vary among populations and between floral morphs and (2) influence pollination and nectar robbing?

MATERIALS AND 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, 1970b; Phillips, 1985). All field sites were located in Athens-Clarke County, Georgia (GA), USA. In this area, *G. sempervirens* blooms from early March into late April, producing up to several hundred yellow, tubular flowers per plant. Each tubular corolla has five petal lobes; individual flowers bloom for 3–5 days. *Gelsemium sempervirens* is distylous. Each plant has either long styles and short filaments (hereafter pin plants) or short styles and long filaments (hereafter thrum plants). Plants and morphs are self-incompatible (Ornduff, 1970b, 1980). In Bulloch County, GA (approximately 240 km south-east of our field sites), thrum flowers have longer, wider corollas than pin flowers, but thrum and pin flowers do not differ in estimates of nectar production or nectar sugar concentration (Leege and Wolfe, 2002). Pollen grains from the two morphs overlap in size, so that the morph of individual pollen grains cannot be identified (Ornduff, 1979).

To date, 45 indole-related alkaloids have been identified from *Gelsemium* species (Schun and Cordell, 1985; Liu and Lu, 1988; Lin et al., 1989a, b, 1991, 1996). Gelsemine, the principal alkaloid of *G. sempervirens*, has been isolated from leaves, flowers, and nectar (Kingsbury, 1964; Adler and Irwin, 2005). We focused on measures of leaf and corolla gelsemine concentrations in this study. We could not measure nectar gelsemine because such analysis requires at least 200 μL of nectar, and it was not possible to collect enough nectar for analysis from individual, wild-growing plants. Gelsemine is deterrent to insects, suggesting that gelsemine may alter plant interactions with a wide variety of leaf and floral visitors.

The dominant animals interacting with *G. sempervirens* flowers in Athens-Clarke County, GA are pollinators and nectar robbers. Unlike the populations of *G. sempervirens* in Bulloch County, GA studied by Leege and Wolfe (2002), we do not find significant levels of floral herbivory or leaf herbivory at our field sites. *Gelsemium sempervirens* is pollinated by a diversity of floral visitors, including *Bombus bimaculatus* (bumble bees, Apidae), *Apis mellifera* (honey bees, Apidae), *Osmia lignaria* (blue orchard bees, Megachilidae), and *Habropoda laboriosa* (blueberry bees, Apidae). In natural populations around Athens-Clarke County, GA, pollen supplementation to *G. sempervirens* increases fruit set by 60% (Adler and Irwin, in press); thus, increased pollen deposition generally translates into higher fruit set per plant. In addition, flowers of *G. sempervirens* are nectar robbed by *Xylocopa virginica* (carpenter bees, Xylocopidae). On some occasions, these bees will enter flowers "legitimately" through the floral opening used by pollinators before robbing; more commonly, however, *Xylocopa* make slits near the base of the corolla through which they steal nectar. Even in legitimate floral visits, *Xylocopa* transfer less pollen than all other legitimate floral visitors (Adler and Irwin, unpublished manuscript). During robbing visits, *Xylocopa* probably do not transfer pollen because it is unlikely that their body parts contact the sexual organs of the flowers. The effects of nectar robbing on plant–pollinator interactions and on male and female plant fitness in this system are unknown. Here we focused on asking whether variation in floral morphology and gelsemine concentrations in leaves and corollas in the two floral morphs were associated with plant interactions with pollinators and nectar robbers. The plant-fitness consequences of such interactions were beyond the scope of this study.

Field methods—We studied five populations (hereafter referred to as sites) of *G. sempervirens* in Athens-Clarke County, GA in March and April of 2002. Sites were separated by at least 1 km, and they were unlikely connected by gene flow via pollen or seeds. In each site, we chose 15–20 plants, comprising all the plants in a defined area that had at least three flowers or buds that we

could see, were distinguishable as individuals, and whose flowers were accessible from a 1.2-m stepladder. On each plant, we measured floral traits and nectar quantity and quality, leaf and corolla gelsemine concentrations, nectar robbing, and pollination.

