ABSTRACT

Title of Document: POLLINATOR SPECIALIZATION AND THE EVOLUTION OF POLLINATION SYNDROMES IN THE RELATED *SILENE*, *S. CAROLINIANA*, *S. VIRGINICA*, AND *S. STELLATA*

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Pollination syndromes are the convergent expression of floral traits in unrelated species reflecting specialized interactions between plants and pollinators exerting similar selection pressures. I addressed the controversial claim that pollinator-mediated selection is unlikely to be a major factor underlying floral evolution because plants often have many functionally different floral visitors. Detailed pollination data and pollinator-mediated selection studies are needed to address the notion that specialized plant-pollinator interactions are a major mechanism of floral evolution. I developed statistical methods to measure the importance of pollinators (Chapter 1). I addressed whether floral morphological differences of the related *Silene* species, *S. caroliniana*, *S. virginica*, and *S. stellata*, correspond to predicted specialized pollination systems (Chapter 2). I asked whether contemporary selection pressures on floral traits were detectable in a population of *S. virginica* (Chapter 3). I investigated

the non-obligate interaction of *S. stellata* and the moth *Hadena ectypa*, that pollinates it and uses its immature seed for the development of larval offspring (Chapter 4).

Using my novel methodology (Chapter 1), I demonstrated that *S. virginica* and *S. stellata* were specialized on hummingbirds and nocturnal moths, respectively (Chapter 2). *S. caroliniana* was least specialized with long-tongued diurnal hawkmoth (*Hemaris sp*) and large bee pollinators (*Bombus spp.* and *Xylocopa virginiana*). These results matched predictions based on interspecific differences in *Silene* floral trait expression and were consistent with the notion that the important pollinators are the major selective agents on floral design. Positive directional but mainly nonlinear hummingbird-mediated phenotypic selection (Chapter 3) on *S. virginica* floral traits was detected through lifetime fitness components, supporting predictions from the syndrome concept. Flowering date predicted the relative density of *H. ectypa* and other moth pollinators of *S. stellata*, and *H. ectypa* density varied by population and year, which may determine the sign of the *H. ectypa-S. stellata* interaction. Both curvature and directional selection in *S. stellata*'s floral trait selection surface were context dependent on the intensity of *H. ectypa* larval fruit predation. Overall pollinators are important sources of selection underlying floral evolution in these *Silene*, and *S. stellata* floral evolution is subject to additional selection pressures from *H. ectypa* larvae.

POLLINATOR SPECIALIZATION AND THE EVOLUTION OF POLLINATION SYNDROMES IN THE RELATED *SILENE*, *S. CAROLINIANA*, *S. VIRGINICA*, AND *S. STELLATA*

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Table of Contents

Chapter 1: Point and interval estimation of pollinator importance: A study using pollination data of *Silene caroliniana*.

Abstract Pollinator importance, the product of visitation rate and pollinator effectiveness, is a descriptive parameter of the ecology and evolution of plant-pollinator interactions. Naturally, sources of its variation should be investigated, but the standard error of pollinator importance has never been properly reported. Here, a Monte Carlo simulation study and a result from mathematical statistics on the variance of the product of two random variables are used to estimate the mean and confidence limits of pollinator importance for three visitors of the wildflower, *Silene caroliniana*. Both methods provided similar estimates of mean pollinator importance and its interval if the sample size of the visitation and effectiveness datasets were comparatively large. These approaches allowed us to determine that bumblebee importance was significantly greater than clearwing hawkmoth which was significantly greater than bee fly. The methods could be used to statistically quantify temporal and spatial variation in pollinator importance of particular visitor species. The approaches may be extended for estimating the variance of more than two random variables. However, unless the distribution function of the resulting statistic is known, the simulation approach is preferable for calculating the parameter's confidence limits.

Keywords Pollinator effectiveness· Pollinator visitation · Variance of product · Floral specialization · Floral generalization

Introduction

Beginning with Stebbins' (1970) assertion that floral traits evolve in response to the most effective *and* abundant pollinators, pollination ecologists have had an interest in quantifying relative pollinator importance, or the product of visitation frequency and pollinator effectiveness, and comparing it across visitor classes. The visitation component is most often measured as a proportion or percent of total visits (e.g. Larsson 2005; Wiggam and Ferguson 2005; Sahli and Conner 2007) but is also measured as a rate (Bloch et al. 2006; Reynolds, Fenster and Dudash unpublished), i.e., number of visits per flower, plant or inflorescence per unit time. Pollinator effectiveness (Inouye et al. 1994) may be measured as per visit pollen grain deposition (e.g., Primack and Silander 1975; Fenster 1991; Reynolds, Fenster and Dudash unpublished) or fruit or seed set (e.g., Schemske and Horvitz 1984; Kandori 2002; Wiggam and Ferguson 2005) or even progeny germination rates (Herrera 2000). As a product of visitation frequency and per visit pollen grain deposition pollinator importance is a measure of a pollinator's total transfer of pollen to the stigmatic surface per unit time. Thus, pollinator importance can suggest the relative strength of the positive effects a pollinator can have on the plant partner (Thomson 2003), and as a measure of the fitness consequences of pollinator service it could indicate which pollinators are likely sources of natural selection on plant traits. For a given plant species relative pollinator importance is useful for interpreting pollination syndromes (Faegri and van der Pijl 1979) and may help resolve the extent of ecological specialization (Fenster et al. 2004) of a plant on a subset of a diverse pollinator

assemblage (Robertson 1928; Waser et al. 1996; Ollerton 1996; Olesen and Jordano 2002).

Waser et. al. (1996) inaugurated a continuing (Johnson and Steiner 2000; Fenster et al. 2004; Waser and Ollerton 2006) controversy among pollination ecologists by criticizing the pollination syndrome as the dominant theme explaining the relationship between flower forms and their visitors and determined the syndrome concept had poor predictive power. Since pollinator importance is one way to assess visitors as pollen grain vectors, it needs to be estimated efficiently and accurately to determine which of the amalgam of visitors are pollinators (Ollerton 1996). However, nearly every study conducted to date fails to present error estimates of pollinator importance. Therefore, we perceive a need to explore the inherent statistical and practical issues many researchers face when measuring the importance of a pollinator.

There are at least three statistical approaches to estimating the mean and variance of a product of random variables, some of which have been successfully applied to studies of demography (e.g., Brown et al. 1993) and mark-recapture population estimation (e.g., Hestbeck et al. 1991). First, the delta method may be used to approximate the variance of the product using the Taylor series expansion (Lynch and Walsh 1998). A simpler method of computing the variance of a product was developed by Goodman (1960) where he presents the exact formula for the variance of the product of two and three independent random variables. Furthermore, he comments on the efficiency of the product of sample means estimator under two different sampling schemes: 1) when observations are made separately (e.g., visitation and effectiveness) and 2) when the sample observations may be paired producing one dataset of products

(e.g., pollinator importance). He proves that the mean of the product is more efficiently estimated (smaller variance of the mean) when the individual sample means are used to estimate the mean of the product (approach 1) rather than if the product is measured directly and the mean of the product estimated from the observations (approach 2). A third method of estimating pollinator importance is to construct its confidence interval by using computer intensive simulations from raw datasets of pollinator visitation rate and effectiveness. The main advantage of this approach is in avoiding the distributional assumptions involved with calculating confidence intervals for population parameters using estimates from the delta method or Goodman exact variance formula. For example, the simulation is preferable when the probability distribution of the estimate of mean importance is unknown and/or when the number of variables is greater than two (see methods below)

The primary objective of this paper is to obtain point and interval estimates of pollinator importance using its components, visitation rate and effectiveness. Because Goodman (1960) showed that approach one produces an estimator with smaller variance, we use approach one to develop a computer intensive simulation method that is novel to studies of pollinator importance: bootstrap the individual visitation and effectiveness datasets, take the bootstrap means and then multiply them to get the resulting product, repeating as many times as possible. In this case, the upper and lower 95^{th} bootstrap confidence intervals are taken from the sampling distribution of mean importance values to estimate the variation in pollinator importance. We also hand calculate the mean, variance and confidence limits of pollinator importance using Goodman's (1960) mathematical statistics result regarding the formula for the exact variance of the product

of two random variables and compare these estimates with estimates from the simulations. We demonstrate the use of these methods with field-collected data of pollinator visitation rate and pollen grain deposition on stigmas for *Silene caroliniana* (Caryophyllaceae).

Materials and methods

Silene caroliniana is a protandrous herbaceous perennial wildflower of the eastern United States. At our study site near the C&O Canal National Park's Billy Goat Trail, Montgomery County, MD, it blooms from mid April to early May. Its corolla is tubular and variable in color, ranging from white to dark pink, but is most commonly light pink. The most common visitors are large bees, *Bombus* spp. (e.g. *B. affinis*) and carpenter bees (*Xylocopa virginiana*), clearwing hawkmoths (*Hemaris* sp.), and bee flies (Bombyliidae), with additional infrequent to rare visits by small bees such as halictids, and lepidopterans such as cabbage whites (*Pieris rapae*) and zebra swallowtails (*Eurytides marcellus*). Hereafter we sometimes refer to large bees, hawkmoths and bee flies as BB, HM, and BF, respectively.

Data collection

To quantify the visitation component of pollinator importance, we estimated the parameter mean visitation rate (# visits per plant per hour), for each of the three common invertebrate visitors during the 2006 field season, using direct observations of 46 separate

patches (each patch = one experimental unit) of *S. caroliniana* individuals in a natural population. Visitation rate is defined here as the sum of visits to all the plants in a patch divided by the number of plants in the patch, and then divided by the time of observation per plant, thus number of visits per plant per hour. Observations were made of five to ten plants per patch for 20 to 30 min, which was appropriate given the relatively frequent visits and easy view of a large number of plants. During each observation the count of visits to each plant and the visitor species was recorded. Every effort was made to keep the experimental units independent, by sampling across the entire flowering period and observing many separate patches in a given day.

The pollinator effectiveness component of pollinator importance was estimated during the 2006 field season by measuring single visit pollen grain deposition for each of the three most common visitors. About 20 plants of the same population used for the visitation study were located and securely caged with fine mesh screening prior to flowering. After the pollinator exclusion cages were removed, female phased flowers were identified and flowers were observed until a visit was noted. Immediately following the visit the flower was collected and its stigmas were fixed on microscope slides with fuschin glycerin jelly (Kearns and Inouye 1993). The number of pollen grains on the stigmas was counted under light microscopy at 40x power. Unvisited stigmas were collected as controls, i.e., pollen grain deposition from sources other than insects.

Data analyses (linear models)

In addition to the major focus described below of quantifying variation in pollinator importance, we also aimed to gain a greater mechanistic understanding of why different pollinators may differ in the components of pollinator importance. Thus, linear models (SAS Institute, 2004) were used to determine if mean pollen grain deposition (pollinator effectiveness) and visitation rate (pollinator visitation) each vary according to visitor species. Pollen grain deposition (PROC GLM) or visitation rate (PROC GENMOD) were modeled as response variables and visitor species as the predictor variable (SAS Institute, 2004). In the case of pollen grain deposition, an additional treatment level, no visitor (control), was used in the model. The pollen grain deposition model was run with square root transformed data, which made the distribution of the response variable more symmetric.

A Poisson regression model was used to model the count variable, number of visits to a patch of plants in a half hour, which ranged between 0 and 17 with a mode of 0. In this model the number of visits was the response variable, species was the predictor, the link function was log and an overdispersion parameter was used and estimated (3.7) as the Pearson chi square divided by its degrees of freedom (135). The model was modified by specifying an offset variable, ln(number of plants*time(h) of observation). The offset variable scales the count-type response data by the time of observation and the number of plants in each patch since actually mean *visitation rate* was the parameter of interest. Because visits of the three species were observed within each experimental unit, the log-linear model was further refined to account for their potential correlation (repeated statement/ corr option unstructured). In using a model without this correlation or without the correction for overdispersion we would have

reported all visitor species were significantly different in visitation rate (BB > HM > BF; analysis not shown). Least squares means were used to estimate the mean values of the predictor variables in both the GLM and GENMOD procedures. In both procedures *a priori* contrasts were used to determine if mean visitation rate differed between species or, for the case of pollen grain deposition, whether each species differed from the control (no visitor). For both models the per-contrast type 1 error rate was controlled by holding the experiment-wise alpha level to 0.05.

Data analyses (simulations and variance calculations)

A Visual Basic routine in Microsoft Excel was developed and used to simulate mean importance values and 95% bootstrap confidence limits (See Appendix A for example code). Simulations were done separately for each visitor species. To correct for pollen on stigmas from sources other than pollinators, the pollen deposition dataset was modified by subtracting the mean number of pollen grains on control stigmas (N = 46) from each observation. If the resulting observation was negative it was replaced with zero. The visitation dataset was left unmodified. For each species it consisted of 46 observations of visitation rate, one from each patch of plants.

To begin, the visitation and deposition datasets were randomly sampled to generate bootstrap samples of visitation and effectiveness each with the same number of observations as the raw datasets. Next, the sample means and variances were calculated, pollinator importance was taken as the product of the means and its variance using the formula described below. A single trial consisted of repeating the above procedure

10,000 times thus generating a distribution of 10,000 mean importance values. After a trial was complete the average of the 10,000 mean importance values and their variances were taken, the dataset was sorted in ascending order, and the 250th and 9,750th simulated observations of mean importance were taken as the estimates of the upper and lower 95% bootstrap confidence limits. In order to investigate the stability of the estimates the whole process was repeated 50 times, and the coefficients of variation (CV) of the mean and upper and lower confidence limits across the 50 trials were calculated. The final mean and upper and lower 95% bootstrap confidence limits were taken as the averages across the 50 trials.

We used the result of Goodman (1960) to make hand calculations of the mean and unbiased pollinator importance variance estimates. In general, using probability theory and the algebra of random variables the mean and variance of the product of two independent (i.e., $COV_{X,Y} = 0$) random variables, $Z = XY$, are $E(Z) = \mu_x \mu_y$ and $Var(Z) = (\mu_X)^2 \sigma_Y^2 + (\mu_Y)^2 \sigma_X^2 + \sigma_X^2 \sigma_Y^2$ where $E(X) = \mu_x, E(Y) = \mu_y, Var(X) = \sigma_x^2, Var(Y) = \sigma_y^2$ (Goodman 1960). Taking the random samples $\{X_1, X_2, ..., X_{n_x}\}$ and $\{Y_1, Y_{2,} ....., Y_{n_y}\}$, an unbiased estimate of the variance of the product of means, $\mu_x \mu_y$, is $\hat{Var}(\overline{xy}) = \bar{x}^2 s_y^2 / n_y + \bar{y}^2 s_x^2 / n_x - s_x^2 s_y^2 / n_x n_y$ where $\bar{x}, \bar{y}, s_x^2, s_y^2, n_x, n_y$ are the respective means, unbiased variances and sample sizes of the two datasets (Goodman 1960). Note that this method does not require any model regarding the probability distribution of the sample observations or sample means. The assumptions are independent observations and no covariance between the random variables, which may be difficult to satisfy under field conditions.

In order to put a probability on the approximate interval containing the population mean importance using the exact variance formula we need to know the distribution of its statistic. If large random samples (e.g., >30) are taken of each variable then the means of the variables may be assumed approximately normal, regardless of the variables' underlying distribution. However, even for large samples of visitation and effectiveness where the means may be assumed normal, a confidence interval for the population mean importance value may not be the sample mean +/- 1.96 times the standard error. Craig (1936) published the distribution function of a product of normal random variates, and under most circumstances it is not normal. Fortunately computational methods for computing the probabilities (Cornwell et al 1978) and statistical tables (Meeker et al. 1981) have been produced. The product of normals distribution, $g_{Z=XY}(z \mid \frac{\mu_x}{\sigma_x}, \frac{\mu_Y}{\sigma_Y}, \rho_{XY})$, has three parameters, the correlation, ρ_{XY} , and the ratios of the means to standard deviations of each variable, $\frac{\mu_X}{\sigma_X}$ and $\frac{\mu_Y}{\sigma_Y}$ (Craig 1936; Meeker et al. 1981). The tables of Meeker et al. (1981) were used to directly calculate an approximate 95% confidence interval for the population mean importance using as parameters the estimates of ratios of the sample means to standard errors to find the appropriate critical values. Bivariate linear interpolation (see Meeker et al. 1981) was used to find critical values corresponding to the appropriate parameter estimates. The approximate 95% confidence interval is $P(\Pi_{\alpha=0.025} < \frac{\overline{xy} - \mu_x \mu_y}{\sqrt{\hat{Var}(\overline{xy})}} < \Pi_{\alpha=0.975}) \approx 0.95$, or $(\overline{xy} \pm \Pi_\alpha \times \sqrt{\hat{Var}(\overline{xy})})$ where $\Pi_{\alpha=0.025,0.975}$ are the critical values corresponding to the 0.025 and 0.975 percentiles of the product of two normals distribution, and $\sqrt{\hat{Var}(\overline{xy})}$ is

the estimate of the standard error of pollinator importance from the exact variance formula (Goodman 1960).

Comparisons were made of the simulated importance values, variances and 95% bootstrap confidence limit estimates to the mean, variance, and confidence limits of importance values calculated directly using estimates from Goodman's (1960) exact variance formula. If the point estimates differ substantially then the approximate 95% confidence limits using the mean and standard error estimates from the exact variance formula may be inaccurate. Such a discrepancy may be due, for example, to violation of the methods' assumptions. For each visitor species, the relative difference of the point or interval estimates from the simulated ones was calculated as

$$\%difference = \left(\frac{Est_{Sim} - Est_{Direct}}{Est_{Sim}}\right) \times 100.$$

Results

Visitation and pollen grain deposition

Overall, the linear models show large bees to be the most frequent and hawkmoths and large bees the most effective pollinator of *S. caroliniana*. The mean (+/- 1 SE) visitation rate for large bees, bee flies and hawkmoths based on the N = 46 observation periods was 1.1 (0.92, 1.2), 0.11 (0.086, 0.15), and 0.25 (0.18, 0.33), respectively. Thus, large bee least squares mean visitation rate was 4.4 times greater than hawkmoth and 10 times greater than bee fly and these differences were statistically significant (BB > HM, χ^2 = 16.54, DF = 1, $p < 0.0001$, BB > BF, χ^2 = 21.52, DF = 1, $p < 0.0001$). Visitation rate of

hawkmoth pollinators was 2.3 times greater than bee flies but this difference was not significant (HM = BF, χ^2 = 2.95, DF = 1p = 0.0858).

