Pollinator specialization and pollination syndromes of three related North American *Silene*

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Abstract. Community and biogeographic surveys often conclude that plant–pollinator interactions are highly generalized. Thus, a central implication of the pollination syndrome concept, that floral trait evolution occurs primarily via specialized interactions of plants with their pollinators, has been questioned. However, broad surveys may not distinguish whether flower visitors are actual pollen vectors and hence lack power to assess the relationship between syndrome traits and the pollinators responsible for their evolution. Here we address whether the floral traits of three closely related hermaphroditic *Silene* spp. native to eastern North America (*S. caroliniana*, *S. virginica*, and *S. stellata*) correspond to predicted specialized pollination based on floral differences among the three species and the congruence of these floral features with recognized pollination syndromes. A nocturnal/diurnal pollinator exclusion experiment demonstrated that all three *Silene* spp. have diurnal pollinators, and only *S. stellata* has nocturnal pollinators. Multiyear studies of visitation rates demonstrated that large bees, hummingbirds, and nocturnal moths were the most frequent pollinators of *S. caroliniana*, *S. virginica*, and *S. stellata*, respectively. Estimates of pollen grains deposited and removed per visit generally corroborated the visitation rate results for all three species. However, the relatively infrequent diurnal hawkmoth pollinators of *S. caroliniana* were equally effective and more efficient than the most frequent large bee visitors. Pollinator importance (visitation × deposition) of each of the animal visitors to each species was estimated and demonstrated that in most years large bees and nocturnal moths were the most important pollinators of *S. caroliniana* and *S. stellata*, respectively. By quantifying comprehensive aspects of the pollination process we determined that *S. virginica* and *S. stellata* were specialized on hummingbirds and nocturnal moths, respectively, and *S. caroliniana* was the least specialized with diurnal hawkmoth and large bee pollinators. Compared across the *Silene* species, divergent floral character states are consistent with increasing the attraction and/or pollen transfer efficiency of their respective major pollinators, which suggests that the pollinators are past and/or contemporary selective agents for floral trait evolution in these three *Silene* species. We conclude that the pollination syndrome concept allows us to effectively relate the functional significance of floral morphology to the major pollinators of these *Silene* species.

Key words: generalization; pollination syndrome; pollinator importance; Silene; *specialization.*

Introduction

Pollination syndromes are suites of traits and trait combinations that are hypothesized to increase the attraction and pollen transfer of specific types of pollinators. Traditionally floral evolution and diversity have been interpreted from the perspective of specialized ecological interactions between flowers and their major

pollinators (Darwin 1862, Grant and Grant 1965, Stebbins 1970, Faegri and van der Pijl 1979, Fenster et al. 2004). From this perspective flowers are considered adaptations, composed of suites of independently evolved correlated traits, in which flowers of similar form (pollination syndromes) reflect selection response to similar pollinators or selective agents (Faegri and van der Pijl 1979, Vogel 2006), i.e., functional groups of pollinators (Fenster et al. 2004). The pollination syndrome concept has support from studies demonstrating natural selection by major pollinators on floral traits (Campbell 1989, Caruso et al. 2003), associating floral polymorphisms with pollination ecotypes (Grant and Grant 1965, Galen et al. 1987), and mapping

pollinator shifts onto phylogenies associated with multiple independent evolution of divergent character states (Fenster et al. 2004, Kay et al. 2005, Wilson et al. 2006, Whittall and Hodges 2007).

The pollination syndrome concept has also proven to be a controversial framework for predicting the important pollinators of plant taxa and for implicating single pollinators as the main sources of floral evolution. Community and geographic surveys of plant–pollinator interactions often show the majority of plant species are "ecologically generalized" or pollinated by multiple animal visitors (Waser et al. 1996, Armbruster et al. 2000, Fenster et al. 2004, Ollerton et al. 2006). Evolutionary stable strategy models demonstrate generalization is favored under certain conditions, such as interannual variation in pollinator density (Waser et al. 1996) or high relative density of focal plant species (Sargent and Otto 2006). Ecological network studies demonstrate plant and pollinator assemblages form highly interconnected webs (Olesen and Jordano 2002). The most common form of pairwise interaction is weak dependence, suggesting generalization on many partners, but the interactions are asymmetric as plants depend more on particular animals than the reverse (Bascompte et al. 2006). These large-scale community-wide surveys suggest that generalization confers stability in mutualistic networks. Furthermore, generalization would seem to lessen the negative demographic consequences of highly variable pollinator density in space and time (Herrera 1988, Waser et al. 1996, Ivey et al. 2003).

A consensus emerging from the debate is that detailed empirical data are needed to evaluate floral specialization and whether pollination syndromes are realistic for describing floral adaptation (Waser et al. 1996, Fenster et al. 2004). In particular, data that describe both the quantity and quality of pollinator visits are needed to distinguish visitors from pollinators, which are potential selective agents on floral form (Schemske and Horvitz 1984, Herrera 1987, 1989). According to Stebbins' (1970) most effective pollinator principle, visitation and effectiveness should both be considered when describing flower adaptations that facilitate pollination. Pollinator importance is the product of visitation frequency and effectiveness (e.g., fruit set, seed set, and pollen grains per visit; reviewed in Reynolds and Fenster 2008). When it is properly estimated statistical comparisons of mean importance can be made among visitor taxa to determine on which pollinators the plant specializes for successful reproduction (Reynolds and Fenster 2008).

Following Fenster et al. (2004), we define specialization from the plant's perspective to mean pollinator service by one pollinator type that accounts for at least three-quarters of total pollinator importance. By this criterion, we conclude that a plant has a specialized pollination system if a pollinator's importance is threefold greater than the next most important pollina-

tor or functional group. However, an all-inclusive approach for evaluating pollinator specialization would weigh evidence from all pollination data including estimates of male reproductive success. We note that indices of pollinator importance commonly rely on female reproductive success, and so our approach here is not unusual in this regard. While the criterion for assessing pollinator specialization or generalization is arbitrary, in the absence of experimental approaches it is a reasonable first step for identifying the agents of selection on floral design.