Floral and nectar characteristics—At the onset of flowering, floral morph (pin or thrum) was recorded for each plant. During peak flowering, we measured the following floral traits using digital calipers (measured to the nearest 0.01 mm) on up to three haphazardly chosen, newly open flowers per plant: corolla length (from the base of the calyx to the corolla opening), corolla width (the width of the floral opening), petal-lobe length and width (of one haphazardly chosen petal lobe per flower), pistil protrusion (for pin plants only, the length of the style outside the corolla), and stamen protrusion (for thrum plants only, the length of the stamens outside the corolla). The dimorphism in pistil and stamen lengths in pin and thrum flowers has been well documented for *G. sempervirens* (Leege and Wolfe, 2002) and was not characterized here. To estimate nectar quantity and quality, we measured 48-h nectar production and sugar concentration. Elongated buds (just prior to opening) were enclosed in fine mesh bags to exclude floral visitors. We returned to flowers approximately 48 h later and measured nectar volume using microcapillary tubes (Drummond Scientific Co., Broomall, Pennsylvania, USA) and sugar concentration (in sucrose equivalents) using a hand-held refractometer (Fisher, Pittsburgh, Pennsylvania, USA). Because measures of nectar sugar concentration required at least 2 μL of nectar on the refractometer for a reliable measurement, we were not able to record sugar concentration for plants that did not produce enough nectar. We measured the above floral and nectar characteristics because these traits are variable in this and other systems and have been shown to influence interactions with a variety of floral visitors (e.g., Campbell, 1991; Mitchell, 1994; Lara and Ornelas, 2001; Leege and Wolfe, 2002). All floral and nectar traits were averaged within plants for statistical analyses (see *Statistical analyses*).

Leaf and corolla gelsemine—We sampled leaves and corollas twice during the flowering season (late March and early April) for gelsemine concentrations. For each plant, we removed up to 20 leaf pairs and up to 20 corollas. Leaves were pinched off at the base of the petioles. Because we sampled such a small amount of leaf tissue per plant, leaf removal is unlikely to have significantly affected plant photosynthetic ability during the blooming season. Corollas were pulled off the plant and included stamens (which are attached to the corolla tube) but not the gynoecium. One concern about corolla removal is that it could have reduced floral display size, especially for small-flowered plants. To address this concern, we only removed one-third of the flowers per plant (up to 20 flowers) on any sampling date. Moreover, mean floral display size (mean number of flowers open per plant measured on three separate census dates) was not correlated with levels of nectar robbing ($r = 0.11$, $N = 90$ plants, $P = 0.31$) or pollination ($r = 0.01$, $N = 89$ plants, $P = 0.90$) in this study. Because we removed only one-third of the flowers per plant, for some plants we did not have enough corolla tissue for the chemical analyses. Thus, the corolla gelsemine measures in this study are biased towards plants that produced more flowers. One additional concern is that leaf and flower collections could have induced secondary compound expression, which could have altered subsequent plant–animal interactions. It is unknown whether tissue damage induces higher alkaloid expression in *G. sempervirens*; however, pinching or cutting tissue from plants typically does not induce defenses in other systems (e.g., Agrawal, 1999; Agrawal et al., 1999). One final concern is that floral emasculation (see *Nectar robbing and pollination*) could have induced secondary compound expression in flowers. However, it seems unlikely that there was an effect of emasculation on corolla gelsemine production because emasculation and corolla collection dates were separated by 3 days, on average, and the number of flowers emasculated was not correlated with corolla gelsemine concentrations ($r = 0.01$, $N = 28$ plants, $P = 0.94$). Tissue was dried for ca. 48 h in a drying oven at 60℃. Dried samples were stored at room temperature until chemical analysis.

Alkaloids were extracted from dry, powdered plant parts that were sonicated in 0.5 N HCl for 10 min, stored overnight and then sonicated again for 10 min. The homogenate was adjusted to pH 12 with 6 N NaOH. Alkaloids were extracted by solid phase extraction using Extrelut columns (EM Science, Gibbstown, New Jersey, USA) and dichloromethane as an eluent. The dichloromethane was evaporated, and the alkaloids were dissolved in 0.5 mL methanol, then analyzed with commercial gelsemine as a standard (Indofine Chemical, Hillsborough, New Jersey, USA) using an HP 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, California, USA) with autosampler and Peak Simple software (Column DB-1; 30 m, 0.25 mm ID, 0.25 μm film thickness, J & W Scientific, Folsom, California, USA; Conditions: carrier gas He at 1 mL/min, split ratio 1 : 25. Program: detector temp. 320℃; injector temp. 300℃; oven temp. initial 200℃, ramp at 20℃ per min for 5 min to 300℃, then hold for 10 min).

There was a positive correlation in gelsemine concentrations between the two sampling dates (late March and early April) for leaves ($r = 0.71$, $N = 58$, $P < 0.0001$), and we found no significant difference in gelsemine concentrations between the two sample dates for leaves ($F_{1,139} = 1.07$, $P = 0.30$) or corollas ($F_{1,31} = 0.80$, $P = 0.38$). These results suggest that gelsemine concentrations do not vary significantly within a season. Therefore, for all statistical analyses, we averaged the gelsemine concentrations across the two sampling dates for each tissue type for each plant.

Nectar robbing and pollination—We estimated robbing levels on plants three times during the blooming season between late March and mid-April in each site. We ensured that at least 4 days separated census dates to avoid censusing the same flowers multiple times. We measured robbing by counting the number of flowers with robber holes, and calculated robbing level for each plant per census as the number of flowers robbed/number of flowers censused. This method of recording may underestimate nectar robbing if bees rob flowers multiple times. However, we were most interested in the relative differences in susceptibility to robbing among plants and sites and not the absolute levels of robbing that plants experienced; therefore, this method of measuring robbing should provide an appropriate estimate for comparison.