Hawkmoths and large bees are the most effective pollinators. The mean (+/- 1 SE) effectiveness for large bees, bee flies and hawkmoths based on the N = 64, N = 9, and N = 29 samples of pollen deposition were 231 (210, 253), 43.3 (25.4, 65.9), and 249 (204, 296), respectively. After adjusting the mean pollen grain deposition values by subtracting the mean pollen grain deposition from control stigmas (no visits, N = 46), on average hawkmoth and large bee pollinators deposited 9.2 times and 8.4 more pollen grains than bee flies. Pairwise contrasts demonstrated that mean pollen grain deposition by hawkmoths and large bees was not significantly different (F = 0.24, DF = 1,144, p = 0.6241). Based on the pollinator effectiveness data, bee flies were insignificant pollinators compared to hawkmoths and large bees. Results from the pairwise means comparisons indicated large bees (F = 103, DF = 1,144, p < 0.0001) and hawkmoths (F = 76.8, DF = 1,144, p < 0.0001) but not bee flies (F = 1.31, DF = 1,144, p = 0.2541) deposit significantly more pollen per visit than there are pollen grains on stigmas in the absence of visitors.

Simulations and exact variance formula

The corrected effectiveness data set was used in the simulations and the Goodman exact formula estimate. The adjusted effectiveness data set of large bees, bee flies, and hawkmoths resulted in a mean (variance, N) pollen grain deposition of 246 (3.55×10^4, N = 64), 47.4 (7.79×10^3, N = 9), and 291 (4.92×10^4, N = 29). In addition, the means for

the visitation rate were the same as those used for the linear models, and the variances that were used for the Goodman exact variance formula were 1.26, 0.0444, and 0.288 for large bee, bee fly and hawkmoth, respectively. The simulation results demonstrate that large bees are the most important pollinators, hawkmoths with intermediate values, followed by bee flies with the lowest importance (Fig. 1). Therefore, in the single season of 2006, high visitation rate by large bees and moderate rate of pollen deposition made them more important than the less frequent but slightly more effective hawkmoths. Mean large bee importance (277) was greater than the mean value (127) of the 97.5^{th} percentile of mean hawkmoth importance after 50 simulation trials. Thus, large bees have *significantly* higher average importance than hawkmoths (73). Although hawkmoth visitation rate was not statistically different from bee fly, the high hawkmoth effectiveness increased its pollinator importance over that of bee flies. Average bee fly importance (5.95) was lower than the mean value of the lower 2.5^{th} percentile of both large bee (190) and hawkmoth (31.2) after 50 simulation trials. The simulations exhibited remarkable stability across the 50 trials for all species. In particular the CVs for mean, LCL and UCL large bee importance were all less than 1% .

It appeared the precision of the estimates between the two methods was associated with the sample size of the effectiveness dataset. The simulated means, variances and confidence intervals were most similar to the estimates computed using the exact variance formula for the large bees (N=64 observations) and most different for bee flies (N=9 deposition observations). There appeared to be no pattern of either method over or underestimating the point or interval estimates of the other (Fig. 1). For example, the upper and lower large bee bootstrap confidence intervals were less than (-2.65%, -3.55%

difference, respectively) the confidence interval from the estimates using the exact variance formula. The simulated hawkmoth upper confidence limit was less than (-7.09%) and the lower confidence limit was greater than (10.3%) the estimates using the exact variance formula.

Discussion

Here we demonstrate two methods, novel in their application to pollinator importance, of estimating the mean and variance for a product of two random samples taken separately. Both methods yielded the same conclusion: using real visitation rate and pollen grain deposition data for three visitor species to *Silene caroliniana* in the 2006 flowering season we find that large bee importance is significantly higher than hawkmoth, which is significantly higher than bee fly. In fact, in no case did a pollinator's upper 95th confidence limit overlap another's lower 95th confidence limit for pollinator importance. The major advance of this paper is that the simulation method and/or the exact variance formula may be used to properly estimate the variance of pollinator importance thereby enabling pollination ecologists to test hypotheses of sources of variation in pollinator importance or any metric that involves the product of means of two random samples. First we discuss our results pertaining to the pollination system of *S. caroliniana*, and then we discuss assumptions and limitations of the methods in estimating pollinator importance and its confidence interval.

Important pollinators.

The simulated point and interval estimates statistically show that pollinators are significant sources of variation in pollinator importance. The separate linear models of the visitation and effectiveness data offer suggestions as to why the importance values differ among the visitors. For example, the difference between large bee and hawkmoth importance was due to the quadruple visitation rate of large bees because the mean effectiveness was not significantly different. Hawkmoths were exceedingly more important than bee flies more because of their very high relative effectiveness than their visitation rates, which were over twice as high as bee fly, but the difference was not statistically significant. However, the linear models of the component variables, visitation and effectiveness, do not sufficiently demonstrate pollinator importance varies among visitors because the standard error of pollinator importance is a function of the mean and variance of both samples.

Pollinator importance as the product of visitation rate and pollen grain deposition can provide some biological insight on the dynamics of pollen transfer. Given that an *S. caroliniana* flower in female phase contains about 30 ovules (Reynolds et al. unpublished data) large bees were delivering pollen at a rate resulting in slightly less than a 10:1 ratio of pollen grains to ovules every hour. It is likely this rate of pollinator service is sufficient to effect maximum seed set per flower since multiple studies have demonstrated seed set as a saturating function of pollen grain deposition on stigmas (Silander and Primack 1978; Mitchell 1997; Brown and Kephart 1999). With 25% the importance of large bees on average it would take hawkmoths four hours to achieve a similar level of pollinator service. Thus while our approaches clearly demonstrate that

large bees are more important pollinators than hawkmoths at our study site in 2006, it is probable that both pollinators are contributing substantially to the stigmatic pollen load. Thus we suggest that large bees and hawkmoths are both important pollinators.

The difference in visitation rate determines the significant variation of pollinator importance between large bees and hawkmoths. Although components of effectiveness may be expected to differ among years (Ivey et al. 2003), yearly variation of pollinator density is an inextricable component of pollination biology (Horvitz and Schemske 1990; Fishbein and Venable 1996; Waser et al. 1996; Fenster and Dudash 2001; Ivey et al. 2003). As pollinator importance fluctuates among years so it may be expected that the dynamics of pollinator mediated selection may also fluctuate. In the case of *S. caroliniana*, if by comparison with large bees, hawkmoth density increases one year such that its importance values overlap or exceed large bees, then in those years we would predict detection of significant selection on moth syndrome traits (e.g. tube length or tube width). In other years selection may correspond more to traits associated with large bee pollination (e.g., sequential anther dehiscence). Spatiotemporal variation in the densities of important pollinators that are selective agents may prevent the evolution of a strictly specialized pollination system (Aigner 2001).

Perhaps then, it is not surprising that the flowers of *S. caroliniana* exhibit traits concordant with the most common visitors. For example, the long narrow tubes, diurnal anthesis, and lack of scent and nectar guides indicate a diurnal moth syndrome (Faegri and van der Pijl 1979). However the syndrome is not exclusively moth as we observe large bees readily forage for nectar located at the base of the tubes (R. Reynolds personal observation). Sequential anther dehiscence has been noted to decrease pollen loss from

large bee grooming behavior (Harder 1990), thus it may represent an example of a large bee syndrome trait for *S. caroliniana*. Since *S. caroliniana* appears to possess floral traits consistent with both large bee and diurnal hawkmoth syndromes, it is particularly relevant to estimate the mean *and* variance of pollinator importance in order to make comparisons between the two pollinators in a hypothesis testing framework.

Estimation issues

The difference between the methods in their point and interval estimates appeared to be associated with sample size of the effectiveness dataset. The estimates were most in agreement for large bees (N=64) and least in agreement for bee flies (N=9), suggesting that small sample size is a serious limitation to the use of both approaches. With larger samples both approaches would yield narrower confidence intervals if the variance was constant among samples of differing size, because the variance of the mean and hence the variance of the product of means is inversely proportional to the sample size. Small sample size is problematic using the exact variance formula for possibly failing to meet the distributional assumption that the sample means of visitation and effectiveness are each normally distributed and therefore that pollinator importance has a product of normals distribution. While no distributional assumptions are required, aside from the observations being identically distributed, the bootstrap statistic's accuracy increases as the size of the samples increases because the sampling distribution then more closely resembles the population distribution (e.g. Chernick 1999). Thus, the point and interval

estimates using the bootstrapping method could potentially be far from population with small sample size.

The interval estimates calculated using the standard error of the exact variance formula are invalid if it can not be safely assumed the mean importance statistic has a product of normals distribution, which is the case when the sample with non-normal data (e.g., visitation rate) is small. Consequently, the accuracy of the bee fly importance measure may be suspect, and additional larger pollen deposition samples should be collected to confirm the very low importance estimates. The central limit theorem of mathematical statistics ensures that when large random samples (the rule of thumb being >30 observations, e.g. p. 236, DeVore 2000) are taken the sample mean becomes normally distributed regardless of the distribution of the individual observations in the sample (p. 246, Hogg and Craig 1995). If the observations are normal then the mean of the sample is normal under any sample size, and the exact variance formula may be used to estimate the standard error for constructing the confidence limits. Pollinator effectiveness data may be modeled as normal if the samples have small variance and relatively large mean (negative values are unrealistic). However this may be an unusual case because pollen grain deposition data can have high variance. It may be more realistic to assume a Poisson distribution for the deposition data, but this probability model may not be appropriate if the data are overdispersed. One way to determine if the data are normally distributed is by examining normal quantile-quantile or probability plots (e.g., p 187, Devore 2000), and Proc Univariate in SAS performs these analyses. Therefore, ideally large samples of both visitation and effectiveness should be taken to

help satisfy the distributional assumptions required for constructing the confidence intervals.

In addition to the problems associated with estimation using small sample size both methods assume no covariance between visitation and effectiveness. Intuitively it seems more likely that visitation and effectiveness should positively covary if pollen is limiting seed production than otherwise. In our study visitation rate was measured on 16 days, effectiveness 10 days, and the two together for large bees, were measured 8 days. The correlation between average visitation rate and pollen grain deposition for those eight days was close to zero ($r = -0.021$, $P = 0.9641$) suggesting minimal covariance between the two pollination measures in this one year study. However, for the rarer pollinators, visitation and effectiveness data coincided for four days and thus a reliable test of the covariance assumption was not possible. Future studies of pollinator importance using the simulation method or the exact variance formula should incorporate a robust test of the no covariance assumption. If there is substantial covariance then it needs to be incorporated in the simulations and/or exact variance formula.

Since both the simulation and exact variance formula yielded similar results, and the exact variance formula is far easier and less time consuming to implement, we suggest using the standard error of importance from the exact variance formula and the appropriate critical values from the distribution of two normals table to construct the confidence intervals. When estimating importance as the product of three random variables an estimate of the standard error is possible using the exact variance formula, but to make a confidence interval the distribution of the statistic must be known, which is not as simple as using the published distribution tables for the product of two normals

(Meeker et al.1981). Therefore, if the number of variables is greater than two the simulation method is preferred. Furthermore, if the sampling distribution of the mean of the two variables can not be safely assumed to be normal then the simulation approach should be used.

Another method of modeling pollinator importance not detailed here is using the framework of hierarchical Bayesian modeling (e.g. Congdon 2003), which is gaining increasing popularity in the ecological literature (Clark 2005). These techniques have proven useful in the demographic literature where vital rates exhibit significant individual, and group level variability that present formidable modeling challenges using classical techniques (e.g., Clark 2003). Pollinators may exhibit much individual variability in visitation and pollen deposition, possibly stemming from body size variation, or nutritional status, and it is conceivable that pollinators may differ in deposition rates by grouping them based on flower gender previously visited, flower plant density, and foraging time. Essentially the hierarchical framework may allow a realistic exploration of the complex relations feeding into variation in pollinator importance.

We applaud Larson (2005) and Bloch et al. (2006) for recognizing the need to add standard errors to their measures of pollinator importance, which motivated this paper, but we argue that our point and/or interval estimates of importance are more accurate. The mean and variance of *both* samples of visitation and effectiveness are functions of the pollinator importance variance (Craig 1936; Haldane 1942; Goodman 1960). Accordingly, scaling each effectiveness observation by the mean (a constant: variance = 0) of the visitation data or the visitation observations by the mean of the effectiveness

dataset (e.g., Larson 2005) underestimates the variance of pollinator importance. Bloch et al. (2006) incorporated a resampling procedure in which each observation of visitation was multiplied by the mean of a random subsample of the effectiveness dataset to generate a single importance dataset. However, the method needed to be repeated numerous times to generate a distribution of mean importance in order to get an estimate of population mean importance and confidence limits with the least bias as possible.

The simulations may be extended to the product of several random variables, and the statistical properties of the product of k independent random variables are known (Goodman 1962). For example one could weight the importance value by its covariance with traits, which would be indicative of its importance as a source of natural selection. Thus, if a rare pollinator that is effective exerts strong selection on a particular trait it may be more important evolutionarily than a pollinator that is frequent, effective but exerts no selection on floral traits. Therefore, the metric could measure the potential for specialization in the plant pollinator interaction (Schemske and Horvitz 1984).

Chapter 2: Pollinator specialization and pollination syndromes of three related North American *Silene*

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 often are unable to distinguish whether flower visitors are pollinators, i.e., actual pollen vectors, hence such surveys may not accurately assess the relationship between floral traits comprising the syndrome and the pollinators responsible for their evolution. Here we address whether the floral traits of three closely related *Silene* species 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, and a multi-year study of visitation rates were performed. Also, pollen grain removal from anthers, pollen grain deposition on stigmas, and pollinator importance (visitation*deposition) of each of the animal visitors of each species were estimated to quantify all aspects of the pollination process. The syndromes were good predictors of the major pollinators. *Silene 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. Nonetheless, *S. caroliniana* was more broadly specialized for diurnal long-tongued

pollinators. Compared across the *Silene* species, divergent floral character states are consistent with increasing the attraction and/or pollen transfer efficiency of subsets of the total pollinator fauna, which suggests that those pollinators featured prominently as selective agents for floral trait evolution in these three species of *Silene.* We conclude that the pollination syndrome concept allowed us to effectively relate the functional significance of floral morphology to the major pollinators of the *Silene* species.
Key Words: Silene, *Pollinator Importance; Pollination Syndrome; Specialization; Generalization*

INTRODUCTION

Quantifying the number and relative value of pollinators provides pertinent information for studies in community ecology, affecting such topics as network theory, diversity and stability, and the extent of generalization vs. specialization. From an evolutionary ecology perspective, these data are required to verify the putative functional relationship between floral traits (e.g., attraction, reward and efficient pollen transfer) comprising pollination syndromes and the most important pollinators, i.e., potential sources of natural selection on floral traits.

Community (Waser et al. 1996, but see Fenster et al. 2004) and geographic (Ollerton et al. 2006) surveys of plant-pollinator interactions often show the majority of plant species are "ecologically generalized" (Armbruster et al. 2000), or pollinated by multiple animal visitors. 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). Drawing from food web and species diversity theory, 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 indicate that generalization confers stability in mutualistic networks. Furthermore, generalization would seem to lessen the negative demographic consequences of lost pollinators (Waser et al. 1996) making it an attractive strategy to cope with highly variable pollination service in space and time (Herrera 1988, Fishbein and Venable 1996, Ivey et al. 2003). Empirical and theoretical studies suggest plant-pollinator interactions are usually generalized, that generalization is a favorable strategy under a wide range of conditions, and that large community size may be required to tolerate the strongly asymmetric strength of specialized interactions.

However these recent conclusions regarding the predominance of generalization conflict with nearly two centuries of observation that flowering plants possess floral features that function to attract and increase the pollen transfer efficiency of particular pollinators (reviewed in Vogel 1996, 2006). 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, Stebbins1970, 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, where flowers of similar form (pollination syndromes) reflect selection response to similar pollinators or selective agents (Vogel 1954, 2006, Faegri and van der Pijl 1979), i.e., functional groups of pollinators (Fenster et al. 2004). In addition to the natural

history observations, the pollination syndrome concept has support from studies demonstrating natural selection by major pollinators on floral traits (Campbell 1989, Caruso 2003, Reynolds et al. in prep), associating floral polymorphisms with pollination ecotypes (Grant and Grant 1965, Galen 1989), 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).

A consensus emerging from the debate is that detailed empirical data are needed to evaluate the extent of floral specialization and whether pollination syndromes are realistic for describing floral adaptation (Waser et al. 1996, Fenster et al. 2004). Here, we define specialization from the plant's perspective to mean significantly greater levels of pollinator service by one pollinator type over others. According to Stebbins' (1970) most effective pollinator principle, the contribution of both visitation and effectiveness (i.e., some measure of the pollination service such as pollen grain deposition or fruit set), should be considered together when describing flower adaptations that facilitate pollination. Thus, a pollinator's importance is best calculated as visitation rate multiplied by effectiveness, thereby concretely describing the dynamics of pollination. Pollinator importance, when properly estimated (Reynolds and Fenster 2008), allows for statistical comparisons of mean importance among taxa to determine which pollinators the plant specializes on for successful reproduction.

Here we quantify the extent of floral specialization and the predictive value of pollination syndromes of three related North American *Silene* species (S. *caroliniana*, *S. virginica*, and *S. stellata)*. Molecular phylogenies indicate these species form a single clade among the nine endemic *Silene* east of the Rocky Mountains (Burleigh and

Holtsford 2003), with two of the species sister to each other (Popp and Oxelman 2007). These three *Silene* species are remarkable in that they are highly divergent from one another in floral traits associated with pollinator attraction, reward, and efficient pollen transfer. Our objectives were (1) to describe completely the floral and breeding system characters among these three *Silene* species, and (2) to determine the degree to which the *Silene* species 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 relationship 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 the *Silene* species.