Finding floral traits and trait combinations congruent with a particular pollination syndrome suggests the corresponding pollinator has been or is an important selective agent for floral evolution. Alternatively, floral specialization may arise without relation to historical plant pollinator interactions and pollinator selection on floral traits. For example, specialized pollination may result from mismatched phenology, with asynchronous flowering and animal activity windows, or spatial isolation as observed in island (more specialized) vs. mainland plant species (Olesen and Jordano 2002, Ollerton et al. 2007). Using a population genetic modeling approach, Sargent and Otto (2006) demonstrated that specialization was a favored outcome under low focal plant density, when visits from different pollinators would likely bear heterospecific pollen. It may be impossible to distinguish between syndrome and non-syndrome ecological factors associated with pollinator specialization when studying plant species in isolation. However, non-syndrome causes of specialization should not confound our ability to assess the relevance of syndromes when the pollinators' role as selective agents on floral traits is considered in a multispecies context. If syndromes predict specialized pollination systems then it is unlikely ecological factors independent of syndromes would be associated with specialized pollination in every case. Therefore a comprehensive analysis of pollination system and syndrome traits in a group of related plant taxa would enable us to assess the predictive power of syndromes and simultaneously consider alternative causes of specialization.

Here we quantify the extent of floral specialization and evaluate the predictive value of pollination syndromes of three related North American *Silene* spp. (*S. caroliniana*, *S. virginica*, and *S. stellata*). Molecular phylogenies indicate these three hermaphroditic species form a single clade among the nine endemic *Silene* east of the Rocky Mountains (Burleigh and Holtsford 2003). *Silene caroliniana* and *S. virginica* are sister species (Popp and Oxelman 2007). These *Silene* spp. are remarkably different in floral traits with respect to pollinator attraction, reward, and efficient pollen transfer. The pattern of interspecific variation among *Silene* spp. floral traits and trait combinations (Appendix A) may be used to predict the most important pollinators according to traditional syndrome defini-

tions. The intermediate sized, pinkish, scentless flowers of *S. caroliniana*, with scant but concentrated nectar and narrow, tubular flowers, are suggestive of both long-tongued bees and diurnal Lepidoptera syndromes (Faegri and van der Pijl 1979, Balkenius et al. 2006, Vogel 2006). The comparatively large, red, scentless flowers of *S. virginica*, with copious and dilute nectar (Baker and Baker 1983), tubular flower shape, and highly exserted stamens and stigmas (Faegri and van der Pijl 1979, Campbell 1989), are traits indicative of hummingbird pollination. The smaller, white, fringed, and nocturnally fragrant flowers of *S. stellata*, with scant nectar reward and bowl-shaped flowers, are indicative of a nocturnal moth syndrome (Faegri and van der Pijl 1979, Vogel 2006).

Our objectives were (1) to fully describe the floral and breeding system characters among these three *Silene* spp. and (2) to determine the degree to which the *Silene* spp. specialize on their predicted pollinators by quantifying flower visitation rate, pollen removal, pollen deposition, and pollinator importance of each of the animal visitors. By comparing the presence or absence of suites of traits across the three species in relation to the degree of specialization or generalization evident from the detailed pollination studies, we can test the usefulness of pollination syndromes in predicting the principal pollinators of these *Silene* spp. With little agreement between the syndromes and observed pollinators we would conclude the syndrome concept lacks predictive power in this system, and we would consider pollinators to have minor roles in floral evolution. Otherwise, correspondence of syndrome and pollinator would implicate single pollinators or pollinator functional groups as the likely past and/or contemporary selective agents for floral diversification. Either finding begs further study of causal selective mechanisms underlying floral trait evolution in this eastern North American clade of *Silene*.

NATURAL HISTORY OF STUDY SYSTEM

Silene caroliniana, *S. virginica*, and *S. stellata* are hermaphroditic, herbaceous, perennial wildflowers of eastern North America. Populations of *S. caroliniana* were studied within the Chesapeake and Ohio Canal National Park, near the Billy Goat Trail and Old Tavern, in Montgomery County, Maryland, USA (77°14′30″ W, 38°58′56″ N, elevation = 150 m). *Silene caroliniana* plants produce one to several bolting stems (10–20 cm) containing 5–10 to dozens of flowers presented in a cymose inflorescence, with flowering occurring from early April to early May. Populations of *S. virginica* (80°33′14″ W, 37°21′20″ N, elevation ~ 1100 m) and *S. stellata* (80°32′36″ W, 37°21′02″ N, elevation ~ 1300 m) were studied near the University of Virginia's Mountain Lake Biological Station (MLBS) in the Southern Appalachian Mountains in Giles County, Virginia. *Silene virginica* plants produce one to several bolting stems (20–40 cm) containing usually one to

seven flowers per cymose inflorescence, with flowering occurring from late May through June. *Silene stellata* plants produce one to many reproductive stems that emerge in early spring and reach up to 120 cm in length. There are typically >20 flowers per panicle inflorescence, with flowering occurring from early July through middle August. All three species are protandrous, with 10 anthers and three stigmas per flower, and are highly outcrossing (Dudash and Fenster 2001; R. J. Reynolds, C. B. Fenster, and M. R. Dudash, *unpublished data*). Unless otherwise noted, all studies described herein were performed in natural populations under field conditions. Anther smut disease, caused by the fungus *Microbotryum violaceum* and sometimes found in flowers of *S. caroliniana* and *S. virginica* (Antonovics et al. 2003), was never observed in our study populations.