We estimated pollination using stigma pollen loads as indices of pollinator visits. On two separate dates in each site (between late March and mid-April), we emasculated 20% of the elongated buds on each plant, up to 10 buds. Approximately 5 days later, we returned to the flowers and collected their stigmas once the corollas abscised. Because flowers were emasculated, it is likely that all pollen deposited on the stigmas was transported by floral visitors (Ornduff, 1979). In previous studies, higher pollinator visitation to *G. sempervirens* increased pollen deposition to plants with pin and thrum flowers ($r = 0.32$, $N = 57$ plants, $P = 0.015$; Adler and Irwin, unpublished data). Stigmas were stained and mounted in a basic fuchsin dye (Kearns and Inouye, 1993), and the number of conspecific and heterospecific pollen grains on each stigma was counted with a compound microscope. We only present analyses with conspecific pollen receipt because heterospecific pollen receipt accounted for less than 50% of pollen deposition. Moreover, analyzing conspecific and total pollen receipt produced qualitatively similar results. Pollen counts were averaged across stigmas per plant for each census period.

Statistical analyses—To understand the degree to which floral attractive and resistance traits were related, we examined the phenotypic correlations among all traits across sites and floral morphs, by site, and by morph. The same general patterns emerged for the correlations across all plants, correlations by site, and correlations by morph; thus, we only present the most comprehensive analysis across sites and morphs. Gelsemine concentrations in the leaves and corollas were square-root transformed to achieve normality, and nectar volume was log $(x + 1)$ transformed. We did not apply the sequential Bonferroni correction to significance levels because this method greatly inflates the Type II error rate (Moran, 2003; Gotelli and Ellison, 2004). Instead, we follow the guidelines provided by Moran (2003) and Gotelli and Ellison (2004) and report unadjusted significance values.

One concern with the correlation analysis is that we are assessing phenotypic correlations, rather than genetic correlations, among traits. The expression of secondary compounds and floral characters can be influenced by environmental factors (Elle and Hare, 2002; Hol et al., 2003). Yet, given that the five sites studied were probably not connected by gene flow and were in fairly different habitat types (forested to open), it is unlikely that spurious,

TABLE 1.   Pearson product moment correlations among floral and resistance traits across sites and floral morphs of *Gelsemium sempervirens* below the diagonal. Plant sample sizes in parentheses. Bold values and asterisks indicate significant correlations[a].

Trait	Leaf gelsemine	Corolla gelsemine	Corolla length	Corolla width	Petal length	Petal width	Pistil/stamen protrusion	48-h nectar production	Sugar concentration
Leaf gelsemine	—								
Corolla gelsemine	**0.48 (27)****	—							
Corolla length	−0.14 (83)	−0.21 (28)	—						
Corolla width	0.02 (83)	−0.22 (28)	**0.22 (90)***	—					
Petal length	−0.09 (83)	−0.23 (28)	**0.32 (90)****	**0.43 (90)*****	—				
Petal width	0.07 (83)	−0.29 (28)	0.18 (90)	**0.63 (90)*****	**0.47 (90)*****	—			
Pistil/stamen protrusion	0.19 (83)	0.30 (28)	**−0.61 (90)*****	0.06 (90)	−0.07 (90)	−0.02 (90)	—		
48-h nectar production	−0.19 (60)	0.04 (23)	−0.07 (65)	−0.18 (65)	**−0.32 (65)****	−0.17 (65)	0.03 (65)	—	
Sugar concentration	−0.15 (36)	**−0.60 (14)***	**−0.38 (37)***	0.11 (37)	−0.08 (37)	0.11 (37)	0.02 (37)	−0.15 (37)	—

[a] Statistically significant coefficients: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

environmentally induced correlations among traits would result in generally the same correlation patterns across all five sites. Nonetheless, we cannot rule out the possibility that environmental variation may induce some of the observed correlations among traits. We are currently measuring genetic correlations among traits in a greenhouse study.

We asked whether expression of floral attractive and resistance traits varied significantly among sites, between floral morphs, and their interaction using MANOVA; factors were considered fixed. A significant MANOVA was followed by univariate tests for each trait (here and for all subsequent analyses). By using a multivariate analysis, we reduced the probability of inflating the Type I error rate and could test how site and floral morph affected several interrelated measures of floral and resistance traits (Rencher, 1995). Because we did not have corolla gelsemine and 48-h nectar production and concentration measurements on all plants, these response variables were not included in the MANOVA but were tested separately with univariate ANOVAs. To assess whether pin and thrum morph frequencies differed from the expectation of 1 : 1 in each site (Ornduff, 1970b), we used *G* tests.