NATURAL HISTORY of STUDY SYSTEM

Silene caroliniana, *S. virginica*, and *S. stellata* are herbaceous perennial wildflowers of eastern North America. Populations of *S. caroliniana* were studied within the C&O Canal National Park, near the Billy Goat Trail and Old Tavern in Montgomery County, MD, 77°14'30"W, 38°58'56"N, elevation=150 meters. 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, VA, 80°33'14"W, 37°21'20"N, elevation≈1,100-1300 meters. Unless otherwise noted, all studies described herein were performed using plants and pollinators in their 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.

The flowers of *Silene caroliniana* are pink and tubular, and are held nearly upright (Fig. 1A). Plants overwinter as basal leaf rosettes and in early spring produce one to several bolting stems (10-20 cm) containing 5-10 to dozens of flowers (R. Reynolds, personal observation) presented in a cymose inflorescence with flowering occurring from early April to early May. The flowers of *Silene virginica* are red and tubular, and are held horizontally (Fig. 1B). Plants overwinter as rosettes and in May produce one to several bolting stems (20 - 40 cm) containing usually 1 - 7 flowers (R. Reynolds, personal observation) per cymose inflorescence with flowering occurring from late May through June. The flowers of *Silene stellata* are white and bowl-shaped with fringed petals and are presented horizontally (Fig. 1C). Plants lack basal rosettes, but they produce one to many reproductive stems that emerge in early spring and reach up to 120 cm in length (R. Reynolds, personal observation). There are typically > 20 flowers per panicle inflorescence at the terminal ends of the reproductive stems with flowering occurring from early July through mid August. All three species are protandrous with 10 anthers and three stigmas per flower, and are highly outcrossing (Dudash and Fenster 2001, Reynolds unpublished).

METHODS

Floral traits: attraction and reward. 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 the Supplemental Methods of Ecological Archives).

Floral traits: breeding system. Pollen presentation and stigma receptivity strategies are traits that directly affect the dynamics of pollen transfer and may be

correlated with other floral traits (Lloyd and Yates 1982, Harder and Thompson 1989, Thomson et al. 2000). Therefore breeding system characters also contribute to pollination syndromes. For each species, timing of anther dehiscence and stigma receptivity were measured by direct observations of flowers from bud stage to receptivity (further details are presented in Supplemental Methods).

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 was performed in April and May 2004 for *S. caroliniana*, June 2002 for *S. virginica*, and July and August 2002 for *S. stellata*. Prior to flowering, 40 plants of each species were randomly selected and each plant was randomly assigned to one of four treatments: (1) total pollinator exclusion, (2) nocturnal pollination, (3) diurnal pollination, and (4) diurnal and nocturnal pollination (further details of are presented in the Supplemental Methods).

Fluorescent dye study. Fluorescent dyes were used as pollen analogs 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). The proportion of plants receiving dye particles on stigmas was measured each night and day. Each day at dawn the anthers of three flowers were labeled on two source plants, pollinators were allowed free access to the plants all day until dusk, and then the treated anthers were removed. At dusk two additional donor plants were chosen with similar floral displays as the source plants selected at dawn, and they were labeled

with different colored dyes than used for the diurnal treatment. Thus, there were 18 experimental units, with two replicate observations in each experimental unit. The dye colors were rotated between the treatments daily. Pollinators were allowed free access to the plants all night and the anthers were removed at dawn. The stigmas of all flowers on every plant within 10 m of the focal plants were checked (mean = 39 plants ± 4.5 SE) for fluorescent dye with a UV lamp. The distance between source and recipient plants was measured with a meter tape. Details of the analyses are presented in the Supplemental Methods.

Visitation data. To investigate how accurately the *Silene* species pollination syndromes predict their animal visitors and to quantify each visitor's pollinator importance (visitation rate*pollinator effectiveness) (Inouye et al. 1994) and the confidence intervals surrounding pollinator importance estimates (Reynolds and Fenster 2008), visitation rate was estimated as the number of plant visits per hour for all the visitors to the flowers of each *Silene* species. Additionally, the proportion of total visits for each visitor was calculated for each *Silene* species. *Silene virginica* plants were observed in a single year (2002) as hummingbird visitation greatly exceeded invertebrate visitation (see results herein) and because a previous study demonstrated hummingbirds were the major pollinators (Fenster and Dudash 2001). Visitation was sampled across five years for *S. caroliniana* (2003 -2007) and *S. stellata* (2002 - 2006), both of which had several candidates for major pollinators. Sampling details and analyses can be found in the Supplemental Methods.

Pollen removal and deposition. To quantify the efficiency of a pollinator (pollen removed vs pollen deposited) and a pollinator's importance (visitation rate*pollen grain

deposition), both pollen removal and deposition were quantified for the floral visitors. The amount of pollen removed or deposited was quantified for a single visit to virgin flowers, which had been excluded from pollinators by exclusion cages. Pollen removal data were collected in 2004 for *S. stellata*, and in 2005 for *S. caroliniana* and *S. virginica*. Due to the low rate of hummingbird visitation, additional data for *S. virginica* were collected using open experimental arrays of potted plants at MLBS and naturally occurring plants from a nearby meadow site, dense with *S. virginica*. Details of methods and analyses for pollen removal and deposition are presented in the Supplemental Methods.

Pollinator importance and pollen loss. Pollinator importance (visitation rate*pollen grain deposition) was 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. The standard error of pollinator importance may be calculated from the variance of a product of random variables (Goodman 1960) or by bootstrapping and a random simulation approach. The methodology, computational details, and results of the approaches using a single year of data for *S. caroliniana* may be found in Reynolds and Fenster (2008).

The cost in terms of male reproductive success of having pollinators that remove high levels of pollen but deposit little, was estimated as the average amount of pollen removed that does not land on the stigmatic surface. By assuming the mean pollen grains removed (R) and deposited (D) are independently and normally distributed, $N(\hat{\mu}_R, \frac{\hat{\sigma}_R^2}{n_R})$ and $N(\hat{\mu}_D, \frac{\hat{\sigma}_D^2}{n_D})$, the amount of pollen lost (L) has distribution,

$N_{L=R-D}(\hat{\mu}_R - \hat{\mu}_D, \frac{\hat{\sigma}_R^2}{n_R} + \frac{\hat{\sigma}_D^2}{n_D})$. Thus, the estimate of the mean loss was calculated as the difference of the mean estimates of deposition from removal. This measure of pollen loss may be considered vector-induced pollen loss, which does not include non-vectorial factors such as wind (Inouye et al. 1994). The pollen loss standard error was taken as the square root of the sum of the variance of the means. An approximate 95% confidence limit on the difference in population mean cost between hawkmoths and large bees for *S. caroliniana* and between diurnal and nocturnal pollinators for *S. stellata* was calculated. If the difference in population means does not overlap zero then we may conclude the sample means are significantly different.

RESULTS

Floral traits: Table 1 contains the floral trait data pertaining to attraction, reward and pollen transfer for the three *Silene* species. The intermediate sized, pinkish, scentless flowers of *S. caroliniana*, with scant but concentrated nectar, and narrowly tubular flowers are suggestive of both long-tongued bees and diurnal lepidoptera syndromes. The comparatively large, red, scentless flowers of *S. virginica*, with copious and dilute nectar, and the tubular flower shape and highly exserted stamens and stigmas are traits that are all 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 nocturnal moth syndrome.

The 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 ten anthers simultaneously at dusk.

Stigmas become receptive during the day for both *S. caroliniana* and *S. virginica*, and *S. stellata* stigmas become receptive at night. Thus, it may be predicted that *S. caroliniana* and *S. virginica* have diurnal pollinators and *S. stellata* has nocturnal pollinators.

Nocturnal-diurnal pollination experiment. The results of the fruit and seed set models were similar, thus we present only the fruit set data (Fig. 2). Mean back-transformed percent 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. carolinina*, $P = 0.0002$, *S. virginica*, $P < 0.0001$, and *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 ($P = 0.4945$). 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. The fluorescent dye study indicated that the probability (±1SE) 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, $P = 0.0305$) between the two groups. On average (SE) nocturnal pollinators moved marked pollen grains 2.2 ± 0.43 meters, which was 50% farther than diurnal pollinators with a mean of 1.2 ± 0.35 meters, but the difference was not statistically significant ($\chi 2 = 2.04$, DF = 1, $P = 0.1529$).

Flower visitation. The three proportionally most common visitors of *S. caroliniana* across the five years of visitor observations (n = 1,057 visits observed) were large bees (0.73), such as bumble bees (*Bombus* spp., e.g., *Bombus affinis*) and carpenter bees (*Xylocopa Virginia*), clearwing hawkmoths (*Hemaris* sp.) (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*) (0.021) and very rarely by cabbage whites (*Pieris rapae)* or juniper hairstreaks (*Callophrys gryneus*). The large bees, 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 is a significant predictor of visitation rate in *S. caroliniana* ($F = 22.85$, $DF = 2, 324$, $P < 0.0001$). Averaged (SE) across the five years of study on *S. caroliniana*, large bee visitation rate was 0.93 ± 0.13, clearwing hawkmoth was 0.12 ± 0.044 and bee fly was 0.10 ± 0.045. Pairwise contrasts indicate large bee visitation rate is significantly greater than both hawkmoth ($F = 27.79$, $DF = 1, 324$, $P < 0.0001$) and bee fly ($F = 22.61$, $DF = 1, 324$, $P < 0.0001$). Hawkmoth and bee fly visitation rates were not significantly different ($F = 0.09$, $DF = 1, 324$, $P = 0.7706$). 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 hawkmoths were rarely observed in 2005 (Fig. 3). Year of sampling was not a significant predictor of overall visitation rate for *S. caroliniana* ($F = 2.30$, $DF = 4, 162$, $P = 0.0614$).

Primarily ruby-throated hummingbirds, *Archilocus 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* ($F = 4.83$, $DF = 1, 85$, $P = 0.0307$). Hummingbirds mean (SE) visitation rate was 0.18 ± 0.043, which was significantly higher than the small bees and flies with a mean of 0.070 ± 0.026.

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*. 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 ($F = 4.66$, $DF = 1, 5$, $p = 0.0834$), although the nocturnal moth mean (SE) visitation rate of 0.93 ± 0.20 was higher than diurnal bees and flies with a mean of 0.51 ± 0.088. Year of sampling was not a significant predictor of visitation rate for *S. stellata* ($F = 0.67$, $DF = 2, 108$, $P = 0.5142$). However, the visitor type by year interaction was a significant predictor of visitation rate ($F = 13.58$, $DF = 2, 5$, $P = 0.0095$) indicating diurnal and nocturnal visitation rate varies depending on the year of observation (Fig. 3).

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 hours following dehiscence are reported in Table 2.

For *S. caroliniana* the mixed model analysis of variance 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) ($F = 42.72$, $DF = 1, 100$, $P < 0.0001$) 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 hawkmoths ($F = 6.15$, $DF = 1, 100$, $P = 0.0148$) 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 hawkmoths ($F = 0.12$, $DF = 1, 100$, $P = 0.7298$) (Table 2).

For *S. virginica* both treatment ($F = 22.27$, $DF = 1, 74$, $P < 0.0001$) and treatment by visitor interaction ($F = 5.65$, $DF = 1, 74$, $P = 0.02$) were significant predictors of the response, number of pollen grains per anther per visit, at the alpha = 0.05 level. 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 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 Bonferonni corrected alpha level = 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 = 0.022) 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 average 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 = 0.5114) (Table 2).

Pollen deposition. The analysis of variance of the *S. caroliniana* pollinator effectiveness data set showed that species of visitor and the completely caged and unmanipulated controls were significant predictors of the pollen grain deposition response variable (F = 34.5, DF = 1, 163, P < 0.0001). Large bees and hawkmoths, but not bee flies, are effective pollinators of *S. caroliniana*. The average 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). Hawkmoths and large bees without the contribution from bee flies deposited significantly more pollen per visit than accumulated on the unmanipulated contols (F = 53.49, DF = 1, 166, P < 0.0001), which suggests that hawkmoths and large bees are effective pollinators and the contribution from bee flies is negligible. Bee fly deposition rates were not significantly greater than mean deposition in the absence of pollinators (F = 1.37, DF = 1, 166, P = 0.2441) (Table 2). Hawkmoth and large bee

pollen deposition effectiveness were not significantly different ($F = 0.25$, $DF = 1, 166$, $P = 0.6167$) (Table 2).

Analysis of variance 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 average pollen grain deposition (Table 2) was significantly higher for nocturnal moth than diurnal bee pollinators ($F = 1\ 5.77$, $DF = 1, 367$, $P < 0.0001$). A second orthogonal contrast indicated that the nocturnal moths still had significantly higher deposition rates than diurnal bees ($F = 3.97$, $DF = 1, 367$, $P = 0.0471$) after the means were corrected by the average pollen deposited on unvisited (completely caged) control stigmas. Furthermore, a third orthogonal contrast showed there was no significant difference ($F = 0.35$, $DF = 1, 367$, $P = 0.5557$) 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 & 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 onto stigmas in flowers of *S. stellata*.

Pollinator importance and pollen loss. Of the three most common visitors of *S. caroliniana,* large bees are the most important pollinators with significantly higher

estimates of pollinator importance than hawkmoths in all years except 2004 (Fig. 3). Hawkmoths and large bees were always significantly more important than bee flies except for 2005, when hawkmoths were rarely observed. Pollen loss by large bees was significantly greater than hawkmoths because the approximate 95% confidence interval containing the difference of population means did not contain zero (Table 2). Nocturnal moths were more important pollinators than diurnal bees on *S. stellata* 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. 3). Pollen loss by diurnal bees was significantly greater than nocturnal moths for *S. stellata* (Table 2). Hummingbird importance and pollen loss are listed in Table 2 for *S. virginica*.

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*. *Silene caroliniana* is the least specialized with large bees and the less important clearwing 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. 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. *Silene caroliniana* has traits consistent with diurnal hawkmoth pollination (Vogel 1954, Faegri and van der Pijl 1979, Balkenius et al. 2006) and large bee pollination (Baker and Baker 1983,Thomson 1986). *Silene*

virginica has traits that increase attraction and efficiency of hummingbird pollination (Faegri and van der Pijl 1979). *Silene stellata* has traits highly indicative of pollination by nocturnal moths and not by diurnal bees (Vogel 1954, Faegri and van der Pijl 1979).

Based on visitation rates and overall floral appearance, *Silene caroliniana* appears to be specialized for large bee pollination but the pollen removal and deposition data suggest that clearwing hawkmoths are also important pollinators. Large bee pollinator importance was significantly greater than 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 hawkmoths. Large bees were consistently the most important pollinators, but the average amount of pollen large bees removed that was not deposited (i.e., lost from the plant's perspective) was three-fold higher than hawkmoths (Table 2). Therefore, from a male reproductive success point of view, hawkmoths would be the more favorable pollinator especially in years with equal visitation rates, and if selection on floral traits is mainly associated with variation in male reproductive success, then hawkmoths may be a very important selective agent on *S. caroliniana* floral traits.

Silene virginica is specialized for hummingbird pollination. Hummingbirds visited at higher rates than the invertebrate visitors, and had higher deposition and removal rates. 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 pollinators fruit and seed set declines by 50%, a result repeated 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 (R. Reynolds, personal observation).

Our work with the pollinators of *S. stellata* demonstrates the value of comprehensively examining all 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. 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. Furthermore, flowers are pollinated first nocturnally, then diurnally. Flowers caged through the night had lost 50% of the pollen grains present on newly dehiscent anthers by 10:00 AM (12 hours post dehiscence), 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. 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 dye dispersal study is overestimated, because equal amounts of dye were available to nocturnal and diurnal pollinators. Although our studies of frequency and effectiveness demonstrate that pollinator importance of nocturnal moths was significantly higher than diurnal bees in two of three years, we believe that the order of pollination, first by nocturnal moths, then by diurnal bees, tips the scale even more towards 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 2000). It follows that if important pollinators are sources of natural selection on syndrome traits the pattern of expression of these traits among the related *Silene* species should be related to pollen transfer efficiency of the important pollinators. 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, *Silene 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 clearwing hawkmoths. It would be too costly for *S. caroliniana* flowers to present all anthers at once because the probability of a pollen grain being successfully transported to a stigma would be lower, due to large bee grooming behavior, than if pollen were packaged in multiple smaller doses. *Silene virginica* also presents pollen sequentially, with five anthers presented simultaneously at flower opening and then again the next day. This pollen packaging strategy could limit

pollen loss associated with pollination by the infrequent (~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 ten 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 the probability of pollen grains removed finding their way to the proper stigmatic surface.

The systematic relationship of the 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 sources of natural selection that have resulted in diversification of the *Silene* species. 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 confirmed experimentally as it has in other systems (Castellanos et al. 2006). The less frequent but highly effective and efficient (in terms of pollen removed versus amount of pollen

deposited) hawkmoths may be the primary sources of selection on other syndrome traits and thus the *S. caroliniana* floral phenotype may represent adaptation to hawkmoth pollination with little or no tradeoff 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. *Silene caroliniana* promises to be a model for research in the evolution of floral traits attracting a mixture of effective pollinators.