METHODS

Floral traits.—To characterize traits comprising the attraction component of pollination syndromes of the *Silene* species, flower morphology, scent, and reward traits were measured on female-phase flowers (methodological details are presented in Appendix B). Pollen presentation and stigma receptivity strategies are also syndrome traits that directly affect the dynamics of pollen transfer and may be correlated with other floral traits (Lloyd and Yates 1982, Harder and Thomson 1989, Thomson et al. 2000). For each species, timing of anther dehiscence and stigma receptivity were measured by direct observations of flowers from bud stage to receptivity (Appendix B).

Nocturnal/diurnal pollinator experiment.—A pollinator exclusion experiment was performed to determine whether the three *Silene* species were pollinated nocturnally and/or diurnally by quantifying the contribution of each group of visitors to seed and fruit set. The experiment consisted of 40 plants divided equally among four pollinator visitation treatments (diurnal, nocturnal, both diurnal and nocturnal, and unvisited) with cages used to restrict pollinator access except during the indicated periods. Seed and fruit set were modeled as response variables and treatment was the predictor. Orthogonal contrasts were used to determine significant differences in mean fruit and seed set among the treatments (Appendix B).

Fluorescent dye study.—Fluorescent dyes were used as pollen analogues to investigate the relative differences between nocturnal and diurnal pollinators of *S. stellata* in successfully dispersing pollen grains from source plants. The efficacy of fluorescent dye in simulating pollen movement for *S. virginica* has been previously shown (Fenster et al. 1996; Appendix B).

Visitation data.—To investigate how accurately the *Silene* species pollination syndromes predict their animal visitors and to quantify each visitor's pollinator importance and the confidence intervals surrounding pollinator importance estimates (Reynolds and Fenster 2008), visitation rate was estimated as the number of

TABLE 1. Floral traits (mean, with SE, CV in parentheses) for each of the three *Silene* species: *S. caroliniana*, *S. virginica* and *S. stellata*.

Trait	*S. caroliniana*	*S. virginica*	*S. stellata*
Attraction			
Color	pink, variable	red	white
Petal length (mm)	12.1 (1.6, 13.1)	18.0 (2.3, 13.0)	9.0 (0.9, 9.9)
Petal width (mm)	6.4 (0.9, 14.1)	5.8 (0.8, 13.2)	11.3 (1.5, 13.0)
Scent	absent	absent	present
Reward			
Nectar (μL)	2.0 (0.2)	15.1 (1.1)	1.1 (0.2)
Sucrose (%)	47.8 (1.9)	22.6 (0.5)	29.5 (2.7)
Pollen transfer			
Stigma exsertion (mm)	2.9 (1.0, 35.1)	7.2 (2.0, 27.4)	10.3 (1.3, 12.4)
Corolla tube length (mm)	21.2 (1.6, 7.4)	24.1 (2.1, 8.8)	9.8 (0.9, 9.1)
Corolla tube diameter (mm)	1.9 (0.4, 19.3)	3.6 (0.5, 14.8)	8.0 (1.0, 12.3)
Anthesis	diurnal, sequential	diurnal, 5 stamens/d	nocturnal, simultaneous

Notes: Numbers in parentheses are SE for reward measurements. Floral morphology measurements: $N = 21$, 73, and 54 plants on *S. caroliniana* (one flower per plant), *S. virginica* (multiple flowers per plant), and *S. stellata* (multiple flowers per plant), respectively. Nectar measurements: *S. caroliniana*, $N = 139$ flowers; *S. virginica*, reported in Fenster et al. (2006); *S. stellata*, $N = 109$ flowers. Populations of *S. caroliniana* were studied within the Chesapeake and Ohio Canal National Park, Montgomery County, Maryland, USA. Populations of *S. virginica* and *S. stellata* were studied near the University of Virginia's Mountain Lake Biological Station (MLBS) in the Southern Appalachian Mountains in Giles County, Virginia, USA.

plant visits per hour for all the visitors to the flowers of each *Silene* species (Appendix B).

Pollen removal and deposition.—To quantify the efficiency of a pollinator (pollen removed vs. pollen deposited) and a pollinator's importance, both pollen removal and deposition were quantified for the floral visitors (Appendix B).

Pollinator importance and pollen loss.—Pollinator importance (visitation rate × pollen grain deposition), its confidence intervals (Reynolds and Fenster 2008), and pollen loss (removal–deposition; Inouye et al. 1994) were calculated for each visitor type and year of study for the three *Silene* species to estimate the amount of pollen each visitor deposits on the stigmatic surface in a one-hour interval (Appendix B).

RESULTS

Floral traits.—Table 1 contains the floral trait data pertaining to attraction, reward, and pollen transfer for the three *Silene* species. Timing of anther dehiscence and stigma receptivity vary among the *Silene* species and were consistent with the syndromes suggested above. *Silene caroliniana* anthers dehisce sequentially during one day, *S. virginica* presents two ranks of five anthers on consecutive days, and *S. stellata* presents 10 anthers simultaneously at dusk. Thus, it may be predicted that *S. caroliniana* and *S. virginica* have diurnal pollinators and *S. stellata* has nocturnal pollinators. The multidimensional scaling (MDS) analysis demonstrated considerable divergence among the species in expression of the floral traits. All species were divergent along dimension 1 but *S. stellata* and *S. virginica* were similar along dimension 2 (Appendix C). After Bonferroni correction, all floral traits were correlated significantly with dimension 1 and/or dimension 2 (Appendix D). For example, corolla tube width was negatively associated
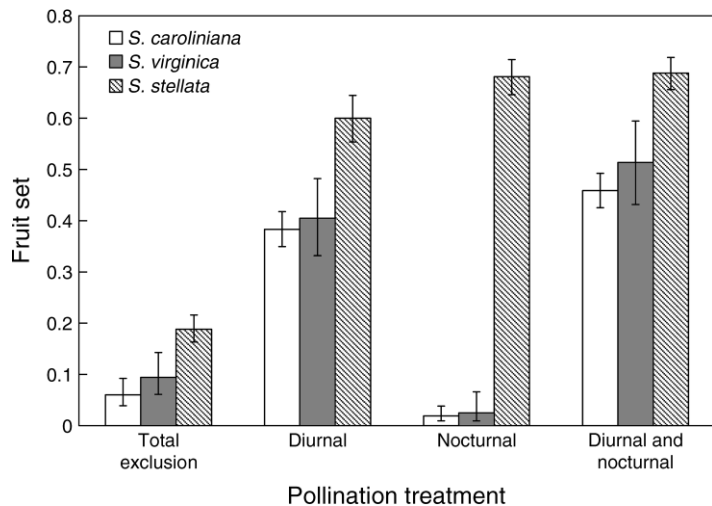
with dimensions 1 and 2 and pink corolla was positively associated with dimension 2.