To examine how patterns of nectar robbing and pollination varied during the season, we used repeated-measures ANOVAs. Proportion of flowers robbed was arcsine-square root transformed; pollen receipt was square-root transformed. To assess the robustness of our results from the repeated-measures ANOVAs, we also used other approaches (von Ende, 1993) to assess whether levels of robbing and pollination varied over the course of the season. Both the repeated-measures ANOVAs and the other approaches yielded similar results; thus, we only report results from the repeated-measures ANOVAs. Finally, to examine how floral and resistance traits influenced nectar robbing and pollination, we used a MANCOVA with site, floral morph, and their interaction as categorical variables, floral and resistance traits as covariates, and nectar robbing and pollination as response variables. In this analysis, nectar robbing and pollen receipt were averaged across the flowering season for each plant. The influence of corolla gelsemine and 48-h nectar production and concentration on robbing and pollination were tested using separate MANCOVAs for each covariate due to smaller sample sizes for these covariates. Prior to analysis, we examined the strength of collinearity among the covariates using variance inflation factors (VIFs). In all cases, VIFs were less than 2.0, suggesting that collinearity among the covariates did not strongly affect the precision or accuracy of the results (Myers, 1990). Finally, one assumption of analysis of covariance is homogeneity of slopes (reviewed in Underwood, 1997); i.e., that the slope of the line between traits and nectar robbing and pollination does not differ among sites or between floral morphs. Therefore, we initially screened for significant trait × site and trait × morph interaction terms for robbing and pollination. We only found one significant interaction term, between site and leaf gelsemine for pollen receipt. Thus, for leaf gelsemine, we removed the trait from the MANCOVA and assessed how leaf gelsemine altered pollination separately in each site. We performed all statistical analyses with JMP (academic version 4.0.4) statistical software (SAS Institute, Cary, North Carolina, USA).

RESULTS

Correlations among floral and resistance traits—Across all sites and floral morphs, we found that many of the floral mor-

phological characters were positively associated (Table 1). Corolla length was negatively correlated with the degree of protrusion of pistils (in pin plants) and stamens (in thrum plants) beyond the corolla tube (Table 1); thus, reproductive structures were more inserted within the corolla in longer flowers. Petal-lobe length was negatively correlated with 48-h nectar production, and corolla length was negatively correlated with nectar sugar concentration (Table 1). There was no significant relationship between 48-h nectar production and concentration (Table 1).

Turning to resistance traits, gelsemine concentrations ranged from 0 to 2052.8 µg/g in the leaves and from 0 to 463.1 µg/g in the corollas. Gelsemine was the only indole alkaloid we could detect in the leaf and corolla tissue. Concentration of gelsemine in leaves was positively correlated with corolla gelsemine (Table 1). In general, leaves expressed 20% higher gelsemine concentrations than corollas (paired *t* test: $t = 2.29$, df $= 26$, $P = 0.03$). Gelsemine concentrations in leaves and corollas were not significantly correlated with any other quantitative floral trait ($P > 0.09$ in all cases; Table 1). However, gelsemine concentration in corollas was negatively correlated with nectar sugar concentration (Table 1).

Variation in floral and resistance traits between floral morphs and among sites—We found significant differences in floral and resistance traits between floral morphs (Wilk's λ = 0.22, $F_{6,68} = 2.48$, $P = 0.03$) and among sites (Wilk's λ = 0.51, $F_{24,238} = 2.09$, $P = 0.003$). However, site did not affect the degree to which floral or resistance characters varied between floral morphs (Wilk's λ = 0.64, $F_{24,238} = 1.35$, $P = 0.13$). Moreover, within each site, the ratio of pin to thrum morphs did not deviate significantly from the 1 : 1 expectation ($G < 1.70$, $P > 0.20$ in all cases; pin : thrum ratio range: 0.63–2.00).

Univariate ANOVAs showed that leaf gelsemine concentration and corolla length varied significantly between floral morphs (Table 2). Thrum plants expressed 30% more gelsemine in their leaves and produced 2% longer corolla tubes than pin plants. Moreover, thrum plants expressed 26% more gelsemine in their corollas, but this difference was not statistically significant (Table 2), probably due to low sample size. We only had a power of 0.41 to detect a statistically significant effect (α = 0.05) of floral morph on corolla gelsemine concentration. To obtain a significant difference in corolla gelsemine between floral morphs (α = 0.05), we needed samples from at least 36 plants (we had 28 plants). No other floral or nectar characters differed significantly between floral morphs (Table 2).

Three floral characters differed significantly among sites:

Table 2. Variation in floral and resistance traits between pin and thrum floral morphs of *Gelsemium sempervirens*. Values are means (±1 SE). Statistical differences in traits between floral morphs came from univariate ANOVAs with morph, site, and morph × site interactions as factors; only the morph factor shown. There were no significant morph × site interaction terms ($P > 0.05$ in all cases). Values in bold indicate statistically significant morph effects.