If pollination generalization means more than one species of visitor pollinates 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 of the principal pollinators as defined by the detailed study of the pollination systems. Some would argue that the pollination syndrome concept is simply a typological construct intended to classify floral systems into neat categories (Ollerton et al. 2007). The comprehensive pollination data described here demonstrate that the syndrome concept is practical for predicting the major pollinators and hence the major selective agents of floral variation. Moreover it suggests many further studies of pollinator specialization and pollinator syndromes with these *Silene* species. For example while *S. caroliniana* has two important pollinators indicating a somewhat generalized syndrome, the species could be interpreted as specialized in that pollen resides in similar locations on the long-tongued insects' heads. Still unresolved is whether the functional

similarity of pollen placement on large bees and hawkmoths translates to selection for traits in the same or opposing direction. The mixture of bee and moth associated traits also suggests that pollination generalization can be accompanied by selection mediated by different pollinators on different traits. Our study shows that specialization is viewed best from the plant's perspective in terms of the important selective agents acting on floral traits. Which of the subset of pollinators are the most important pollinators, and are there floral traits expressed by these plants that are functionally related to increasing the efficiency of pollination by the major pollinators? The answers are that all three related species appear to be specialized on a subset of the potential pollinators, and the plants exhibit floral traits concordant with the most important pollinators acting as the selective agents responsible for either the origin or maintenance of the measured trait variation across these three *Silene* species.

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 tradeoffs may also contribute to floral evolution (reviewed in Galen 1999, Strauss and Irwin 2004, Strauss and Whittall 2006). Seed predation by *Hadena* moth larvae (Kephart et al 2006; Reynolds et al. in prep.) 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 to the three *Silene* species 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.

TABLE 1. Average values (SE, CV) of floral traits for each of the three *Silene* species, *S. caroliniana, S. virginica* and *S. stellata*. Numbers in parentheses are SE for reward measurements. Attraction and pollen transfer measurements in mm.

TRAIT	***S. CAROLINIANA***	***S. VIRGINICA***	***S. STELLATA***
ATTRACTION			
COLOR	Pink, variable	Red	White
PETAL LENGTH	12.1 (1.6,13.1)	18.0 (2.3, 13.0)	9.0 (0.9, 9.9)
PETAL WIDTH	6.4 (0.9, 14.1)	5.8 (0.8, 13.2)	11.3 (1.5, 13.0)
SCENT	Absent	Absent	Present
REWARD			
NECTAR ML	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	2.9 (1.0, 35.1)	7.2 (2.0, 27.4)	10.3 (1.3, 12.4)

EXSERTION			
COROLLA TUBE LENGTH	21.2 (1.6, 7.4)	24.1 (2.1, 8. 8)	9. 8 (0.9, 9.1)
COROLLA TUBE DIAMETER	1.9 (0.4, 19.3)	3.6 (0.5, 14.8)	8.0 (1.0, 12.3)
ANTHESIS	Diurnal, sequential	Diurnal, 5 stamens/day	Nocturnal, simultaneous

TABLE 2. Average (SE) visitation rate, pollen removal, pollen deposition and pollen loss of visitors to *Silene caroliniana, S. virginica* and *S. stellata*. Visitation rate is the number of visits per plant per hour. Pollen removal is number of grains removed per anther per visit. Pollen deposition is the number of pollen grains deposited per visit. Pollen production is amount of pollen per anther. Old females are flowers in female phase collected from plants in their natural population. Pollen loss is the difference between pollen removed and pollen deposited.

	SILENE CAROLINIANA	***SILENE VIRGINICA***	***SILENE STELLATA***
POLLEN PRODUCTION			
	2,870 (115)	4,820 (409)	Nocturnal 1,340 (169); Following day 756 (184)
OVULES			

	39 (0.8)				46 (2.8)		25 (0.4)			
VISITOR	LARGE BEES	HAWK-MOTHS	BEE FLIES	NO VISITS	HUMMING-BIRDS	NO VISITS	BEES	NO DAY VISITS	MOTHS	NO NIGHT VISITS
POLLEN REMOVAL										
	2,000 (200)	800 (420)	--	640 (240)	3,300 (500)	1,100 (800)	780 (670, 910)	120 (-46, 210)	490 (400, 580)	240 (180, 300)
POLLEN DEPOSITION										
	230 (209, 253)	249 (206, 297)	43 (25, 66)	18 (15, 22)	302 (267, 338)	54 (42, 67)	34 (5)	34 (4)	74 (7)	28 (9)

POLLEN ON OLD FEMALES										
	168 (143, 195)				--		86 (14)			
POLLEN LOSS										
	1,770	551	--	--	3000	--	416	--	746	--
	(204)	(421)			(501)		(91)		(130)	

FIG 1. Color plate of three closely related *Silene* species from eastern North America: (A) *S. caroliniana*, (B) *S. virginica*, and C) *S. stellata*. All three species are shown in male phase.

FIG 2. Mean (± 1SE) fruit set per plant in the diurnal-nocturnal pollinator exclusion experiment demonstrating the extent of nocturnal vs. diurnal animal pollination for *Silene caroliniana, S. virginica* and *S. stellata*.

FIG 3. Visitation rate (visits/plant/hour) and pollinator importance (pollen grains deposited/ hour) of the pollinators of *Silene caroliniana* and *S. stellata* for each of five years. Key for *S. caroliniana*: open bars-large bees, shaded bars-hawkmoths, cross hatched bars-bee flies. Key for *S. stellata*: open bars-diurnal bees, shaded bars-nocturnal moths. Visitation rates are mean (± SE) and pollinator importance values are mean (± approximate 95% confidence intervals). Visitation rates of diurnal pollinators were not quantified in either 2005 or 2006.

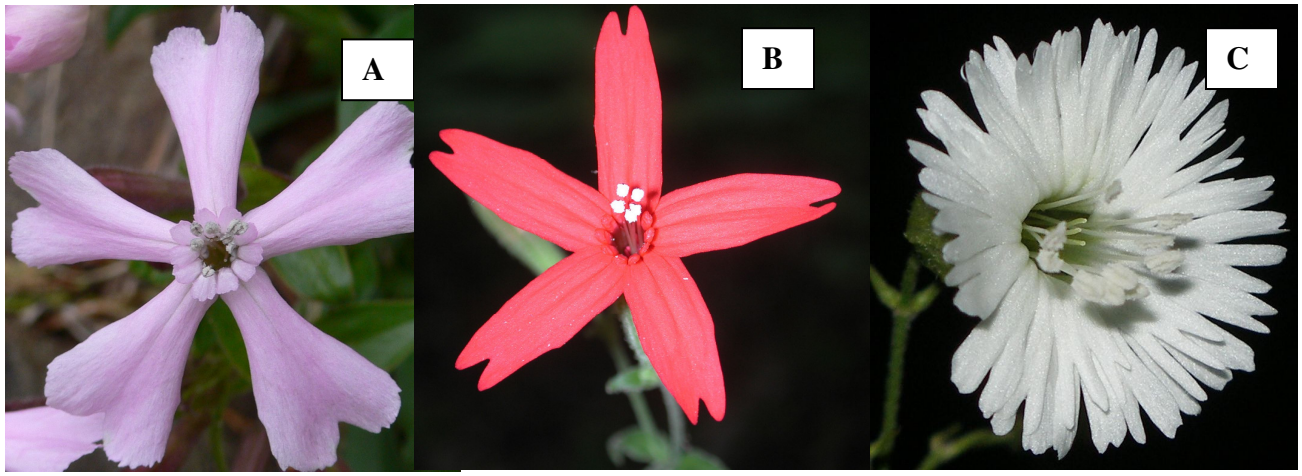

FIG. 1

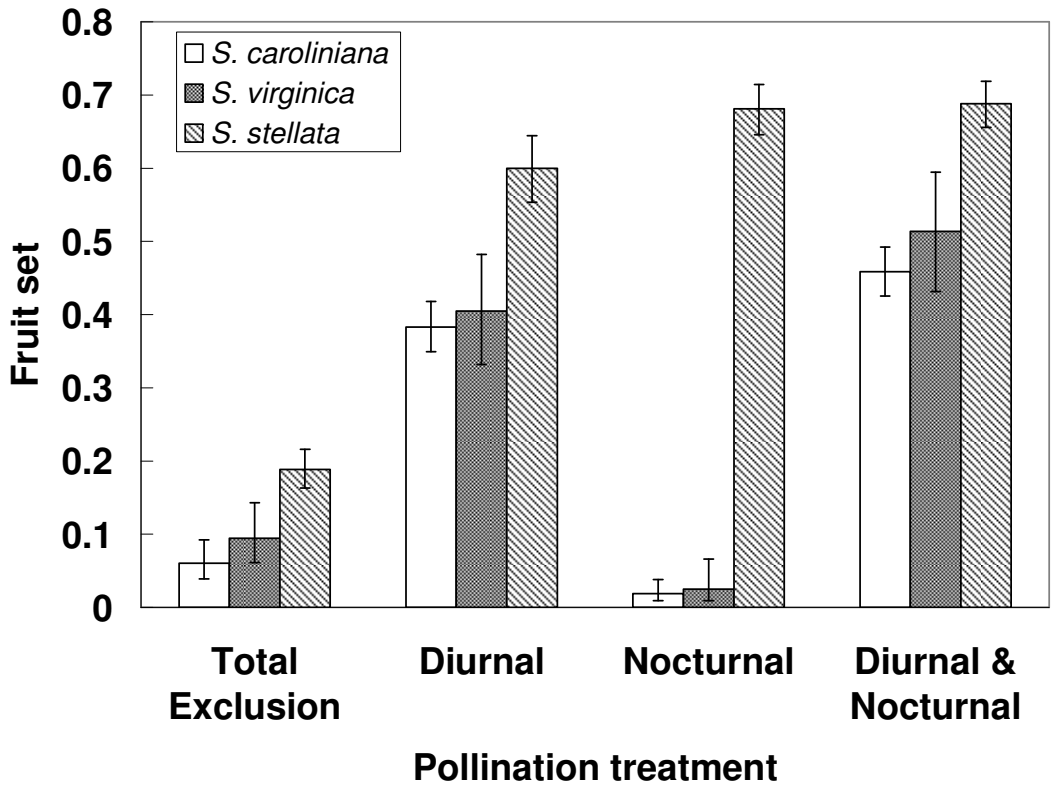
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fruit set
S. caroliniana
S. virginica
S. stellata
Total Exclusion
Diurnal
Nocturnal
Diurnal & Nocturnal
Pollination treatment

FIG. 2

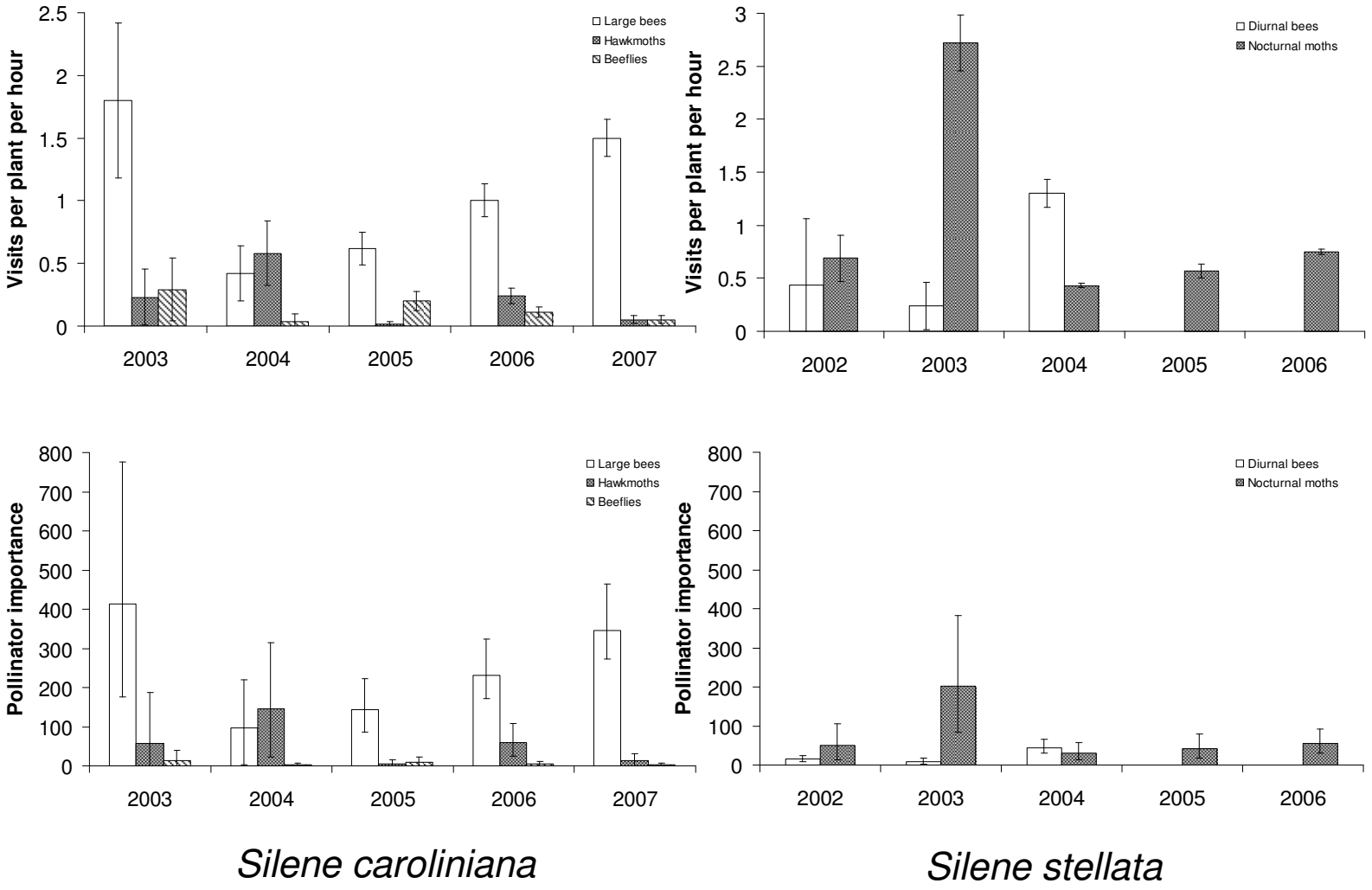
Visits per plant per hour
Large bees
Hawkmoths
Beeflies
2003
2004
2005
2006
2007
Diurnal bees
Nocturnal moths
2002
Pollinator importance
Silene caroliniana
Silene stellata

FIG.3

SUPPLEMENTARY METHODS

Floral traits: attraction and reward. The morphological traits were measured in the field with dial calipers to 0.1 mm on randomly selected plants from natural populations. Measurements were taken in one population for each of the three *Silene* species. Corolla tube length was measured from the base of calyx to the tube opening. Corolla tube width was measured as the widest length across the corolla tube opening. Petal length was measured as the length of the largest of the five petal limbs, which spread outward perpendicularly from the corolla tube. Petal width was measured as the widest portion of the petal measured for its length. *Silene caroliniana* floral measurements were made on one flower from each of 21 plants. For *S. virginica* and *S. stellata* multiple flowers were measured and averaged on each of 73 and 54 plants, respectively and averages and coefficients of variation were taken across plants (Table 1).

Chemical descriptions of floral scent produced by the *Silene* species in the field were obtained using the dynamic headspace/GC-MS method and will be presented elsewhere (S. Dötterl et al. unpublished). Here we report presence or absence of scent. For *S. virginica* and *S. stellata* scent samples were obtained from male and female flowers at dawn and dusk. For *S. caroliniana* samples of male and female flowers were taken during the day.

The reward component of pollination syndromes was measured as the volume and sucrose concentration of nectar from flowers in female phase caged with fine mesh screening to prevent pollinator visitation. Nectar measurements were collected only if > 24 hrs had elapsed without rain. A Hamilton microsyringe (10 µl) was used to draw nectar from *S. carolinana* and *S. stellata.* *Silene caroliniana* nectar volume

measurements were made on 30 flowers from 15 plants and 109 flowers from 10 plants in 2006 and 2007, respectively. *Silene stellata* nectar volume measurements were made on 11 flowers from 5 plants in 2004 and 2005 and 87 flowers from 10 plants in 2007.

Nectar concentration (mg/ml) was measured with a temperature-controlled hand-held refractometer (Sugar/Brix Refractometer 0 to 32% w/ATC SPER SCIENTIFIC). *Silene caroliniana* nectar concentration measurements were made on nectar of 2 pooled samples from several flowers of 1 plant and each of 65 flowers from 10 plants in 2006 and 2007, respectively. *Silene stellata* nectar concentration measurements were made from several flowers together from 1 plant in 2004 and 2005 and from each of 37 flowers from 10 plants in 2007. Nectar volume and concentration methods for *S. virginica* were described in Fenster et al. (2006).

Floral traits: breeding system. The number of dehiscent anthers was recorded on marked flowers that were visited at three-hour intervals during a 24 hour period. When flowers advanced to neuter stage, the stigmas were observed with a loupe (10X) to note swelling and papillae development. Stigma receptivity was confirmed with hand pollinations, as flower wilting indicates successful pollen germination. Stigma-nectary distance was measured as the distance between the base of the nectaries where the floral tube and pedicel join and the tip of the stigmas. The sample size was thirty flowers representing between 15-30 plants per species (reported in Dudash and Fenster 2001 for *S. virginica*).

Nocturnal-diurnal pollinator experiment. To generate treatment (1) (total pollinator exclusion), cages constructed of poultry wire covered with fine mesh screening were placed over ten plants during the entire flowering period. To generate treatments

(2) and (3) (nocturnal and diurnal pollination, respectively), cages were removed from ten nocturnally pollinated plants at dusk and placed on the ten neighboring diurnally pollinated plants. At dawn the cages on the diurnally pollinated plants were returned to the nocturnally pollinated plants and the cages were switched daily for the duration of flowering. To generate treatment (4) (diurnal and nocturnal pollination), ten plants were left uncaged. Fruits, with and without seed, were removed from the 40 plants of each species between two to three weeks following flowering (fruit open and disperse their seeds about 3 weeks post-pollination). Fruits were individually scored in the lab under a dissecting scope as fruit set, no fruit set, or eaten by noctuid larvae, and all seeds were counted. In addition, the mean number of ovules per flower was estimated as the average of the sum of the number of seeds and unfertilized ovules per fruit.