Nocturnal/diurnal pollination experiment.—The results of the fruit and seed set models were similar, thus we present only the fruit set data (Fig. 1). Mean back-transformed percentage of fruit set in the unmanipulated control treatments was 46% for *S. caroliniana*, 51% for *S. virginica*, and 69% for *S. stellata*. Fruit set in the pollinator exclusion control was comparatively low, averaging 6%, 9%, and 18%, respectively, and contrasts showed the two treatments were significantly different for each species (*S. caroliniana*, $P = 0.0002$; *S. virginica*, $P < 0.0001$; *S. stellata*, $P < 0.0001$). Thus all three species require pollinators for full fruit set. *Silene caroliniana* and *S. virginica* are exclusively diurnally pollinated. Only *S. stellata* has nocturnal pollinators. For *S. stellata* there was no significant difference in mean fruit set between the diurnal and nocturnal pollination treatments ($P = 0.49$). For *S. caroliniana* ($P < 0.0001$) and *S. virginica* ($P < 0.0001$), the only significant component to pollination was from diurnal animals.

Fluorescent dye study.—Dye used as a pollen analogue indicated that the probability (mean ± SE) a *S. stellata* individual received pollen from a single source plant by nocturnal pollinators was 0.12 (0.096, 0.16). This was about 2.5 times greater than diurnal pollinators, with a mean of 0.05 (0.038, 0.059). The difference in mean probabilities of pollen receipt was significant ($\chi^2 = 4.68$, df = 1, $P = 0.03$) between the two groups. On average nocturnal pollinators moved dye particles 2.2 ± 0.43 m or 50% farther than diurnal pollinators (1.2 ± 0.35 m), but the difference was not statistically significant ($\chi^2 = 2.04$, df = 1, $P = 0.15$).

Flower visitation.—The three proportionally most common visitors of *S. caroliniana* across the five years of visitor observations ($n = 1057$ visits observed) were

Fig. 1. Fruit set per plant (mean ± SE) in the diurnal/nocturnal pollinator exclusion experiment demonstrating the extent of nocturnal vs. diurnal animal pollination for Silene caroliniana, S. virginica, and S. stellata. Populations of S. caroliniana were studied within the Chesapeake and Ohio Canal National Park, Montgomery County, Maryland, USA. Populations of S. virginica and S. stellata were studied near the University of Virginia's Mountain Lake Biological Station (MLBS) in the Southern Appalachian Mountains in Giles County, Virginia, USA.

large bees (0.73), such as bumble bees (*Bombus* spp., e.g., *Bombus affinis*) and carpenter bees (*Xylocopa virginica*), diurnal clearwing hawkmoths (*Hemaris* sp., Lepidoptera: Sphingidae) (0.081), and bee flies (Diptera:Bombyliidae) (0.064). Visits were also observed by honeybees (0.053), halictid bees (Hymenoptera: Halictidae), and hoverflies (Diptera: Syrphidae) (0.041), zebra swallowtails (*Eurytides marcellus*, Lepidoptera: Papilionidae) (0.021), and very rarely by cabbage whites (*Pieris rapae*, Lepidoptera: Pieridae) or juniper hairstreaks (*Callophrys gryneus*, Lepidoptera: Lycaenidae). The large bees, diurnal hawkmoths, and bee flies were most consistently observed across years and populations, thus the visitation rate model included data on these species and not the rarer visitors. Visitor type was a significant predictor of visitation rate in S. caroliniana ($F = 22.85$, df = 2, 324, $P < 0.0001$). Averaged across the five years of study on S. caroliniana, large-bee visitation rate was 0.93 ± 0.13 plants/h, diurnal hawkmoth was 0.12 ± 0.044 plants/h, and bee fly was 0.10 ± 0.045 plants/h. Pairwise contrasts indicated large-bee visitation rate was significantly greater than both diurnal hawkmoth ($F = 27.79$, df = 1, 324, $P < 0.0001$) and bee fly ($F = 22.61$, df = 1, 324, $P < 0.0001$). Diurnal hawkmoth and bee fly visitation rates were not significantly different ($F = 0.09$, df = 1, 324, $P = 0.77$). However, the visitor type effect was dependent on the year of sampling for S. caroliniana ($F = 3.95$, df = 8, 324, $P < 0.0002$) as diurnal hawkmoths were rarely observed in 2005 (Fig. 2). Year of sampling was not a significant predictor of overall visitation rate for S. caroliniana ($F = 2.30$, df = 4, 162, $P = 0.06$).

Primarily Ruby-throated Hummingbirds, *Archilochus colubris*, and halictid bees and syrphid flies were observed visiting S. virginica from our sample of visitors ($n = 89$ visits observed) during 2002. Additionally, bumble bees (*Bombus* spp.) (Fenster and Dudash 2001) and very rarely pipevine swallowtails (*Battus philenor*) have been casually observed. Hummingbirds (0.71) were proportionally the most common visitors of S. virginica

compared to the small bees and syrphid flies. Visitor type was a significant predictor of visitation rate in S. virginica ($F = 4.83$, df = 1, 85, $P = 0.03$). Hummingbird visitation rate was 0.18 ± 0.043 plants/h, which was significantly higher than small bees and flies, with a mean of 0.070 ± 0.026 plants/h.