Character (units)	Pin	Thrum	df	F	P
Leaf gelsemine (μg/g)	**189.16 (25.35)**	**344.27 (63.06)**	**1,73**	**7.16**	**0.009**
Corolla gelsemine (μg/g)	119.38 (35.59)	202.73 (36.26)	1,22	3.31	0.08
Corolla length (mm)	**22.12 (0.30)**	**23.36 (0.30)**	**1,80**	**5.59**	**0.02**
Corolla width (mm)	12.97 (0.20)	13.26 (0.24)	1,80	2.90	0.09
Petal length (mm)	9.79 (0.16)	9.86 (0.17)	1,80	0.26	0.61
Petal width (mm)	9.48 (0.17)	9.61 (0.18)	1,80	0.58	0.45
Pistil/stamen protrusion (mm)	3.02 (0.28)	2.38 (0.17)	1,80	1.76	0.19
Nectar production (μL/48 h)	1.07 (0.13)	1.15 (0.26)	1,55	2.48	0.14
Nectar concentration (%)	49.97 (1.84)	46.67 (2.07)	1,29	0.26	0.61

corolla width ($F_{4,80} = 9.95$, $P < 0.0001$), petal-lobe width ($F_{4,80} = 4.94$, $P = 0.0013$), and petal-lobe length ($F_{4,80} = 2.47$, $P = 0.05$). Plants at the Research Drive site generally produced wider corollas and petal lobes and longer petal lobes than flowers at other sites (Fig. 1A–C). No other floral characters or leaf or corolla gelsemine differed among sites ($P > 0.15$ in all cases). Nectar traits differed significantly among sites; flowers at the Whitehall site produced marginally more nectar ($F_{4,55} = 2.22$, $P = 0.08$; Fig. 1D) that was less concentrated by about 10% than flowers at other sites ($F_{3,29} = 3.27$, $P = 0.04$; Fig. 1E).

Variation in nectar robbing and pollination—Nectar robbing ranged from 0 to 100% of flowers per plant per census
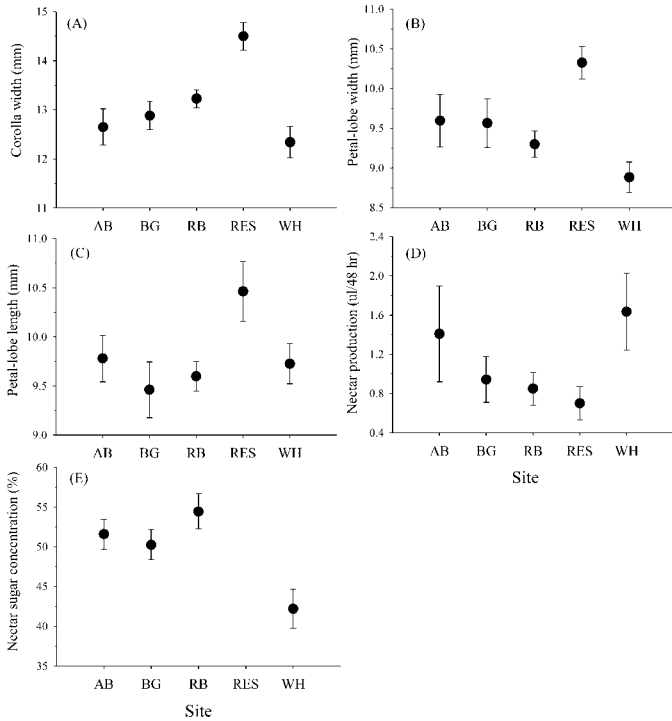

Fig. 1. Variation in (A) corolla width, (B) petal-lobe width, (C) petal-lobe length, (D) 48-h nectar production, and (E) nectar sugar concentration in *Gelsemium sempervirens*. Values are means ± 1 SE. Site abbreviations are as follows: AB = Abi site, BG = Botanical Garden, RB = Riverbend Road, RES = Research Drive, and WH = Whitehall Forest. Because so little nectar was produced by flowers at RES, nectar sugar concentration could not be measured at the site.

period. Robbing level varied significantly across the three census periods (repeated-measures ANOVA season effect: $F_{2,110} = 11.29$, $P < 0.0001$). Robbing levels were highest at the first census period (mean percentage robbing ± 1 SE: 27.9 ± 3.1%), lowest at the second census period (4.2 ± 4.0%), and intermediate at the third (16.6 ± 3.9%). Mean pollen receipt per stigma per plant (an estimate of pollination) ranged from 0–1324 pollen grains. Pollen receipt varied significantly between the two census periods (repeated-measures ANOVA season effect: $F_{1,43} = 44.20$, $P < 0.0001$), decreasing 3× between the first (mean pollen receipt per stigma per plant ± 1 SE: 310.4 ± 24.6 pollen grains) and second (106.0 ± 33.9 pollen grains) census periods. Averaged across the season, there was no significant correlation between levels of robbing and pollination plants received ($r = 0.009$, $N = 89$ plants, $P = 0.93$).