A generalized linear model (SAS institute, 2004: Proc Genmod) was used to model the response variables, fruit and seed set, with treatment as the predictor. Fruit set per plant (proportion fruits with seed of total number of flowers; however, if seeds were later eaten by *H. ectypa* larvae we still counted the fruit as successful because here we are interested in successful pollination) was modeled as a binomial response with logit link function, and Pearson's χ^2 divided by n-p degrees of freedom, where n is the number of observations and p is the number of estimated parameters (= 4), was used to account for overdispersion. Seed set (seeds per fruit) was modeled as a normal variate and identity link function. Orthogonal contrasts were used to compare average fruit set and seed set between the combined treatment levels, nocturnal and diurnal vs. open and closed, diurnal vs. nocturnal and closed vs. open.

Fluorescent dye study. Generalized linear models (Proc Genmod) were used to test the effect of the predictor, diurnal or nocturnal pollination, on the response variables, proportion of plants receiving dye particles (distribution = binomial, link = logit), and distance between source and recipient plants (dist = normal, link = identity). The covariance of the two repeated measures (dye source plants) in each experimental unit was modeled using the unstructured option in Proc Genmod.

Visitation data. *Silene caroliniana* observations were made of patches with five to ten plants per patch. Because insect visitation was frequent, the patches were observed for approximately 0.5 hours. During each observation the count of visits to each plant and the visitor species was recorded. Care was taken to keep the observations independent. Patches were not sampled consecutively, rather they were sampled at different times during the day, and the sampling occurred across the entire flowering season. The number of *S. caroliniana* patches (and total hours of observation) sampled were 10 (3.3), 13 (5.3), 45 (22.2), 48 (24.8), and 51 (25.5) for years 2003-2007, respectively.

Visitors to *S. virginica* and *S. stellata* flowers were observed with digital camcorders (Sony Digital Handycams: model #TRV17) for up to 2 hours (the maximum running time of the video tapes). Video cameras were used for *S. virginica* because hummingbird visitation rate was low and for *S. stellata* because direct continuous observations of nocturnal visitors with flashlights altered visitation. Plants were randomly chosen and the video cameras were focused on a single inflorescence. The inflorescence architecture and the field of view of the camera limited the number of flowers observed to a maximum of four and 12 flowers for *S. virginica* and *S. stellata*,

respectively. Visitor species, number of visits, and time and duration of visits were recorded. The same precautions were used to keep the observations independent as were used with *S. caroliniana*. Diurnal insect visitation rate to *S. stellata* was not measured in 2005 and 2006 as we judged the species to be specialized for nocturnal moth pollination (see results and discussion). We observed 86 *S. virginica* plants for a total of 344 hours in 2002. The number of *S. stellata* plants sampled (total hours of observation) was 58 (98), 23 (37), 51 (82), 18 (28), and 24 (36) for years 2002 - 2006, respectively.

Generalized linear mixed models were used to analyze the visitation datasets (Proc Glimmix in SAS). The number of visits was modeled as a Poisson response variable with log link function and the predictor variables were year, visitor and their interaction for *S. caroliniana*, visitor for *S. virginica*, and year and treatment and their interaction for *S. stellata* where treatment was nocturnal or diurnal visitor. The covariate, number of flowers per observation, was not used in the *S. caroliniana* or *S. stellata* models because in both cases the visitor and visitor by year interaction effect were not significant predictors of flower number. Therefore the visitor and visitor by year effects as sources of visitation rate variation were not confounded by the variation due to flower number. The random effect modeled was the residual error, which in Glimmix corrects for overdispersion of the Poisson response using the variance components covariance structure. Additionally, since multiple responses were measured within each experimental unit, e.g., the visitation of the different vector species, the repeatedly measured subject was modeled as the plant for *S. virginica* or *S. stellata*, and patch for *S. caroliniana*. The model was modified by specifying an offset variable, ln (number of plants*time (hr) for *S. caroliniana* or ln (time (hr)) for *S. virginica* and *S. stellata*. The

offset variable scales the count-type response data by the time of observation and the number of plants in each patch since mean visitation rate was the actual parameter of interest. If the main effect of treatment or visitor was significant, pairwise contrasts were performed to determine significant differences among the visitors. The family-wise error rate was held at the alpha = 0.05 level by using a sequential Bonferroni correction for type 1 error at each contrast. Sequential Bonferroni conservatively controls type one errors by testing the first contrast at the alpha level divided by the number of contrasts, and if the first contrast was significant, the second contrast at an alpha level by the remaining number of contrasts and so on.

Pollen removal and deposition. Pollen grain removal was measured on newly dehiscent male phase flowers. Insect visitation to flowers before the trials was prevented by caging study plants with metal screening. A trial consisted of removing two anthers prior to visitation, and the remaining anthers were used to estimate the amount of pollen in anthers following a visit, if one occurred. Upon removal, anthers were placed into microcentrifuge tubes with 200 μL of lactophenol with 0.1% aniline blue. The two anthers collected first were used to estimate standing crop of pollen and the anthers removed following the trial were used to estimate the amount of pollen in anthers following a visit, if one occurred. The difference between the amount of pollen before and after a visit equals the amount of pollen removed by the visitor, or if there was no visitor, the amount of pollen shed from anthers due to abiotic sources for the duration of the trial (e.g., physical handling, natural wind-driven shedding). During the trials the flowers were observed with video cameras for at most two hours, for *S. virginica*, or *S. stellata*, or by a human observer until a visit was noted for *S. caroliniana*, and the species

of visitor and number of visits was recorded. In the laboratory, ten replicate counts of pollen grains were made using hemacytometers under light microscopy at 40X power from each sample anthers before and after a visit, totaling 20 counts per flower. The counts of pollen grains were made on samples varying in anther number because the species differed in the number of anthers presented to pollinators at a given time. Therefore, to standardize the pollen removal data for comparison among species, the observed pollen counts were divided by the number of anthers per samples and the subsequent statistical models were performed on pollen grains per anther.

General linear mixed models (Proc Mixed) were used to model the response, pollen grains per anther, as a function of the predictors, visitor, treatment and their interaction for each species of *Silene.* For the *S. stellata* model the response was square root transformed to homogenize the variance of the predicted means corresponding to the treatment levels. The two levels of treatment are before or after a visit, or if no visit occurred, then the amount of pollen remaining on anthers at the end of the trial. The levels of visitor are the visitor types and control (no visit). The flower was treated as the experimental unit of observation because 20 repeated observations of pollen grains were made within each experimental unit. A compound symmetry correlation structure, which estimates a constant variance and constant covariance among the observations, was used to model the repeated measures. Additionally, the model was fit assuming the correlation structure differed within the two samples of anthers from each flower, before and after visitation. The *S. virginica* removal data were pooled across sites since the predictors site ($F = 2.43$, $DF = 2, 70$, $P = 0.0953$), site by treatment ($F = 0.51$, $DF = 2, 70$, $P = 0.6000$) or

site by treatment by visitor (F = 0.43, DF = 2, 70, P = 0.6496) had no significant effect on pollen grains per anther.

Pairwise contrasts were made for the *S. caroliniana* and *S. stellata* models to determine significant differences between the predicted means of combinations of levels of treatment and visitor. Sequential Bonferroni adjustment of type 1 error was used to ensure the family-wise error rate was held at 0.05. The predicted mean of the number of pollen grains per flower before a visit was used to estimate the standing crop of pollen in flowers of the three *Silene* species. Because *S. stellata* pollen dehisces at night, pollen may be shed with time in an unvisited flower, thus two pollen standing crops were estimated: (1) for flowers at night shortly after pollen dehiscence and (2) for flowers the morning following dehiscence and were compared using a Z-test for the difference between two large sample means. The number of measurements of the response, number of pollen grains per anther, and the number of experimental units was 2,200 across 117 flowers for *S. caroliniana*, 1,521 across 76 flowers for *S. virginica*, and 1,737 across 89 flowers for *S. stellata*. Too small a sample was collected of bee flies for *S. caroliniana*, syrphids and small bees for *S. virginica*, and bumble bees for *S. stellata* to estimate robustly pollen removal of these visitors.

Pollen deposition was quantified by removing stigmas following a visit by a pollinator and fixing the stigmas with fuschin glycerin jelly. The number of pollen grains on the three stigmas was then counted under light microscopy at 40x power. Unvisited stigmas were collected as controls to determine the background amount of pollen that falls on stigmas from sources other than pollinators, e.g. wind, handling. As an additional control for *S. stellata* and *S. caroliniana*, stigmas were collected from

randomly selected late stage female phase flowers to estimate the cumulative pollen grain deposition by all pollinators under unmanipulated conditions. The pollen deposition data were collected for three seasons (2004-2006) for *S. caroliniana* and *S. stellata* and in 2005 for *S. virginica*. Stigmas were collected for only nocturnal pollinators of *S. stellata* in 2005-2006. The sample size of flowers with stigmas was 168 for *S. caroliniana*, 97 for *S. virginica*, and 372 for *S. stellata*.

General linear models (Proc GLM) were used to model the response, pollen grains deposited per visit, as a function of the predictor, visitor type or control (no visit). The flower was treated as the experimental unit in the pollen grain deposition models. For the *S. caroliniana* and *S. virginica* models the square root of pollen grain deposition was used to control the residual variance among visitor groups. No transformation was necessary for the *S. stellata* pollen grain deposition model. The *S. caroliniana* data were not pooled among years. Although a GLM indicated a non-significant year effect ($F = 0.37$, $DF = 2, 293$, $P = 0.6906$), a year by visitor interaction effect ($F = 9.99$, $DF = 6, 293$, $P < 0.0001$) had a strong effect on the response, square root of pollen grains deposited per visit. Thus, data were analyzed only for the 2006 field season, when the sample sizes of both large bee and hawkmoth deposition was high. The *S. stellata* data were pooled across years as a GLM demonstrated year ($F = 0.29$, $DF = 2, 124$, $P = 0.7488$) was not a significant predictor of pollen grains on stigmas for the nocturnal pollinators. The *S. virginica* data were pooled across sites as a GLM demonstrated no significant site ($F = 0.04$, $DF = 2, 91$, $P = 0.9588$) or site by visitor effect ($F = 1.08$, $DF = 2, 91$, $P = 0.3455$) on square root of number of pollen grains deposited. For the *S. caroliniana* deposition model, pairwise contrasts were used to test hypotheses of differences among treatment

groups. The family-wise type 1 error was controlled by sequential Bonferroni correction. Orthogonal contrasts were used to determine if average pollen removal was significantly different among the visitor species and significantly different from control (no visitor) in the *S. stellata* model. Using the orthogonal contrasts insures a per contrast error rate held at the alpha = 0.05 level.

Chapter 3: Multi-year study of multivariate linear and nonlinear phenotypic selection on floral traits of hummingbird-pollinated *Silene virginica*

Pollination syndromes suggest that convergent evolution of floral traits reflects similar selection pressures. Interpreting flowers as suites of floral trait combinations that attract and maximize the pollen transfer efficiency of specific pollinators leads to the prediction that the contemporary signal of selection should be correlational and/or stabilizing. Furthermore, if directional selection is detected it should be oriented in directions consistent with floral character state differences of related species with different syndromes. We present evidence that Ruby-Throated hummingbird pollinators of *Silene virginica* select for floral traits in ways that are consistent with pollination syndrome differences compared to its sister species, *S. caroliniana*, and that stabilizing selection is prominent. We measured individual variation in six floral traits and yearly and lifetime total plant seed and fruit production of 758 plants across eight years of study in natural populations of the perennial, iteroparous *S. virginica*. Statistically significant directional selection gradients were rarely detected. When significant, positive directional selection was found to operate on floral display height and stigma exsertion and was in the direction predicted from floral trait differences of its sister species, bumblebee and hawkmoth pollinated *S. caroliniana*. By comparison, convex selection, estimated from canonical rotation of the matrix of correlational and quadratic selection gradients, was the most common form of curvature in the selection surface. By most indications contemporary selection by hummingbirds on floral traits and trait combinations is

stabilizing. Therefore, we found that intermediate floral variants with respect to attraction and pollen transfer efficiency are favored, and the pattern of directional selection was oriented in the direction indicated by floral trait differences away from its sister species, *S. caroliniana*, thereby confirming two central tenets of the pollination syndrome concept.

KEY WORDS: pollination syndrome, stabilizing selection, correlational selection, lifetime fitness, canonical analysis.

A major corollary of the pollination syndrome concept is that floral trait evolution occurs in response to selection by a plant's important pollinators. Past studies have found evidence of directional selection on floral traits influencing the efficiency of pollen transfer of major pollinators such as tube length (Maad 2000), corolla width (Campbell 1989), and nectary-stigma distance (Caruso et al. 2003), or features of attraction such as display height (Johnston 1991). Selection is often found to be context-dependent operating only in some years or populations (e.g., Caruso 2000; Caruso et al. 2003), or correlated with abiotic factors such as drought (Maad 2000), or biotic factors such as interspecific competition for pollinators that are independent of the putative evolutionary mechanisms thought to have generated the floral divergence (Caruso 2000). Few studies in the evolutionary ecology literature have demonstrated evidence of pollinator-mediated natural selection on floral traits corresponding to the predicted pattern based on measured trait variation across different pollination syndromes of closely related taxa (Fenster et al. 2004). More studies are needed to shed light on the role of pollinators as selective agents

promoting floral trait divergence among closely related species, which would address a central tenet of the pollination syndrome concept.

Viewed through the lens of pollination syndromes, flowers are complex multivariate structures that consist of suites of correlated characters increasing the attraction and pollen transfer efficiency of their major pollinators (Darwin 1862; Stebbins 1951). If flowers are adaptations to the best pollinator environment, then we might expect particular floral character states that maximize attraction and efficient pollen transfer of specific pollinators. Thus, stabilizing selection on flowers is the expected contemporary signal from selection studies rather than directional selection (Fenster et al. 2004). However, quantitative genetic theory predicts generations of enforced stabilizing selection should decrease genetic and phenotypic variation for floral traits (Lynch and Walsh 1998). Therefore, the selective surface may be broad in the region of the optimum phenotype making it very difficult to detect stabilizing selection, even if it exists. In fact, when stabilizing selection is found in phenotypic selection studies it is usually weak (Kingsolver et al. 2001; Blows and Brooks 2003). It is not surprising then that experimental manipulation may be required to convincingly demonstrate stabilizing selection (Cresswell 2000) or nonadditive selection on floral trait combinations (Fenster et al. 2006).

Since pollination syndromes are suites of characters and character combinations that are organized and associated with particular pollinators (Vogel 1954, 1996, 2006; Faegri and Van der Pijl 1979; Fenster et al. 2004), correlational selection may be common. Correlational selection is selection on the positive or negative correlation of pairs of floral traits indicating certain floral character combinations are favored over

others. For example, if selection is acting on the positive association of petal length and petal width, then wide and long petals or narrow and short petals are the favored floral character combinations. Correlational selection differs from selection on a single character that is phenotypically and/or genetically correlated with another character. If many traits are measured, as many often are, correlational selection has the potential to mask the pattern of quadratic selection indicating stabilizing or disruptive selection on floral traits in unexpected ways (Blows and Brooks 2003). Phillips and Arnold (1989) and later elaborated on by Blows and Brooks (2003) and Blows (2007), have indicated the most informative and efficient way to detect nonlinear selection (*sensu stricto* Phillips and Arnold 1989), in multivariate phenotypic selection studies is to conduct a canonical transformation of the matrix describing correlational and quadratic selection in order to detect curvature in the selection surface. Canonical analysis of the matrix of quadratic and correlational selection gradients is potentially a powerful tool in studies of phenotypic selection on floral traits because, rather than making *ad hoc* explanations for each correlational selection gradient, the question can be distilled to whether nonlinear selection is occurring on latent axes describing the joint action of selection on the original floral traits. A convex relationship implies that selection is acting to decrease the variance of linear floral trait combinations, e.g., stabilizing selection, while a concave relationship indicates that selection is acting to increase that variance, e.g., disruptive selection. We apply this approach here in our investigations of selection on *S. virginica* floral traits.

It is reasonable to assume that hummingbirds are sources of selection on *S. virginica* floral traits. First, hummingbirds are the most common visitors (about 2 plant

visits/ day versus < 1 plant visits/ day for small bee and fly visitors), they deposit six times the number of pollen grains as ovules per visit (Reynolds et al. *in review,* chapter 2), and not surprisingly plants are not pollen limited for seed production, only fruit production (Dudash and Fenster 1997). Furthermore, in the absence of hummingbird pollination, fruit and seed production are significantly lower relative to open pollinated plants (Fenster and Dudash 2001). The pollination data and experimental manipulations strongly suggest hummingbirds are the most important pollinators (Reynolds et al. *in review,* chapter 2). Second, published (Fenster et al. 2006) and unpublished studies have demonstrated that hummingbirds exhibit preferences among manipulated floral phenotypes and plant display attributes.

Because selection operates within generations for iteroparous plant species such as *S. virginica*, lifetime fitness data are needed to quantify phenotypic selection. We present approximate estimates of lifetime fitness by integrating the combined effects of selection across multiple flowering seasons for the hummingbird-pollinated, perennial wildflower species, *S. virginica*. Few studies have documented selection through lifetime female reproductive success on an iteroparous plant species (but see Herrera 1993). Here we present the results of a long-term phenotypic selection study on floral traits of hummingbird-pollinated *S. virginica* (Caryophyllaceae), a perennial, iteroparous wildflower of eastern North America. A novel aspect of the study is that linear and nonlinear selection on floral traits was estimated using canonical analysis in eight separate years, and additional estimates were made using lifetime seed and fruit production of two pedigreed cohorts. The following two major questions were addressed. 1) Can we detect a contemporary pattern of selection that is oriented in directions

consistent with differences in the pattern of measured trait variation relative to its sister species *S. caroliniana* and 2) Can we detect multivariate nonlinear selection on the suite of traits comprising the species pollination syndrome?