For S. stellata the total number of visits observed across the five years of study for nocturnal and diurnal insects was 438. The nocturnal visitors of S. stellata include the noctuid moths *Hadena ectypa* (a nursery pollinator: see Kephart et al. 2006), *Amphipoeaea americana*, *Feltia herelis*, *Autographa precationis*, and *Cucullia asteroids*, the arctiid *Halysidota tessellaris*, and the notodontid *Lochmaeus manteo*. Holarctic notodontids commonly have reduced mouthparts (Weller 1992). Our voucher specimens of L. manteo have well-developed proboscises, which indicates the species actively feeds and is long-lived (Weller 1992). The diurnal visitors are primarily halictid bees, syrphid flies, and bumble bees. Visitor type (nocturnal or diurnal) was not a significant predictor of visitation rate in the S. stellata model ($F = 4.66$, df = 1, 5, $P = 0.08$), although the nocturnal moth visitation rate of 0.93 ± 0.20 plants/h was higher than diurnal bees and flies with 0.51 ± 0.088 plants/h. Year of sampling was not a significant predictor of visitation rate for S. stellata ($F = 0.67$, df = 2, 108, $P = 0.51$). However, the visitor type × year interaction was a significant predictor of visitation rate ($F = 13.58$, df = 2, 5, $P = 0.01$), indicating diurnal and nocturnal visitation rate varies depending on the year of observation (Fig. 2).

Pollen production and removal.—The average number of pollen grains produced per anther for newly dehiscent flowers of S. caroliniana and S. virginica as well as newly dehiscent flowers at dusk for S. stellata and 12 h following dehiscence are reported in Table 2: *Notes*.

For S. caroliniana the mixed-model ANOVA demonstrated that visitor species and lack of visitation (control; $F = 11.90$, df = 2, 100, $P < 0.0001$), treatment (pollen grains before or after a visit; $F = 42.72$, df = 1,
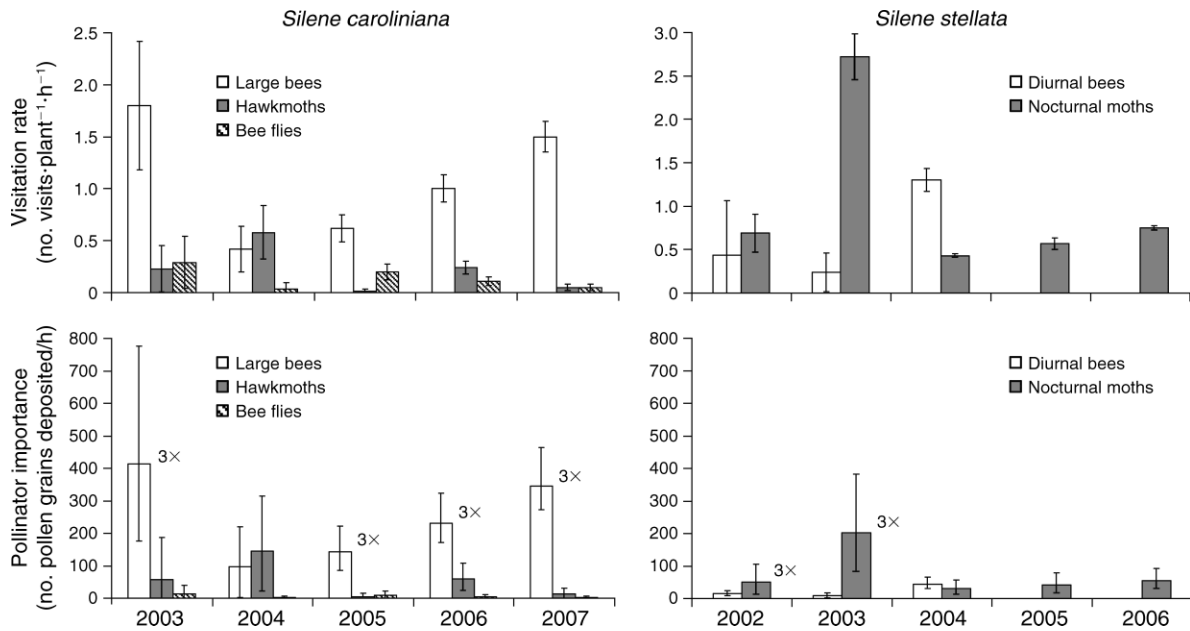
FIG. 2. Visitation rate and pollinator importance of the pollinators of *Silene caroliniana* and *S. stellata* for each of five years (*S. caroliniana*: open bars, large bees; shaded bars, diurnal hawkmoths; cross-hatched bars, bee flies) (*S. stellata*: open bars, diurnal bees; shaded bars, nocturnal moths). Visitation rates are means ± SE, and pollinator importance values are means ± approximate 95% CI. Visitation rates of diurnal pollinators were not quantified in either 2005 or 2006. The abbreviation "3×" indicates mean pollinator importance was three times greater than the next most important pollinator.

100, $P < 0.0001$), and their interaction ($F = 9.54$, df = 2, 100, $P = 0.0002$) were all significant predictors of number of pollen grains per anther. Pairwise contrasts showed that on average large bees removed significantly more pollen per anther per visit than diurnal hawkmoths ($F = 6.15$, df = 1, 100, $P = 0.01$) and more than controls or pollen that sheds freely in the absence of visitation ($F = 17.25$, df = 1, 100, $P < 0.0001$; Table 2). No significant difference was found between pollen shed in the absence of a visit and pollen removed by diurnal hawkmoths ($F = 0.12$, df = 1, 100, $P = 0.73$; Table 2).

For *S. virginica* both treatment ($F = 22.27$, df = 1, 74, $P < 0.0001$) and the treatment × visitor interaction ($F = 5.65$, df = 1, 74, $P = 0.02$) were significant predictors of

the number of pollen grains per anther per visit. The significant interaction effect demonstrated that hummingbirds removed significantly more pollen per visit than control or pollen that sheds freely in the absence of visitation (Table 2).