Using a MANCOVA, we found that floral morph and petal-lobe length influenced floral visitation (i.e., robbing and pollination; Table 3). We also found that floral visitation differed among sites and that site affected the degree to which floral morph influenced visitation (Table 3). No other floral characters (corolla length or width, petal-lobe width, and pistil/stamen protrusion) were associated with floral visitation (Table 3). We then used univariate ANCOVAs (results described next) to assess whether the subset of floral characters and sites influenced nectar robbing, pollination, or both types of visitation.

Nectar robbing did not vary among sites or between floral morphs; there was no site × floral morph interaction, and petal-lobe length did not influence levels of nectar robbing (Table 3). Significant factors observed in the MANCOVA were driven by differences in pollination. We found significant differences among sites in pollen receipt and the degree to which pollen receipt varied among sites in pin versus thrum morphs (Table 3; Fig. 2). Across all floral morphs, pin plants received two times more pollen than thrum plants per stigma (Table 3). In addition, plants with shorter petal lobes received significantly more pollen (Table 3).

Nectar characters and corolla gelsemine were analyzed separately from the other floral traits, due to lower sample sizes. Nectar production had no effect on nectar robbing or pollination (Wilk's λ = 0.06, $F_{2,52} = 1.64$, $P = 0.20$). However, nectar sugar concentration did influence floral visitation (Wilk's λ = 0.36, $F_{2,27} = 4.80$, $P = 0.02$). Robbers foraged on plants that produced more concentrated nectar ($F_{1,27} = 7.16$, $P = 0.01$), whereas nectar sugar concentration was not associated with pollen receipt ($F_{1,27} = 1.61$, $P = 0.22$).

TABLE 3. Effect of site, floral morph, and covariates (floral characters) on levels of nectar robbing and pollination in *Gelsemium sempervirens*. In the factor column, S × M denotes site by floral morph interaction. Bold values and asterisks indicate MANCOVA or univariate ANCOVAs that are statistically significant[a].

Factor	MANCOVA			Nectar robbing			Pollination		
	df	Wilks' λ	F	df	SS	F	df	SS	F
Site	**8,146**	**0.73**	**3.12****	4,75	0.75	2.08	**4,74**	**434.93**	**4.47****
Floral morph	**2,73**	**0.36**	**12.96*****	1,75	0.04	0.44	**1,74**	**637.08**	**26.19*****
S × M	**8,146**	**0.80**	**2.10***	4,75	0.36	1.01	**4,74**	**340.56**	**3.50****
Corolla length	2,73	0.04	1.31	1,75	0.15	1.71	1,74	16.22	0.67
Corolla width	2,73	0.05	1.81	1,75	0.04	0.47	1,74	75.13	3.09
Petal length	**2,73**	**0.11**	**3.91***	1,75	0.01	0.16	**1,74**	**191.42**	**7.87****
Petal width	2,73	0.01	0.32	1,75	0.01	0.14	1,74	13.03	0.54
Pistil/stamen	2,73	0.01	0.36	1,75	0.04	0.45	1,74	3.16	0.13

[a] Statistically significant factors: * $P \le 0.05$, ** $P \le 0.01$, *** $P \le 0.001$.

Concentration of gelsemine in corollas had no effect on nectar robbing or pollination (Wilk's $\lambda = 0.07$, $F_{2,20} = 0.74$, $P = 0.49$). Moreover, leaf gelsemine had no effect on nectar robbing ($F_{1,75} = 0.21$, $P = 0.65$). However, in two sites, higher concentrations of leaf gelsemine were marginally associated with lower pollen receipt (Botanical Garden: $F_{1,12} = 9.51$, $P = 0.009$; Riverbend Road: $F_{1,11} = 3.49$, $P = 0.088$; Fig. 3). In the other three sites, concentrations of leaf gelsemine and pollen receipt were not significantly related ($P > 0.26$ in all cases).

DISCUSSION

There is growing recognition that traits associated with pollination might not evolve independently from traits associated with herbivore resistance (Strauss and Irwin, 2004). Here we add to studies linking pollination and herbivory by showing that traits associated with pollination (distyly) and that traits associated with herbivore resistance (secondary compounds) may not be independent. For example, plants with thrum flowers were more heavily defended with alkaloids in their leaves than plants with pin flowers, and there was a negative phenotypic correlation between nectar sugar and corolla gelsemine concentrations. Thrum flowers received two times less pollen than pin flowers, as is common in many distylous species