Materials and Methods

STUDY SYSTEM

Silene virginica is a short-lived herbaceous perennial wildflower. Seeds germinate in the early spring, and plants over-winter as rosettes of basal leaves, growing a minimum of 2-3 years prior to flowering. In April - May of the following year plants may produce one to several reproductive stems each holding one to several flowers, which bloom from late May to early July. The flowers are protandrous with a male phase lasting two days (five new dehiscent anthers each day, exserted beyond the corolla tube opening), followed by a non-sexual phase with elongating style, and ending in a female phase with receptive stigmas exserted well beyond the corolla tube opening. Male and female flowers may occupy the same inflorescence, but the incidence of geitonogamous pollinations is low as *S. virginica* is highly outcrossing (Dudash and Fenster 2001). The flowers are red, with long corolla tubes formed from unfused petals, and they provide a dilute and copius nectar reward (Fenster et al 2006; Reynolds et al. *in review*, chapter 2). These characters correspond to the hummingbird pollination syndrome, and differ from the closely related nocturnal moth-pollinated, *S. stellata* and *S. virginica*'s sister species, the hawkmoth and large bee-pollinated *S. caroliniana* (Reynolds and Fenster 2008; Reynolds et al. *in review*, chapter 2). Flowers do not autonomously set seed and vegetative reproduction has not been observed. A study of the demography of the population is on-going but we

know from monitoring hundreds of plants across many years that all plant stage class transitions are possible except for germination to flowering. After germination the juvenile plants become non-reproductive, non-reproductives may become reproductive, reproductives may become non-reproductive, or reproduce again. Multiple reproductive episodes are possible, although two or fewer reproductive bouts before death are most common.

STUDY SITE AND DESIGN

The study was performed near Mountain Lake Biological Station (Giles County, Virginia) at one site (elev~1,100 m, 80°33'14"W, 37°21'20"N) during two separate periods of four (1992-1995) and five (2003-2007) consecutive years. The site is located in a mixed oak-hickory forest with heterogeneous light environment due to tree falls and gaps in the canopy, and it is on a steep grade on Bean Field Mountain, adjacent to Salt Pond Mountain. Naturally occurring *S. virginica* is common at the site. Three types of designs were used to quantify phenotypic selection on floral traits. The first consisted of flowering plants randomly selected along transects. The second and third consisted of maternal sibships or paternal half sibs, which will also be used for estimating floral trait genetic variance and covariance (Reynolds et al. in prep).

For design 1 all flowering plants in each year were marked along two 20 m wide and 100 m long parallel transects. Thus, plants in each year of study are the number of individuals flowering in a 0.2 hectare area of forest. Because they are perennial and iteroparous, plants found flowering in any given year may or may not flower in any or all subsequent years. In all 443 individuals were marked and measured: 261 flowered once,

138 flowered twice, 38 flowered three times, and 6 flowered every year. The study was primarily cross-sectional as many new plants were added each year, but there was a longitudinal component as plant measurements were taken on plants flowering in multiple years. Plants marked in any given year may have reproduced prior to the beginning of the study. Therefore, estimates of selection were made in each year, but estimates using maternal fitness of individuals pooled across multiple flowering episodes were not attempted.

For design 2 plants were grown from seed collected from individual plants in their natural population in summer 2001. They were cold stratified and then germinated in spring 2002 on standard greenhouse soil (Sunshine HCI, Sun Gro Horticulture). In the greenhouse, seedlings grew individually through the summer under natural light, were watered as needed, and were transplanted back into their home site in fall 2002. In all 180 individuals in this maternal sib design were planted with one meter spacing into 3 blocks of 60 plants with each block consisting of 6 rows and 10 columns. The plants originated from 43 maternal source plants. The maternal sibs from each of the 43 maternal families were randomly assigned to the three blocks and then they were randomized to position within the block. Flowering began in May 2003.

For design 3 plants were grown from seed as in design 2, but some plants were kept in the greenhouse and allowed to flower in May 2002. Individual seedlings were transplanted into 6" pots, and randomly placed onto greenhouse benches. During this period 43 plants flowered and hand-pollinations were conducted to generate paternal half sibships, maternal half sibships and full sibships using a partial circulant diallel design (Kempthorne and Curnow 1961). Seed were collected, cold-stratified in fall 2003 and

germinated in 2004 as described for design 2. In all 38 paternal families sired at least eight offspring from matings with four dams. Within each paternal family there were 4 half sibs, with each half sib replicated twice representing full sibships. The offspring from the 38 paternal families were planted in their home field environment during early June 2004 as seedlings, and they flowered for the first time in summer 2005. Seedlings were planted in eight blocks of 40 plants each, arranged in 5 rows and eight columns separated by one meter. Single individuals of each of the 38 paternal families were randomly positioned in each block and two additional seedlings randomly selected from two of the 38 paternal families were planted to fill the remaining positions in the block.

DATA COLLECTION

Because we know that hummingbirds are the most important pollinator of *S. virginica* (Fenster and Dudash 2001; Reynolds et al. *in review*, chapter 2) we quantified phenotypic selection on traits that differ between *S. virginica* and its closely related non-hummingbird-pollinated *Silene* species. Consequently, phenotypic selection analyses were made on the following floral traits presumed to be associated with hummingbird pollinator attraction (e.g., Johnston 1991; Fenster et al. 2006): petal length, petal width, and flower height above the ground; or efficiency of pollen transfer (e.g., Campbell 1989): corolla tube length, corolla tube width, and exsertion of the stigma. The morphological traits were measured with dial calipers to 0.1 mm. Height of the flower above the ground (DHT) was measured with a meter ruler to 1 cm. Corolla tube length (TL) was measured from the base of calyx to the tube opening. Corolla tube width (TD) was measured as the widest length across the corolla tube opening. Petal length (PL) was

measured as the length of the largest of five petal limbs, which spread outward perpendicularly from the corolla tube. Petal width (PW) was measured as the widest portion of the petal measured for its length.

Plants were monitored daily during flowering. When a new flower was noted, it was marked with a jewelers tag, and floral trait measurements were taken. When flowers became female the nectary-stigma distance was measured from the base of the flower tube to the end of the stigma. Stigma exsertion (SE) was taken as the difference between the nectary-stigma distance and the corolla tube length. This process was repeated throughout the flowering period until all flowers were marked and measured on the plants. To reduce the potential for systematic bias in floral measurements, multiple investigators took measurements each day and the same person did not measure plants of the same block on consecutive days. All fruits were collected when seed matured and the fruit dehisced, about 18 days following female phase, and were stored for processing in the laboratory. After fruit collection ended, the vegetative characters, number of bolting stems, stem length, and the length and width of the largest basal leaves were measured. These measurements were used as covariates to account for plant vigor as a possible environmentally induced factor of floral trait and fitness covariation. In the laboratory, number of fruits and the number of seeds per fruit were counted, and the incidence of noctuid seed predation was noted using a dissecting scope.

STATISTICAL ANALYSES

We studied selection on plants in eight separate flowering years, and each year two maternal fitness components (total fruit-set, total seed production) were measured. Thus,

16 statistical models were used to analyze the linear phenotypic selection on floral traits. To obtain nonlinear selection estimates, 16 additional models were run for the yearly analyses. Floral trait values were averaged and fruit and seed production was summed across female flowers within plants for phenotypic selection analyses at the individual plant level. Our original intention was to perform genotypic selection analysis (Rausher 1992) as a way to control for environmental sources of covariation between traits and fitness. Ultimately, the small sample size of 43 maternal families and 38 paternal half-sib families precluded the detection of significant selection gradients. Thus, all analyses presented henceforth are based on phenotypic analyses using individuals as the level of replication. While genotypic selection analysis is preferred, we are confident that we were able to factor out environmental sources of covariation by using plant vigor and block as covariates (see below).

To obtain standardized selection gradients, floral trait values were z-transformed. Fitness data was scaled by mean fitness of all plants in the year of analysis. As a maternal fitness component, flower production correlated strongly with fruit and seed production, and it was correlated with vegetative characters. Thus, number of flowers per plant was used as a covariate in the analyses, and the direct effects of selection on floral traits were analyzed holding plant vigor constant. The possibility of an attractiveness component to floral display beyond a simple linear increase in fruit production was addressed by modeling fruit production as a second order polynomial regression on flower number for all eight years of study. Fruit production was never found to increase nonlinearly with flower number (analysis not shown). We used the false discovery rate (FDR) approach of Benjamini and Hochberg (1992) to control the proportion of type 1

errors at Q = 0.05 for the eight replicate measurements of selection on each fitness component and floral trait performed for each year of study. The FDR approach was used to guard against the possibility in each model of failing to reject the null hypothesis of no selection when in fact there was significant selection on a floral trait, by tolerating a slight increase in erroneously finding a signal of selection, when there was none. Additionally, a Q = 0.10 threshold was used to determine if more traits would become significant,or if the traits significant at the Q = 0.05 level would be significant more often.

The above analysis was repeated again using only the plants of the two studies that flowered between 2002 and 2007. The weighted average of floral traits was taken across years for individual plants, weighting by the number of flowers produced each year. Maternal fitness components were summed across years for each individual and then scaled by the mean fitness among the plants of each study. Linear and nonlinear selection was estimated for the 2002 and 2004 cohorts using seed and fruit production as proxies for lifetime fitness. Because the probability of at least one type one error for performing two replicate selection analyses on each trait and fitness component of the 2002 and 2004 *S. virginica* cohorts increases to 0.0975 the Bonferroni corrected alpha level of 0.025 was used for the lifetime fitness models.

Linear and nonlinear selection gradients were estimated using two approaches. First, a general linear model was fit to obtain estimates of the vector of linear selection gradients and the matrix of quadratic and correlational selection gradients. Second, we used the approach outlined by Phillips and Arnold (1989) and Simms (1990) and more recently fully described by Blows

(2007) in following the original models with a canonical analysis of the matrix of standardized quadratic and correlational selection gradients. Essentially, the canonical analysis calculates latent axes in multivariate space from the diagonalized, symmetric matrix of quadratic and correlational selection gradients. The resulting eigenvalues represent the strength of nonlinear selection on the new orthogonal axes and the eigenvectors explain the degree to which the combination of original traits is related to the new latent axes. The method increases the power to detect nonlinear selection by reorganizing the pattern of correlational and quadratic selection into a new summary statistic describing curvature along the new axes (Blows and Brooks 2003). It is particularly relevant here for two reasons. First, the traits were measured from our reference frame, and it is difficult to predict in the absence of an experimental approach exactly which traits and trait combinations are important for hummingbird attraction and pollen transfer efficiency. Second, natural selection acts on the total phenotype, thus adaptations are inherently multivariate, and so is the composition of pollination syndromes. With six traits we have 15 correlational selection gradients to test, but we have no a priori hypothesis as to which of the 15 are most important. However, we do expect stabilizing selection to be strong as extreme floral variants might be expected to alter the fit of the pollinator and flower, the efficiency of pollen transfer, and successful fruit and seed production. Therefore, it seems appropriate to investigate nonlinear selection on linear combinations of traits.

The models we used were the following and were analyzed using the GLM and RSREG procedures in SAS v. 9.1.2 (SAS 2004). We first fit models 1 & 2 to estimate

the directional and then quadratic and correlational selection gradients, respectively (Lande and Arnold 1983; Phillips and Arnold 1989; Blows and Brooks 2003),

$$w_i / \overline{w} = \alpha + \eta f + \sum_{j=1}^{6} z_{ij}\beta_j + \varepsilon \quad (1)$$

$$w_i / \overline{w} = \alpha + \eta f + \sum_{j=1}^{6} z_{ij}\beta_j + 1/2\sum_{j=1}^{6} z_{ij}^2\gamma_j + \sum_{j=1}^{6}\sum_{k=1}^{6}\gamma_{jk} z_j z_k (j \neq k) + \varepsilon \quad (2)$$

where w is the measured fitness component, $\overline{w}$ the mean fitness, α the intercept, f the covariate flowers, z_j the jth floral trait, η, β, γ, the parameters estimated by least squares for the covariate, linear and nonlinear coefficients of selection, respectively. Equation (2) can be reformulated in matrix notation as,

$$w = \alpha + \eta + z'\beta + z'\gamma z + \varepsilon \quad (3)$$

and then following Phillips and Arnold (1989) the matrix of quadratic and correlational estimates, $\boldsymbol{\gamma}$, was diagonalized,

$$M\gamma M' = \Lambda \quad (4)$$

where $\boldsymbol{M}$ is the 6 x 6 matrix of eigenvectors and $\boldsymbol{\Lambda}$ is the diagonal matrix containing the eigenvalues from the canonical rotation. The significance of the resulting eigenvalues was determined by transforming the original data matrix z according to the matrix M,

$$y = zM' \quad (5)$$

and then the transformed data were analyzed with a similar model as equation (3) (Bisgaard and Ankenman 1996). The new model may be written as,

$$w = \alpha + y'\theta + y'\Lambda y \quad (6)$$

The parameters estimated in equation 6 describe the direction and magnitude of linear and nonlinear selection acting on the new orthogonal axes of the selection surface.

Here we use the terminology of Phillips and Arnold (1989). For example, if all the eigenvalues are negative/positive then selection is convex/convave and nonlinear selection is acting on major axes of the selection surface. If there is a mix of negative and positive estimates then the surface describes a saddle. If there is an intermediate peak or valley in the range of measured data then we may speak of stabilizing or disruptive selection, which are special cases of convex/concave selection (Phillips and Arnold 1989).

The models were also run with transect (1992-1995) or block (2003-2006) as covariates. The block effect was significant in one of the 16 models of yearly selection (seed production, 2005) and in none of the four models of selection on lifetime fitness. In the one case the signs of the selection gradients remained the same for all characters, and for only display height did the magnitude of selection increase causing it to become significant. Therefore, due to the almost exclusively null effect of block on fitness variation, the models were run without block.

To visualize the pattern of selection realized by the canonical analyses, thin plate spline analyses were performed using TPSPLINE in SAS. The response variable was lifetime relative seed production for the 2002 and 2004 cohorts (fruit production was not significant in the 2004 cohort) and the predictors were axes with significant nonlinear selection. The data corresponding to these axes were the raw data transformed into the space of the respective eigenvectors as indicated in equation 5.

Mitchell-Olds and Shaw (1987) emphasized that the limitation of the selection models is that phenotypic correlations among characters can affect significance testing in serious ways. Also, unmeasured characters that are phenotypically correlated and covary

with fitness will bias the estimates. Lande and Arnold (1983) suggested performing selection analyses on the principal components representing the phenotypic covariance matrix when the characters are highly correlated indicating linear dependence. We felt this approach was unwarranted as we never observed a pairwise floral trait correlation above 0.51 and the median correlation for the floral traits was never higher than 0.2. We acknowledge that unmeasured traits, if measured, could change the estimated selection surface. However, our choice of traits reflected our biological intuition of their adaptive significance in terms of attraction; reward and efficient pollen transfer and the experimental manipulation of these traits do have an effect on attraction of hummingbirds and efficient pollen transfer (Fenster, Reynolds and Dudash, unpublished). In addition, a consistent pattern of directional and correlational selection was detected in different years and different studies. Thus we are confident that the selection surfaces generated by our analyses have biological meaning.

Results

YEARLY ANALYSES

Linear Selection

The means, standard deviations and sample sizes for *S. virginica* plants of the yearly studies are reported in Appendix 1. Significant (false discovery rate, FDR = 0.05) positive directional selection gradients through fruit production were detected on stigma exsertion in 1993 and 2005 and display height in 1992 and 1995 (Appendix 2). Controlling the (FDR) at Q = 0.10 resulted in additional significant positive directional

selection gradient estimates on stigma exsertion in 1995, 2003, and 2004 and significant negative directional selection on petal length in 2005. Significant positive directional selection gradients were detected through seed production on display height, after controlling the FDR at Q = 0.05, in 1992 and 1995 (Appendix 3), and after controlling the FDR at Q = 0.10, in 2004.

Nonlinear Selection

No significant selection gradients were detected in any year after controlling the FDR at Q = 0.05 or Q = 0.10 for any floral traits from the second order polynomial model. However viewing the results per table yielded numerous significant quadratic and correlational selection gradients below the alpha = 0.05 level (Appendices 2 and 3).

Canonical analysis produced a number of significant nonlinear selection estimates indicating multivariate stabilizing or disruptive selection was acting on floral trait combinations. The eigenvalues and eigenvectors are reported in Appendices 4 and 5. Through fruit production, nonlinear convex selection was detected on one latent axis in 1992, 1995, and 2003-2006, and two latent axes in 1993 and 1994 (Appendix 4). Nonlinear concave selection was detected on one axis in 1995, 2003, and 2004 (Appendix 4). Through seed production, convex selection was detected on one latent axis in 1992, 1993, 1995, and 2006, and two axes in 1994 and 2005 (Appendix 5). Nonlinear concave selection was detected on one axis in 1995, 2003, and 2006 (Appendix 5).

SELECTION THROUGH LIFETIME MATERNAL FITNESS COMPONENTS

There was a decline in survivorship and probability of flowering for plants in the 2002 and 2004 cohorts (Figure 1). By the end of the study (2007) the probability of flowering was 0.02 for the 2002 cohort and 0.18 for the 2004 cohort, indicating that the combined fruit and seed production of individual plants is a close approximation to lifetime maternal fitness components.

Linear Selection

The means and the standard deviations and sample sizes for *S. virginica* plants of the cohort studies are reported in Table 1. Significant positive linear selection was detected on display height through fruit production using the 2002 cohort (Table 2) and through seed production for the 2004 cohort (Table 3).

Nonlinear selection

Significant nonlinear selection was detected on the negative correlation between petal length and petal width through fruit production in the 2002 cohort (Table 2) and on the positive correlation between petal length and petal width through seed production in the 2004 cohort after Bonferroni correction (Table 3). Viewing the results per table, at the alpha = 0.05 level, yielded significant stabilizing selection on corolla tube diameter through fruit and seed production in the 2002 cohort (Tables 2 & 3).

Canonical analysis produced a number of significant nonlinear selection estimates indicating multivariate stabilizing or disruptive selection was acting on latent axes representing selection on combinations of floral traits. Significant concave selection was detected on one latent axis and convex selection was detected on another axis through

fruit and seed production in the 2002 cohort (Table 4 & 5; Figure 2). Significant convex selection on two axes was detected using the plants of the 2004 cohort, but only through seed production (Table 5; Figure 3).