The average number of pollen grains per anther for *S. stellata* flowers shortly following dehiscence at dusk was significantly greater than for flowers the following morning (caged and not visited by pollinators; $Z = 2.37$, $P = 0.0089$; Table 2). Treatment ($F = 17.44$, df = 1, 81, $P < 0.0001$) and visitor type ($F = 13.01$, df = 1, 85, $P < 0.0001$) and their interaction ($F = 6.05$, df = 1, 81, $P = 0.0009$) were all significant predictors of pollen grains per anther per visit. Pairwise contrasts demonstrated

TABLE 2. Pollen removal, pollen deposition, and pollen loss of visitors to *Silene caroliniana*, *S. virginica*, and *S. stellata*.

	Silene caroliniana				*Silene virginica*	
Pollen fate	Large bees	Hawkmoths	Bee flies	No visits (control)	Hummingbirds	No visits (control)
Removal	2000 (200)	800 (420)	ND	640 (240)	3300 (500)	1100 (800)
Deposition	230 (209, 253)	249 (206, 297)	43 (25, 66)	18 (15, 22)	302 (267, 338)	54 (42, 67)
Loss	1770 (204)	551 (421)	ND		3000 (501)	

Notes: Values are means with SE in parentheses, some of which are asymmetrical (in which case two values are reported for SE). Pollen removal is the number of pollen grains removed per anther per visit. Pollen deposition is the number of pollen grains deposited per visit. Pollen loss is the difference between pollen removed and pollen deposited. Pollen production, the amount of pollen per anther, was, for *S. caroliniana*, 2870 ± 115; for *S. virginica*, 4820 ± 409; and for *S. stellata*, 1340 ± 169 (nocturnal) and 756 ± 184 (next day). The numbers of ovules per plant were, for *S. caroliniana*, 39 ± 0.8; for *S. virginica*, 46 ± 2.8; and for *S. stellata*, 25 ± 0.4. The numbers of pollen grains on old females (flowers in female phase collected from plants in their natural population) were, for *S. caroliniana*, 168 (143, 195), and for *S. stellata*, 86 (14); no data were available for *S. virginica*. The abbreviation "ND" means that no data were available.

that nocturnal moths on average remove fewer pollen grains per anther per visit than diurnal bees ($F = 8.81$, df $= 1, 81$, $P = 0.0039$; Table 2), which was significant at the sequential Bonferroni-corrected alpha level of 0.0125. A second contrast, after correcting for the control or pollen that sheds freely in the absence of visitation, demonstrated the effect remained significant ($F = 5.45$, df $= 1, 81$, $P = 0.02$) at the sequential Bonferroni-corrected alpha level of 0.025. A third contrast demonstrated that on average diurnal bees remove more pollen than control, although the contrast was marginally significant at the sequential Bonferroni-adjusted alpha level of 0.017 ($F = 5.83$, df $= 1, 81$, $P = 0.018$; Table 2). The mean amount of pollen removed by nocturnal moths was greater than the control but the difference was not significant ($F = 0.44$, df $= 1, 81$, $P = 0.51$; Table 2).

Pollen deposition.—The ANOVA of the *S. caroliniana* pollinator effectiveness data set showed that species of visitor and the completely caged and unmanipulated controls were significant predictors of pollen grain deposition ($F = 34.5$, df $= 1, 163$, $P < 0.0001$). Large bees and diurnal hawkmoths, but not bee flies, are effective pollinators of *S. caroliniana*. The mean deposition of all visitors, correcting for the amount of pollen deposited on unvisited (completely caged) controls, was significantly greater than the unmanipulated controls ($F = 29.29$, df $= 1, 166$, $P < 0.0001$; Table 2). Diurnal hawkmoths and large bees without the contribution from bee flies deposited significantly more pollen per visit than accumulated on the unmanipulated controls ($F = 53.49$, df $= 1, 166$, $P < 0.0001$), which suggests that diurnal hawkmoths and large bees are effective pollinators and the contribution from bee flies is negligible. Bee fly deposition rate was not significantly greater than mean deposition in the absence of pollinators ($F = 1.37$, df $= 1, 166$, $P = 0.24$; Table 2). Diurnal hawkmoth and large-bee pollen deposition were not significantly different ($F = 0.25$, df $= 1, 166$, $P = 0.62$; Table 2).

An ANOVA demonstrated that hummingbird pollen grain deposition on *S. virginica* stigmas was significantly higher than the mean of stigmas not visited by any pollinators ($F = 38.03$, df $= 1, 95$, $P < 0.0001$; Table 2).

Nocturnal moths were more effective pollinators of *S. stellata* than diurnal bees. The pollen grain deposition model demonstrated that type of pollinator, nocturnal or diurnal, and the unmanipulated and unvisited (completely caged) controls were significant sources of variation ($F = 11.93$, df $= 4, 367$, $P < 0.0001$). Orthogonal contrasts demonstrated mean pollen grain deposition (Table 2) was significantly higher for nocturnal moth than diurnal bee pollinators ($F = 15.77$, df $= 1, 367$, $P < 0.0001$). A second orthogonal contrast indicated that the nocturnal moths still had significantly higher deposition rates than diurnal bees ($F = 3.97$, df $= 1, 367$, $P = 0.047$) after the means were corrected by the average pollen deposited on unvisited (completely caged) control stigmas. A third orthogonal contrast showed there was no significant difference ($F = 0.35$, df $= 1, 367$, $P = 0.56$) between the sum of nocturnal moth and diurnal bee deposition and the amount of pollen accumulating on unmanipulated stigmas. Because moths deposit significantly more pollen per visit than bees (contrasts 1 and 2), but there is no significant difference between combined deposition by moths and bees and the unmanipulated controls (contrast 3), moths are responsible for the majority of pollen grain deposition for *S. stellata*.