(Ganders, 1979). Moreover, in two sites across both floral morphs, pollen receipt was lower in plants with higher alkaloid expression in their leaves. It is important to note that the effects of leaf gelsemine and floral morph on pollen deposition are confounded in this study, and teasing apart the relative importance of each character requires larger sample sizes (within and among sites) and experimental manipulation. However, two lines of evidence suggest that leaf gelsemine may be important in influencing pollination independent of any effects of floral morph. First, we found no significant interaction between leaf gelsemine and floral morph for pollen receipt, suggesting that floral morph does not alter the degree to which leaf gelsemine influences pollen receipt. Second, within the two sites in which leaf gelsemine was negatively related to pollen deposition, both pin and thrum morphs exhibited a wide range of leaf gelsemine expression (Fig. 3), suggesting that the negative relationship between leaf gelsemine and pollen receipt was not solely driven by floral morph. Given the relationships among traits associated with pollination (distyly) and herbivore resistance (secondary compounds) and their effects on pollination, our results imply that traits associated with herbivore resistance and pollination may not evolve independently in this system. Addressing such a hypothesis further requires knowledge of genetic correlations among traits, which we are currently assessing.

Our study was not designed to tease apart mechanisms driving the relationships between traits and interactions; we were most interested in assessing whether phenotypic correlations exist among traits associated with pollination and herbivore resistance and how these traits influence pollination and nectar robbing. However, given our findings, future studies that experimentally tease apart the mechanisms will provide addi-
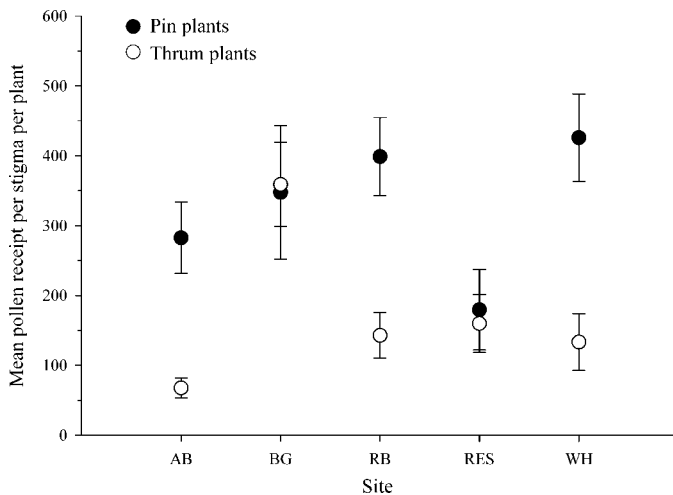

Fig. 2. Site affects the degree to which floral morph (pin versus thrum) alters pollen receipt in *Gelsemium sempervirens*. Values are mean pollen grain receipt per stigma per plant ± 1 SE. Site abbreviations as in Fig. 1.
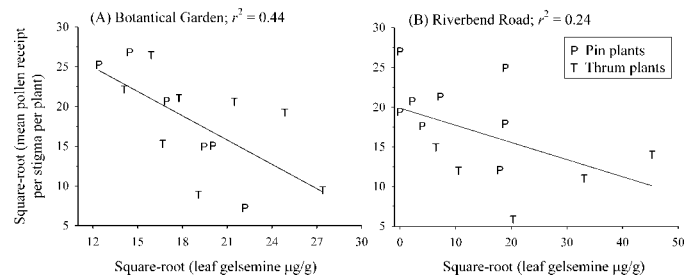

Fig. 3. Pollen load per stigma per plant is negatively associated with concentrations of gelsemine in the leaves of *Gelsemium sempervirens* at two sites: (A) Botanical Garden and (B) Riverbend Road.

tional ecological and evolutionary insight. For example, why do thrum flowers express higher concentrations of leaf gelsemine? One plausible hypothesis is that their larger, showy flowers make thrum plants more attractive to antagonists (Contreras and Ornelas, 1999), which could select for increased gelsemine expression in this morph. Such a hypothesis warrants further attention. In addition, it is surprising that floral display size did not positively affect levels of nectar robbing or pollination, as shown in previous systems (Mitchell, 1994; Mitchell et al., 2004). One hypothesis is that plants with larger floral displays may attract more total visits but fewer visits per flower than plants with smaller floral displays (Brody and Mitchell, 1997). Such a distinction is important because in this study, we estimated both nectar robbing and pollination as means per flower per plant. Finally, it is unknown why the WH site produced generally smaller flowers with copious amounts of dilute nectar compared to other sites. Trait differences among sites could be the result of selective forces, such as pollinator or robber preferences, and/or nonselective forces, such as variation in resources that alter flower size and nectar production and concentration.

The only other character aside from leaf gelsemine and floral morph that influenced pollination was petal-lobe length. Plants with shorter petal lobes experienced higher levels of pollen receipt. While this result was initially surprising, petal-lobe length was negatively correlated with 48-h nectar production; thus, pollinators may be attracted to flowers with shorter petal lobes and/or may use petal-lobe length as a reward cue. In other systems, nectar reward has been associated with visible floral characters (Stanton and Preston, 1988; Cresswell and Galen, 1991), and pollinators have been shown to use floral traits as cues for reward status (Blarer et al., 2002). The negative correlation between nectar production and a floral size character could suggest a tradeoff between reward production and the production of larger flower parts; however, additional experimental work would be required to test such a hypothesis.