Discussion

We have demonstrated significant linear and nonlinear phenotypic selection on *S. virginica* floral traits with datasets collected across 8 years. Natural selection on floral traits in *S. virginica* could come from non-pollinator sources such as herbivores (Strauss and Irwin 2004) and environmentally and physiologically induced variation could cause floral trait (Galen 1999) or fitness variation. In a four year study of selection through cumulative seed production in the perennial violet, *Viola cazorlensis*, soil substrate was a much stronger predictor of fitness variation than floral traits (Herrera 1993). However, because Ruby-throated hummingbirds are the most important pollinators of *S. virginica* (Fenster and Dudash 2001, Reynolds et al. *in review*, chapter 2) and are known to prefer particular floral trait combinations in experimental trials (e.g., Fenster et al. 2006), we attribute the pattern of selection generated to pollination. The pattern of linear selection observed on *S. virginica* floral traits was consistent with predictions based on floral trait variation corresponding to pollination syndrome differences with the closely related large bee and hawkmoth-pollinated *S. caroliniana*. Furthermore, multivariate stabilizing selection was acting on major axes of the selective surface, each axis jointly associated with several floral traits. Therefore, we detected a contemporary pattern of linear and nonlinear selection that suggests hummingbirds were a major selective force underlying *S. virginica* floral evolution.

LINEAR SELECTION

If hummingbird-mediated phenotypic selection is a plausible evolutionary mechanism for the origin and/or maintenance of floral trait divergence of *S. virginica* from its sister species (*S. caroliniana*) then the pattern of selection revealed by our analyses should parallel the direction of floral trait differences among the taxa. We found evidence of directional selection for flowers held high above the ground. Directional selection on display height (2 of 8 years) was detected in the yearly analyses through fruit production and seed production, and through fruit production in cohort 2002 and seed production in cohort 2004. By comparison, closely related *S. caroliniana* holds its flowers near the ground. It is remarkable that we were able to detect a contemporary microevolutionary signal of selection on display height for *S. virginica*, which was in a similar direction to the pattern of interspecific variation in floral display height between *S. virginica* and *S. caroliniana.* This correlative evidence for hummingbird preference for plants with flowers held high above the ground corroborates evidence from *Lobelia* (Johnston 1991), in which directional hummingbird-mediated selection was found for *L. cardinalis* plants with flowers held high, but not for the bee-pollinated congener, *L. siphilitica*. Experimental preference trials and examining the covariance of bee or hummingbird pollination and display height in genera with multiple independent origins of hummingbird pollination such as *Penstemon* would be fruitful avenues of research to study if the microevolutionary process of pollinator-mediated selection scales up to macroevolutionary patterns of trait variation among closely related taxa.

In addition to display height, stigma exsertion is another trait with significant positive directional selection in two of eight years. This trait should be associated with pollen transfer efficiency in that higher stigma exsertion would increase the probability that pollen is transferred from hummingbirds via the stigma contacting the forehead, where pollen is deposited. This trait is also much more exserted than the much less exserted character state of its close relative *S. caroliniana* again suggesting that positive directional selection by hummingbirds has and continues to be a mechanism maintaining the divergence between the two related species with differing pollination/floral syndromes.

NONLINEAR SELECTION

Flowers as adaptations to their most important pollinators may represent the optimal contemporary evolutionary solution. In this scenario, floral variants attracting and maximizing pollen transfer by important pollinators with subsequent successful plant reproduction are favored. Our experimental manipulations have revealed that floral traits are selected in a non-additive way (Fenster et al. 2006; Fenster et al. unpublished). Therefore, we expect plants in natural populations should most likely be under nonlinear selection including stabilizing or correlational selection. Thus, it may not be surprising that directional selection on *S. virginica* floral traits was infrequently detected because in general, finding any directional selection on floral traits may be unexpected if the traits are presumed to be at an optimum phenotype. Berg (1960) hypothesized and found support for the concept that the traits that compose flowers, and are *a priori* expected to be associated with specialized pollination, should be genetically and phenotypically

integrated for attracting pollinators. The covariance of floral traits should be distinct from vegetative traits, which have little to do with the fit of flowers and pollinators, and plant reproduction. Her ideas about flowers as tightly coupled multivariate phenotypes may not be completely generalizable (Armbruster et al. 1999), but they do suggest we should find evidence for nonlinear selection on flowers by pollinators, including correlational and stabilizing selection. It is possible that floral ecologists and evolutionists rarely detect a signal of stabilizing or optimizing selection on flowers because trait variance is low (Cresswell 2000), or the pattern of nonlinear selection is not quadratic, but correlational. Kingsolver (2001) found that nonlinear selection, if reported at all, was weak compared to directional selection.

Evidence of correlational selection using natural phenotypic variation in floral traits is very limited. Using a large sample of plants, O'Connell and Johnston (1998) found evidence of negative correlational selection acting though male and female reproductive success in pink lady slipper orchid populations, which was interactively related to the attraction and pollen transfer efficiency of queen bumble bees. Correlational selection may be related to attracting bees to tall flowers, but the increased success was only seen when combined with smaller labellums, which might be necessary for efficient pollen transfer. Maad (2000) in a study of hawkmoth-pollinated *Platanthera bifolia* also found selection on the negative correlation between flower number and plant height in a single year, but it was attributed to physiological trade-offs due to drought and not to pollinator-mediated selection. In our study, correlational selection on pairs of individual traits was uniformly weak. The yearly analyses demonstrated several instances where selection was acting on the correlation between floral traits through fruit

production and seed production, but after controlling the FDR level at 0.05, they were not significant. Significant selection was detected on the negative correlation between petal length and petal width through fruit production and the positive correlation between petal length and width through seed production from the 2002 cohort analysis.

As the number of traits measured increases from 3 as in O'Connell and Johnston's (1998) study to 6, the number of correlational terms to estimate and interpret increases from 3 to 15. Blows and Brooks (2003) argue that nonlinear selection is underestimated since it often is correlative and oriented in directions away from the original measured traits. With many traits the problem of correlational selection manifesting in predictable ways is a difficult one, but the canonical approach has proved to be a good solution (Simms 1990; Blows and Brooks 2003). Results from our canonical analysis demonstrated that flowers were under nonlinear selection by pollinators and in the majority of cases it was stabilizing. In most cases the significant eigenvalues were negative, which indicated convex or stabilizing selection along latent axes of the selection surface. Consistent with the findings of Blows and Brooks (2003), performing the canonical rotation of the gamma matrix increased the ability to find evidence for nonlinear selection. Less support for the role of concave or disruptive selection on the latent axes of the selection surface was found.

What these new axes represent in terms of the original traits has probably been the major stumbling block to the widespread use of the canonical approach in selection analyses (Blows 2007). Multivariate stabilizing selection was detected on the flowers of the 2004 cohort through lifetime seed production with two latent axes having negative eigenvalues. The matrix of eigenvectors indicated petal length and width were associated

with the first axis and tube diameter and stigma exsertion with the second axis. The pattern of multivariate stabilizing selection operating on the cohort from 2004 was associated with floral traits of both the attraction (axis M1) and pollen transfer efficiency (axis M2) components of pollination syndromes. This result is therefore evidence that stabilizing selection is operating to favor intermediate floral phenotypes over extreme variants. In addition, stabilizing selection occurred on axes with joint associations with floral traits. The finding that hummingbirds select for *S. virginica* floral trait combinations supports the pollination syndrome concept, namely that particular flower morphologies consist of unique trait combinations owing to selection by particular animal pollinators.

It seems intuitive that the absence of directional selection on *S. virginica* floral traits could be attributed to lack of pollen limitation for the measured maternal fitness components (Dudash and Fenster 1997), which suggests selection may be more likely through male reproductive success (Wilson et al. 1994) instead of female. For example Wright and Meagher (2004) found significant selection on *S. latifolia* floral traits through male function but not female. However, no pollen limitation does not preclude the detection of maternal fitness and trait covariation, which means that fitness variation is organized with respect to variance in the trait. No pollen limitation suggests that the variance of maternal fitness is limited which would only decrease the opportunity to detect selection on floral traits through female reproductive success (Arnold and Wade 1984). Perhaps nonlinear selection was detected through seed production but not fruit production in the 2004 cohort because the variance in seed production was nearly 50 times greater. Also directional selection was found through fruit production in the 2002

cohort, but not the 2004 cohort. The latter had half the variance as the 2002 cohort. Future study of selection through male function are needed to determine if the pattern of selection differs through male or female function.

The long term consequence of consistent multivariate stabilizing selection is to reduce the genetic variance for those traits under selection (Johnson and Barton 2005). However, it is unusual not to find genetic variance for single traits (Lynch and Walsh 1998). What maintains genetic variance for these traits under stabilizing selection is an active area of theoretical research, but much of the disconnect may involve unsuitable analytical methods to detect selection and genetic variance on complex multivariate phenotypes (Brooks et al. 2005; Chenowith and Blows 2006; Hunt et al. 2007). Hunt et al. (2007), in an empirical analysis of multivariate stabilizing selection and genetic variance for male cricket call properties, generally found less genetic variation for those trait combinations that comprise the latent axes under stabilizing selection. Measuring additive genetic variance for the major axes representing linear combinations of *S. virginica* floral traits (Reynolds et al. *in prep*) combined with estimates of selection presented here would provide more empirical data to address the paradox of ample genetic variance and strong stabilizing selection (Blows and Hoffman 2005).

Table 1. Means (SD) of the fitness data and floral traits (mm) across years of study for two cohorts of *Silene virginica* plants, the first planted in 2002 and flowering from 2003-2007 and for the other planted in 2004 and flowering from 2005-2007.

	Flowers	Fruit	Seed	Corolla tube length	Petal length	Petal width	Corolla tube diameter	Stigma exsertion	Display height
2002 cohort (N = 115)	10.4 (11.1)	4.5 (4.6)	175 (182)	23.8 (1.46)	18.4 (1.92)	6.02 (0.710)	3.49 (0.392)	7.71 (1.88)	313 (74.3)
2004 cohort (N = 250)	5.2 (4.6)	2.8 (2.7)	101 (99)	23.9 (1.81)	18.9 (2.15)	6.15 (0.775)	3.16 (0.448)	6.95 (1.83)	307 (67.3)

Table 2. The vector of standardized selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) estimated using fruit production as the lifetime fitness component for *Silene virginica*. Estimates in bold were significant after Bonferroni correction of type 1 error for the two models run for each cohort. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	β	γ					
		TL	PL	PW	TD	SE	DHT
2002 cohort							
Corolla tube length (TL)	0.0536	-0.00604					
Petal length (PL)	-0.0007	-0.0482	0.00114				
Petal width (PW)	0.000488	0.0106	**-0.212***	0.0816			
Corolla tube diameter (TD)	0.0469	-0.0884	0.129	0.0114	-0.131*		
Stigma exsertion (SE)	0.107	-0.0236	0.0995	-0.0449	0.111	-0.0239	
Display height (DHT)	**0.144***	-0.0344	0.0495	-0.0256	0.00145	0.105	0.0295
2004 cohort							
Corolla tube length (TL)	0.00801	-0.00145					
Petal length (PL)	-0.0162	0.00475	0.0193				

	β	γ					
		TL	PL	PW	TD	SE	DHT
Petal width (PW)	0.0179	0.0235	0.0603	-0.0370			
Corolla tube diameter (TD)	-0.0227	-0.0256	0.00188	0.0142	-0.0142		
Stigma exsertion (SE)	0.00205	-0.0644	0.0202	-0.0298	0.00732	-0.0307	
Display height (DHT)	0.0616	-0.00661	-0.00069	-0.0201	-0.00283	0.00436	0.00635

Table 3. The vector of standardized selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) estimated using seed production as the lifetime fitness component for *Silene virginica*. Estimates in bold were significant after Bonferroni correction of type 1 error for the two models run for each cohort. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	β	γ					
		TL	PL	PW	TD	SE	DHT
2002 cohort							
Corolla tube length (TL)	0.0694	-0.00095					
Petal length (PL)	0.0456	-0.0253	0.0219				
Petal width (PW)	0.0248	0.00356	-0.214 *	0.0611			
Corolla tube diameter (TD)	0.0711	-0.0900	0.112	0.120	-0.162 *		
Stigma exsertion (SE)	0.0513	-0.00518	0.0905	-0.0506	0.0797	-0.05585	
Display height (DHT)	0.117	-0.0610	0.127	-0.0379	0.0579	-0.0306	-0.0204
2004 cohort							
Corolla tube length (TL)	0.0214	-0.0101					
Petal length (PL)	-0.0185	0.0168	-0.0099				

	β	γ					
		TL	PL	PW	TD	SE	DHT
Petal width (PW)	0.0765	-0.0685	**0.138** *	-0.0302			
Corolla tube diameter (TD)	-0.0618	0.115 *	-0.0331	0.00351	-0.0252		
Stigma exsertion (SE)	-0.0506	-0.0450	0.0614	-0.0432	-0.0446	-0.0253	
Display height (DHT)	**0.0837 ***	-0.0394	-0.00328	-0.0255	0.0243	0.00104	0.0237

Table 4. The M matrix of eigenvectors from the canonical rotation of γ containing the quadratic and correlational estimates of selection using fruit production as the lifetime fitness component for *Silene virginica*. In the last two columns are the estimates of linear (θ) and nonlinear selection (λ) on the new latent axes described by the eigenvectors. The nonlinear selection gradient is the eigenvalue of each eigenvector. * P < 0.05; ** P < 0.01; *** P < 0.001.

	TL	PL	PW	TD	SE	DHT	λ	θ
2002 cohort								
M1	0.144	-0.371	-0.320	0.831	-0.207	0.0765	-0.202**	0.139
M2	0.191	0.592	0.252	0.135	-0.687	0.243	-0.0846	-0.0310
M3	0.736	0.285	0.191	0.215	0.462	-0.285	-0.0277	0.0956
M4	0.505	-0.310	-0.100	-0.325	0.0300	0.730	0.0106	0.0809
M5	-0.350	0.0681	0.566	0.367	0.416	0.495	0.0748	0.147 *
M6	-0.155	0.574	-0.683	0.0720	0.313	0.277	0.181 **	0.0933
2004 cohort								
M1	0.179	-0.331	0.696	-0.0898	0.600	0.08650	-0.0634	0.0545

	TL	PL	PW	TD	SE	DHT	λ	θ
M2	0.583	0.126	-0.516	0.271	0.546	-0.0753	-0.0471	-0.0312
M3	0.0920	-0.149	0.226	0.898	-0.267	0.203	-0.0153	-0.00161
M4	0.196	0.177	-0.0523	-0.209	-0.0664	0.938	0.00631	0.0516
M5	-0.672	0.470	-0.00966	0.262	0.489	0.144	0.0262	-0.0253
M6	0.361	0.775	0.442	-0.0213	-0.166	-0.214	0.0355	-0.0028

Table 5. The M matrix of eigenvectors from the canonical rotation of γ containing the quadratic and correlational estimates of selection using seed production as the lifetime fitness component for *Silene virginica*. In the last two columns are the estimates of linear (θ) and nonlinear selection (λ) on the new latent axes described by the eigenvectors. The nonlinear selection gradient is the eigenvalue of each eigenvector. * P < 0.05; ** P < 0.01; *** P < 0.001.

	TL	PL	PW	TD	SE	DHT	λ	θ
2002 cohort								
M1	0.155	-0.283	-0.315	0.871	-0.184	-0.0566	-0.220 *	0.157
M2	0.0609	-0.559	-0.171	-0.0909	0.614	0.519	-0.109	0.0736
M3	0.651	0.196	0.244	0.00606	-0.330	0.607	-0.0316	0.0981
M4	0.390	0.335	0.344	0.285	0.664	-0.307	-0.0235	0.112
M5	-0.618	0.169	0.504	0.386	0.0498	0.429	0.0505	0.0660
M6	-0.117	0.654	-0.663	0.0526	0.190	0.284	0.1778*	0.0753
2004 cohort								
M1	0.432	-0.506	0.604	-0.282	0.313	0.123	-0.127*	0.118 *
M2	-0.412	-0.242	0.228	0.710	0.437	-0.156	-0.0688 *	-0.0903 *

	TL	PL	PW	TD	SE	DHT	λ	θ
M3	0.393	0.344	-0.388	0.0227	0.755	0.0742	-0.0125	-0.0526
M4	0.187	0.389	0.333	0.405	-0.186	0.710	0.0184	0.0708*
M5	0.446	0.384	0.342	0.239	-0.173	-0.67	0.0400	-0.0106
M6	-0.505	0.517	0.452	-0.441	0.277	-0.0383	0.0732	0.0328

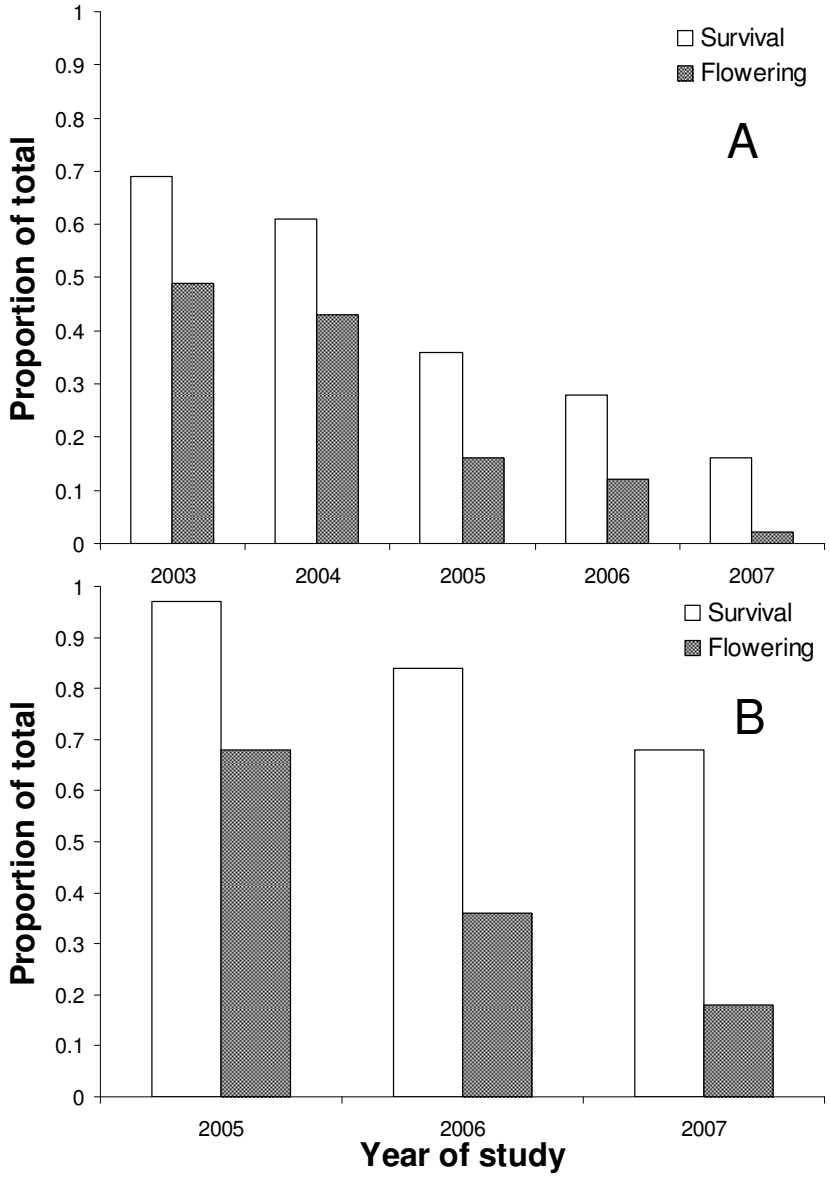

Figure 1. Probability of *Silene virginica* survival and flowering across the years of study for two cohorts. The first cohort was planted in 2002 (A) and the second planted in 2004 (B).