Pollinator importance and pollen loss.—Of the three most common visitors of *S. caroliniana*, large bees were the most important pollinators, with significantly higher estimates of pollinator importance than diurnal hawkmoths in all years except 2004 (Fig. 2). In those years large bees were at least three times more important than diurnal hawkmoths and beeflies combined. Diurnal hawkmoths and large bees were always significantly more important than bee flies except for 2005, when diurnal hawkmoths were rarely observed. Pollen loss by large bees was significantly greater than diurnal hawkmoths because the approximate 95% CI containing the difference of population means did not contain zero (Table 2).

We were unable to accurately estimate small-bee and small-fly pollen removal and deposition for *S. virginica* (see Appendix B: Pollen removal and deposition). Thus, pollinator importance and pollen loss were not compared between hummingbirds and small bees and flies. However, hummingbird pollen loss was estimated and is reported in Table 2.

Nocturnal moths were more important pollinators of *S. stellata* than diurnal bees in two of three years, with significantly higher estimates of pollinator importance. However, in 2004 the importance values were not significantly different due to the extremely high visitation rates of the diurnal pollinators (Fig. 2). Pollinator importance of nocturnal moths was three times greater than diurnal bees in both 2002 and 2003, but not in 2004. Pollen loss by diurnal bees was significantly greater than that by nocturnal moths for *S. stellata* (Table 2).

TABLE 2. Extended.

	Silene stellata		
Bees	No day visits (control)	Moths	No night visits (control)
780 (670, 910)	120 (−46, 210)	490 (400, 580)	240 (180, 300)
34 (5)	34 (4)	74 (7)	28 (9)
746 (130)		416 (91)	

DISCUSSION

We found the pollination syndrome concept to be an effective rubric for predicting the major pollinators in the eastern North American *Silene* clade consisting of *S. caroliniana*, *S. virginica*, and *S. stellata*. Relative to the other sister species, the traits expressed by each *Silene* species appear to operate functionally to increase the attractiveness and the efficiency of pollination by the major pollinators as demonstrated by our comprehensive pollination studies. *Silene caroliniana* is the least specialized, with large bees and the less important diurnal hawkmoths as major pollinators, though one might consider *S. caroliniana* specialized on long-tongued diurnal pollinators. *Silene virginica* and *S. stellata* are specialized to pollination by hummingbirds and nocturnal moths, respectively (see Plate 1).

Based on visitation rates and overall floral appearance and by our criterion for defining specialization using pollinator importance, *S. caroliniana* appears to be specialized for large-bee pollination but the pollen removal and deposition data suggest that diurnal hawkmoths are also important pollinators. Large-bee pollinator importance was significantly greater than diurnal hawkmoth importance in four of five years such that the probability of a pollen grain arriving at a stigma ranged between 4 and 40 times higher for large bees than diurnal hawkmoths. Large bees were consistently the most important pollinators, but the average amount of pollen lost by large bees was threefold higher than diurnal hawkmoths (Table 2). Therefore, from a male reproductive success point of view, diurnal hawkmoths would be the more favorable pollinator, especially in years with equal visitation rates. If selection on floral traits is mainly associated with variation in male reproductive success, then diurnal hawkmoths may be a very important selective agent on *S. caroliniana* floral traits. Additionally, *S. caroliniana* floral design may reflect potentially different selection pressures exerted by bees and diurnal hawkmoths through female and male reproductive success, respectively.

Silene virginica is specialized for hummingbird pollination. Hummingbirds visited at higher rates than the invertebrate visitors. Because the invertebrate visitors were infrequent, we could not obtain a suitable sample for effectiveness or removal and direct comparison of pollinator importance and pollen loss between visitors cannot be made. Nevertheless the results are consistent with previous studies of *S. virginica* pollination. Fenster and Dudash (2001) demonstrated that without hummingbird pollinator visitation, fruit and seed set declined by 50%, across several years. Furthermore, hummingbird pollination is sufficient to ensure full fruit set and in most years full seed set, relative to pollen augmentation by hand-pollinations (Dudash and Fenster 1997). Invertebrate visitors rarely contacted the *S. virginica* stigmas and most likely acted as pollen thieves.

Our work with the pollinators of *S. stellata* demonstrates the value of examining comprehensive aspects of pollination. For example, simply relying on the exclusion experiment and failing to measure the schedule of anther presentation or visitation of nocturnal pollinators would have led to the erroneous conclusion that the species is generalized to both diurnal and nocturnal insect pollinators. However, under our criterion of specialization based on the pollinator importance data, *S. stellata* was specialized for nocturnal moth pollination in two of three years. Furthermore the pollen removal and deposition data also indicate specialization by nocturnal moths. The nocturnal/diurnal exclusion experiment demonstrated that both visitor types can potentially perform equal pollinator service in terms of fruit set, which indicates that flowers unvisited by moths at night may be secondarily pollinated by diurnal bees. However, the temporal order of pollination, nocturnal first then diurnal, was unaccounted for in the exclusion experiment, and thus fruit set in the diurnal treatment was overestimated. Because the anthers simultaneously dehisce pollen at dusk, the pool of pollen available to moths is substantially larger than to diurnal bees the following dawn. Flowers caged through the night had lost 50% of the pollen grains present on newly dehiscent anthers by early the next morning due to abiotic causes (Table 2). Additionally uncaged flowers randomly selected at dawn the following day had lost 75% of the pollen grains due to abiotic factors plus nocturnal moth pollination (results not shown). Therefore, fruit set by diurnal insects may be overestimated because pollen grains on stigmas from nocturnal moths may first fertilize ovules, thereby preempting fertilization from diurnal pollinators. In addition, pollen dispersal by diurnal pollinators as inferred through the fluorescent dye dispersal study is overestimated, because equal amounts of dye were available to nocturnal and diurnal pollinators. The order of pollination, first by nocturnal moths, then by diurnal bees, tips the scale even more toward specialization on nocturnal moth pollination.