Surprisingly, secondary compounds in leaves and corollas did not influence nectar robbing. Even though gelsemine was expressed in petal tissue and gelsemine (in nectar) has been shown to be deterrent to carpenter bees in a previous study (Adler and Irwin, 2005), gelsemine expression in corollas did not deter carpenter bees from robbing flowers. One possible explanation for the lack of effect of corolla gelsemine on robbing levels is that when carpenter bees use their maxillary galeae to make slits in the corollas (Gerling et al., 1989), they do not taste gelsemine in the corolla tissue. Moreover, corolla gelsemine may deter other floral visitors (i.e., florivores in other regions) or other floral inhabitants (such as flower mites or ants); such a hypothesis has not been tested. The only character that was associated with nectar robbing was nectar sugar concentration. Nectar robbers foraged more often on plants that produced more concentrated nectar. The preference of bees for more concentrated nectar has been shown in other systems as well (Bolten and Feinsinger, 1978; Wiegmann et al., 2003; Irwin et al., 2004).

One important aspect of our results is that many of the floral traits positively scaled with one another, as did leaf and corolla secondary compounds. Positive correlations among floral size traits are common in many flowering species (e.g., Irwin, 2000; Wolfe, 2001), and positive scaling relationships in body parts is common in animals as well (e.g., Frankino et al., 2005). These correlations could be driven by natural selection

as well as by developmental constraints. With respect to the secondary compounds, relatively few studies have provided detailed comparisons of secondary compound expression in multiple tissue types. Those that have done so have generally found positive correlations for secondary compound expression (e.g., Ehrlén and Eriksson, 1993). On one hand, the correlated expression of gelsemine in leaf and corolla tissue found in our study could be driven by correlated selection from foliar and floral herbivory, respectively. This would be the case if plants that receive leaf herbivory are also more likely to receive floral damage, driving the evolution of both traits in concert. On the other hand, gelsemine expression in corollas may be a pleiotropic consequence of gelsemine expression in the leaves, in which case the evolution of traits involved in floral interactions may be constrained by leaf interactions, and vice versa. The notion that secondary compound expression in different tissue types might be a pleiotropic consequence of leaf secondary compound expression has been hypothesized to explain the existence of both secondary compounds in nectar (Adler, 2000) and in ripe fruits (Ehrlén and Eriksson, 1993) and may be important here as well.

Gelsemium sempervirens has been the subject of previous work assessing variation in floral morphology among pin and thrum morphs (Ornduff, 1970b; Leege and Wolfe, 2002) and how floral morphology influences flower susceptibility to herbivores (Leege and Wolfe, 2002). Our results with respect to floral morphology generally match previous work in this and other distylous plant systems. In particular, thrum flowers are longer than pin flowers (reviewed in Ganders, 1979), potentially due to developmental constraints (Dulberger, 1992); thrum and pin flowers do not differ in 48-h nectar production or concentration (Leege and Wolfe, 2002); and pin flowers receive more pollen than thrum flowers in *Gelsemium* spp. (Ornduff, 1970b) as well as in other distylous systems (Ganders, 1979). Our work adds to these previous studies by explicitly assessing the relationships among floral traits and secondary compound concentrations. One major difference between our work and previous work, particularly by Leege and Wolfe (2002), is that their study sites around Bulloch County, GA had high levels of floral herbivory, whereas our study sites around Athens-Clarke County, GA were dominated by pollinators and nectar robbers, but not floral herbivores. Interestingly, *G. sempervirens* near Bulloch County, GA do not have significant levels of nectar robbing (L. M. Leege, Georgia Southern University, personal observation). Spatial variation in both the abundance and the identity of species interactors is common (Thompson, 1994). If these species are important agents of selection on traits, geographic variation in the extent of species interactions could serve to create differentiation in traits among populations.

In conclusion, traits associated with herbivory and pollination may not be independent in *Gelsemium sempervirens*. Some resistance and attractive traits were phenotypically correlated, including a negative correlation between nectar sugar and corolla gelsemine concentrations. Plants with thrum flowers were more heavily defended with alkaloids in their leaves. In addition, thrum flowers received significantly less pollen than pin flowers, and across both floral morphs, pollen receipt was lower in plants with higher leaf alkaloids in two sites. Our next step in this system is to link trait associations and species interactions to plant fitness to understand the evolutionary ecology of trait expression in a multispecies context. Given that almost all plants interact simultaneously with multiple

species, including both mutualists and antagonists, correlations between characters related to mutualism and antagonism may be ubiquitous and may have widespread importance to our understanding of the evolution of traits in natural systems.

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