Pollen presentation and packaging are pollination syndrome traits as they directly affect the dynamics of pollen transfer by the important pollinators (Thomson et al. 2000). Pollen presentation theory (PPT) predicts high pollinator visitation rate and low pollen transfer efficiency to be associated with sequential anther dehiscence, a pollen packaging strategy that reduces the cost to male reproductive success of having frequent but wasteful pollinators (Thomson 2003). Conforming with PPT, *S. caroliniana* anthers present sequentially, and the most important pollinator, large bees, are by far most frequent and lose more pollen than the next most common pollinator, diurnal hawkmoths. *Silene virginica* also presents pollen sequentially, with five anthers presented simultaneously at flower opening and then another five anthers the next day. This pollen packaging strategy could limit pollen loss associated with pollination by the infrequent (approximately two visits per day) hummingbirds if a flower in male phase goes unvisited by any pollinator. Assuming flowers are visited each day

at least once, sequential anther dehiscence may also serve to limit the cost of pollen loss by hummingbirds. *Silene stellata*, on the other hand, presents 10 anthers at once, and frequent nocturnal moths are less wasteful, more effective, and more important than the diurnal pollinators. Therefore, the divergent packaging strategies of the three *Silene* species are consistent with response to selection by the major pollinators in maximizing pollen grain movement to the proper stigmatic surfaces.

The close systematic relationship of these three *Silene* species makes the interpretation of the relationship between pollinator specialization and syndromes clearer. The different expression of pollination syndromes congruent with different important pollinators implies that pollinators are the likely past and/or contemporary sources of natural selection that have resulted in diversification of these *Silene* spp. While the approaches presented here are a powerful test of the relationship between pollinator syndrome traits and principal pollinators and of the predictive power of syndromes, we cannot demonstrate that the pollinators select for the syndrome traits. For this line of direct evidence phenotypic selection or experimental selection studies need to be performed. For example we know that large bees are the most important pollinators of *S. caroliniana*, and we indicate that sequential anther dehiscence appears associated with limiting the cost of pollen loss for these pollinators. That this pollen presentation strategy is adaptive for bee pollination could be tested experimentally as it has been in other systems (Castellanos et al. 2006). The less frequent but highly effective and efficient (in terms of pollen removed vs. amount of pollen deposited) diurnal hawkmoths may be the primary sources of selection on other syndrome traits. From an optimality standpoint (Aigner 2001) the *S. caroliniana* floral phenotype may represent adaptation to diurnal hawkmoth pollination with little or no trade-off in utilizing large bees. Finding floral specialization on one of a subset of many effective pollinators (i.e., an ecological generalist) is not unprecedented. Schemske and Horvitz (1984) demonstrated *Calathea ovandensis* specialization on bees while most visitation was by ineffective Lepidopteran visitors. Further investigation to determine whether large bees and diurnal hawkmoths exert differential or similar selection pressures on *S. caroliniana* floral traits will help determine whether the syndrome corresponds to a long-tongued pollinator functional group or rather is more adequately described as generalized.

If pollination generalization means that more than one species of visitor is an effective pollinator, then our results indicate that the *Silene* species are generalists and floral evolution in this *Silene* clade has favored generalist pollination systems. However, this proposition is at odds with our conclusions regarding the function of the floral traits that together constitute the different pollination syndromes, i.e., the pollination syndromes are predictive

PLATE 1. (Top to bottom) *Bombus* sp. visiting *Silene caroliniana*, Ruby-throated Hummingbird visiting *Silene virginica*, and *Hadena ectypa* visiting *Silene stellata*. Photo credits (top to bottom): S. Konkel, R. J. Reynolds, C. B. Fenster.

of the principal pollinators as defined by the detailed study of the pollination systems. It is unlikely that asynchronous visitor activity and plant flowering, sampling artifacts, and plant isolation (which may occur if plant populations were located on the edge of their species range) have caused the appearance of specialized pollination systems. For one, large bees visit *S.*

caroliniana and *S. stellata*, and they frequent a spiderwort (*Tradescantia ohiensis*), which coflowers with *S. virginica*. However, in the present studies large bees were never observed visiting *S. virginica*. It is unlikely that finding few pollinators and low visitation rates was due to low sampling effort. *Silene virginica*, with the lowest visitation rates, was observed in one year (344 hours on 86 plants) longer than each of the other two species combined across all five years (*S. caroliniana*, 81.3 hours on 167 patches; *S. stellata*, 281 hours on 174 plants). All study populations were located near the center of the species ranges so it is unlikely that we were observing plant populations associated with depauperate pollinator fauna.

Surely selection by agents other than pollinators may be factors that reinforce or disrupt a specialized or generalized syndrome. For example, alternative selection pressures exerted by floral herbivores and physiological trade-offs may also contribute to floral evolution (reviewed in Galen 1999, Strauss and Whittall 2006). Seed predation by *Hadena* moth larvae (Kephart et al. 2006; R. Reynolds, C. Fenster, and M. Dudash, *unpublished manuscript*) and infection by anther smut fungus (e.g., Giles et al. 2006) are specific candidate sources of selection on floral traits of *Silene*. The pattern of ecological generalization indicated by the various insect visitors in addition to any non-pollinator source of selection on the three *Silene* spp. would appear to obscure the pattern of specialization attributable to the major pollinators. Nonetheless, here we document a clear evolutionary signal of pollinator specialization manifested as floral traits comprising the alternative pollination syndromes associated with the predicted important pollinators.

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APPENDIX A

A series of flower photographs of the study *Silene* spp. with anterior and lateral views (*Ecological Archives* E090-144-A1).

APPENDIX B

Detailed methods for all studies (*Ecological Archives* E090-144-A2).

APPENDIX C

The multidimensional scaling analysis of all measured floral traits for individual *Silene* plants (*Ecological Archives* E090-144-A3).

APPENDIX D

Correlation table of floral traits with the transformed multidimensional scaling scores for individual *Silene* plants (*Ecological Archives* E090-144-A4).