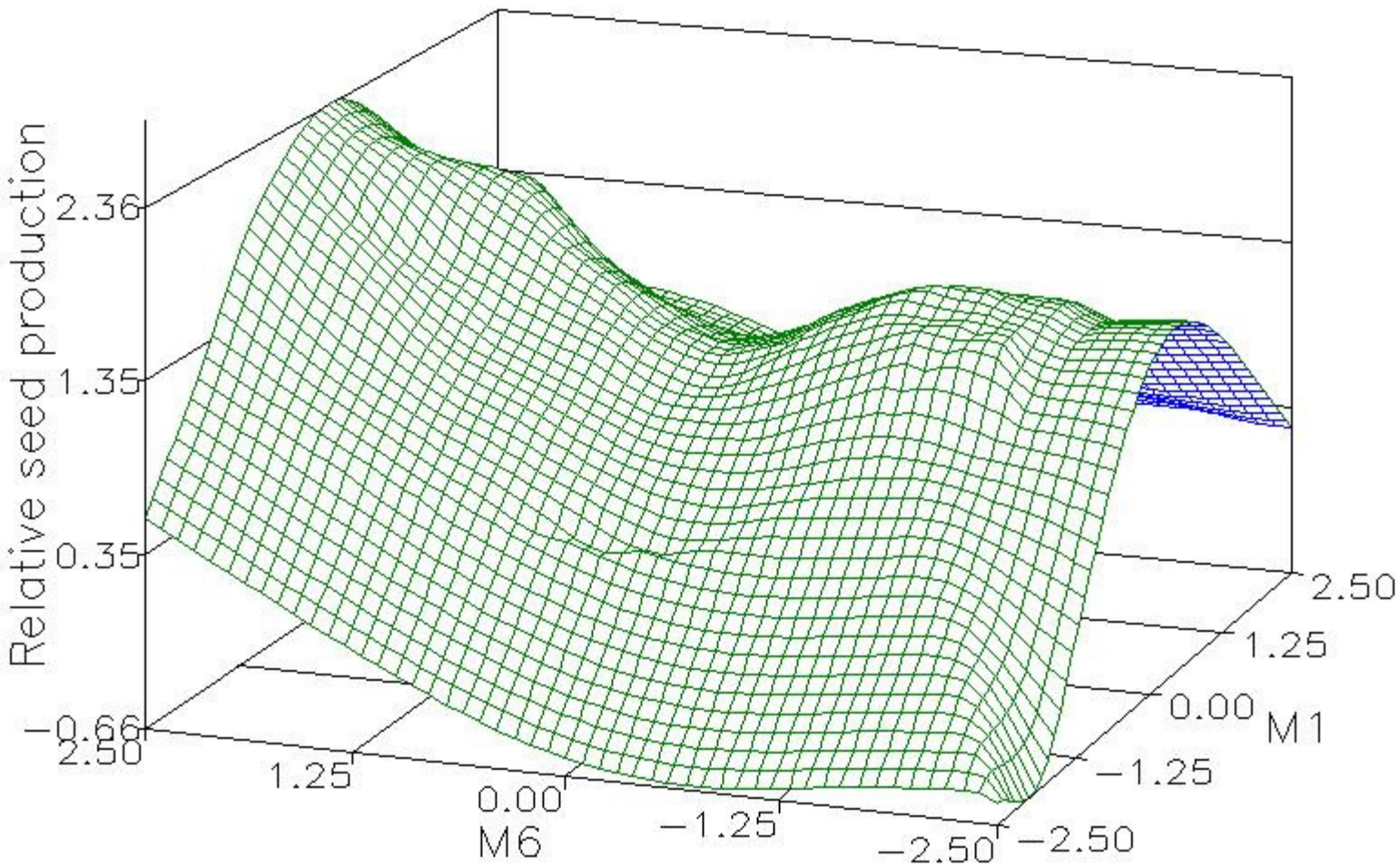
Relative seed production
2.36
1.35
0.35
−0.66
2.50
1.25
0.00
−1.25
−2.50
M6
2.50
1.25
0.00
−1.25
−2.50
M1

Figure 2. Thin plate spline analysis showing multivariate selection on the 2002 cohort of *Silene virginica* on two major axes, M1 and M6, representing the joint action of selection on combinations of traits with lifetime seed production as the fitness variable.

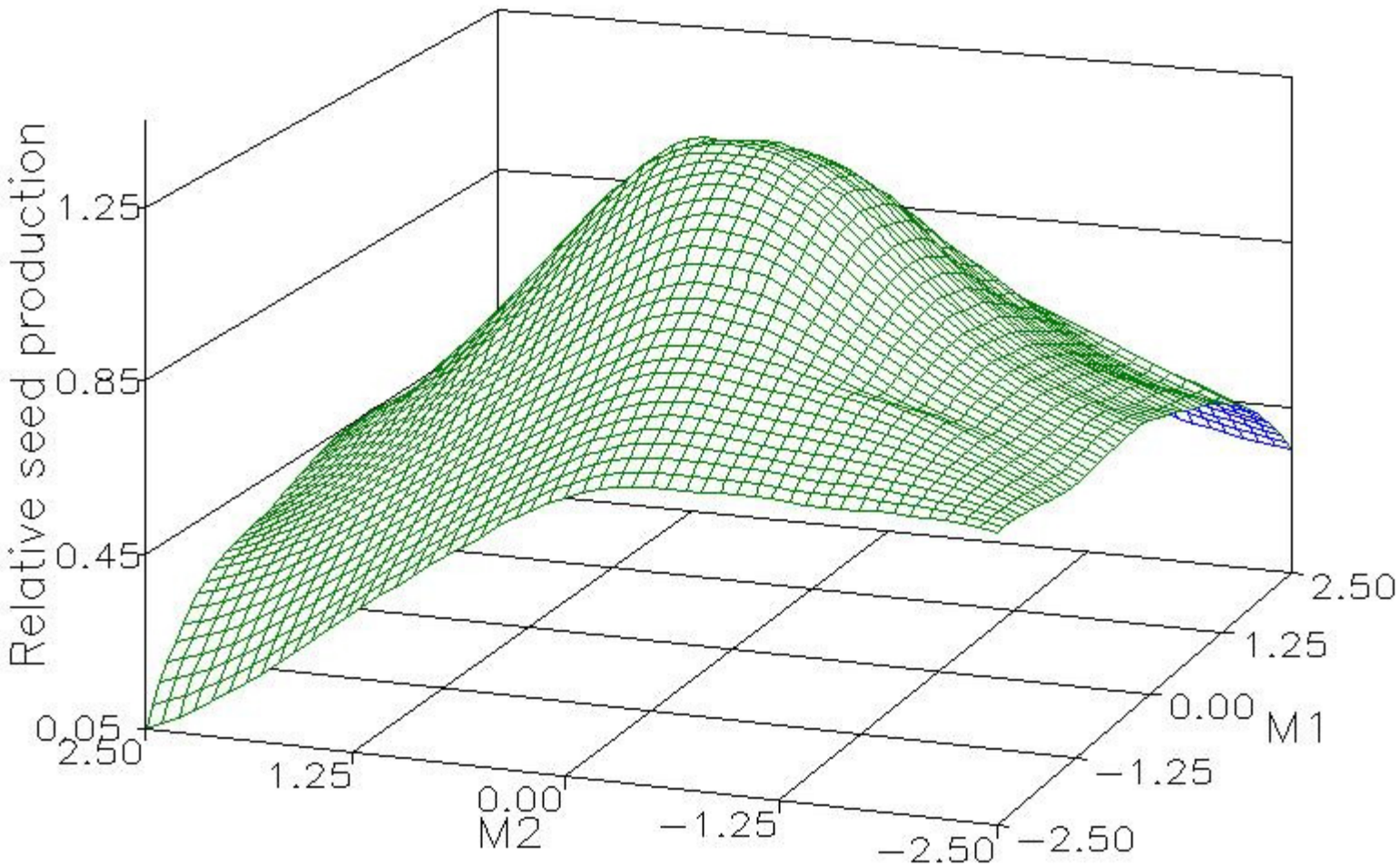
Relative seed production
1.25
0.85
0.45
0.05
2.50
1.25
0.00
−1.25
−2.50
M2
2.50
1.25
0.00
−1.25
−2.50
M1

Figure 3. Thin plate spline analysis showing multivariate stabilizing selection on the 2004 cohort of *Silene virginica* on two major axes, M1 and M2, representing the joint action of selection on combinations of traits through lifetime seed production.

Appendix 1. Means (SD) of the fitness data and floral traits (mm) for *Silene virginica* taken across all plants in each year of study.

	Flowers	Fruit	Seed	Corolla tube length	Petal length	Petal width	Corolla tube diameter	Stigma exsertion	Display height
1992 N = 193	4.1 (3.0)	1.6 (1.5)	65.1 (68.6)	23.9 (1.64)	20.0 (2.53)	6.50 (0.)	--	7.29 (2.35)	361 (87.9)
1993 N = 175	3.2 (2.2)	1.2 (1.2)	45.8 (49.2)	23.5 (1.67)	18.7 (2.03)	5.95 (0.847)	4.42 (0.725)	7.10 (3.32)	345 (90.9)
1994 N = 130	3.4 (2.8)	1.4 (1.8)	57.2 (75.4)	24.0 (1.58)	18.7 (2.27)	6.21 (0.880)	4.23 (0.570)	7.81 (2.07)	324 (71.1)
1995 N = 164	4.7 (3.7)	1.4 (1.6)	60.9 (74.2)	23.6 (1.49)	18.8 (2.00)	5.94 (0.760)	3.93 (0.461)	7.95 (2.17)	297 (87.1)
2003 N = 88	8.5 (7.4)	3.7 (3.6)	135 (131)	23.5 (1.45)	18.4 (1.68)	6.09 (0.727)	3.53 (0.350)	8.20 (1.85)	306 (78.5)
2004	3.7	1.6	72.2	24.2	18.1	5.74	3.65	7.11	312

N = 72	(3.7)	(1.7)	(86.0)	(1.84)	(2.22)	(0.712)	(0.536)	(2.00)	(91.5)
2005 N = 213	3.8 (3.0)	2.1 (1.8)	90.9 (82.3)	24.0 (1.73)	18.7 (2.15)	6.17 (0.799)	3.04 (0.417)	6.67 (1.86)	312 (75.4)
2006 N = 107	4.3 (3.3)	1.5 (1.6)	52.7 (61.2)	23.6 (1.95)	19.0 (2.11)	6.05 (0.790)	3.48 (0.443)	7.72 (1.92)	287 (74.5)

Appendix 2. The vector of standardized selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) estimated using fruit production as the fitness component for *Silene virginica*. Estimates in bold or underlined were significant at the FDR adjusted level Q = 0.05, or Q = 0.10, respectively, for the 8 models run for each year of study. * P < 0.05; ** P < 0.01; *** P < 0.001.

	β	γ					
		TL	PL	PW	TD	SE	DHT
1992							
Corolla tube length (TL)	-0.0484	0.00339			--		
Petal length (PL)	-0.0263	-0.0412	0.0158		--		
Petal width (PW)	0.0322	0.0618	-0.169*	-0.0224	--		
Stigma exsertion (SE)	0.0771	-0.0166	0.0603	-0.0138	--	0.0151	
Display height (DHT)	**0.140****	-0.0709	0.0228	0.0647	--	-0.0965	0.0299
1993							
Corolla tube length (TL)	0.0721	-0.106					
Petal length (PL)	-0.0141	0.0418	0.0451				

	β	γ					
		TL	PL	PW	TD	SE	DHT
Petal width (PW)	-0.0154	0.0611	-0.0564	0.0284			
Corolla tube diameter (TD)	-0.0113	-0.0402	-0.00088	0.0168	-0.0250		
Stigma exsertion (SE)	**0.166****	-0.0687	-0.0523	-0.00414	-0.0991	-0.0600	
Display height (DHT)	0.0656	-0.0988	0.0124	-0.0259	0.0976	0.0777	0.0124
1994							
Corolla tube length (TL)	0.175	-0.114					
Petal length (PL)	-0.202*	-0.133	0.192*				
Petal width (PW)	-0.0138	-0.0731	-0.201	-0.0669			
Corolla tube diameter (TD)	0.0320	0.151	-0.0282	0.0457	-0.045		
Stigma exsertion (SE)	0.0979	-0.0153	0.0933	-0.102	-0.0724	-0.0802	
Display height (DHT)	0.0356	-0.132	0.00545	-0.0800	0.242	0.0232	-0.169*
1995							
Corolla tube length (TL)	0.0487	0.0200					

	β	γ					
		TL	PL	PW	TD	SE	DHT
Petal length (PL)	-0.0911	0.236*	0.0146				
Petal width (PW)	0.153	-0.211	0.0140	0.0115			
Corolla tube diameter (TD)	-0.0116	0.00305	-0.0253	-0.107	0.0768		
Stigma exsertion (SE)	0.181*	-0.169	-0.179	0.0385	0.141	-0.00776	
Display height (DHT)	**0.275*** **	-0.0507	-0.234*	0.265*	0.0596	0.226*	-0.00134
2003							
Corolla tube length (TL)	0.0874	0.0163					
Petal length (PL)	0.0160	-0.0717	-0.0470				
Petal width (PW)	-0.0371	0.0321	-0.261	0.174			
Corolla tube diameter (TD)	-0.00089	-0.0327	0.0713	-0.0590	-0.0890		
Stigma exsertion (SE)	0.159*	-0.0456	0.0973	0.0436	0.000693	-0.139*	
Display height (DHT)	0.127	0.165	0.0429	-0.201	0.200	0.330**	-0.00449
2004							

	β	γ					
		TL	PL	PW	TD	SE	DHT
Corolla tube length (TL)	0.0292	-0.0754					
Petal length (PL)	-0.0172	0.108	-0.0276				
Petal width (PW)	0.0615	0.0547	-0.0558	-0.0635			
Corolla tube diameter (TD)	0.0572	-0.0674	0.0772	0.0720	-0.0557		
Stigma exsertion (SE)	0.125*	-0.0445	-0.0750	0.168	-0.0564	0.0322	
Display height (DHT)	0.107	0.0471	-0.204	0.0342	-0.0661	0.0706	-0.0265
2005							
Corolla tube length (TL)	0.0720*	0.0243					
Petal length (PL)	-0.0997**	0.00809	-0.0372				
Petal width (PW)	0.0433	-0.0153	0.109	-0.0596			
Corolla tube diameter (TD)	-0.0277	-0.00444	-0.0137	-0.0136	0.000606		
Stigma exsertion (SE)	**0.0863****	-0.0423	0.0450	-0.0233	0.0292	-0.0177	
Display height (DHT)	0.0588	0.0356	-0.0533	0.0514	0.00631	-0.00514	0.00794

	β	γ					
		TL	PL	PW	TD	SE	DHT
2006							
Corolla tube length (TL)	-0.0808	0.104					
Petal length (PL)	0.106	-0.223	0.0627				
Petal width (PW)	-0.0554	-0.273	0.352*	-0.206			
Corolla tube diameter (TD)	0.0356	0.220	-0.250	0.418*	-0.176		
Stigma exsertion (SE)	-0.122	0.124	-0.141	-0.0561	0.0500	-0.0478	
Display height (DHT)	0.120	0.0116	0.145	-0.195	-0.0262	-0.112	-0.0263

Appendix 3. The vector of standardized selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) estimated using seed production as the fitness component for *Silene virginica*. Estimates in bold or underlined were significant at the FDR adjusted level Q = 0.05, or Q = 0.10, respectively, for the 8 models run for each year of study. * P < 0.05; ** P < 0.01; *** P < 0.001.

	β	γ					
		TL	PL	PW	TD	SE	DHT
1992							
Corolla tube length (TL)	-0.052	0.00369			--		
Petal length (PL)	0.0119	0.00121	-0.00379		--		
Petal width (PW)	0.0518	0.0711	-0.191*	-0.00945	--		
Stigma exsertion (SE)	0.0361	-0.0436	0.0101	0.0103	--	-0.00184	
Display height (DHT)	**0.188****	-0.0635	0.148	-0.0022	--	-0.0719	0.00541
1993							
Corolla tube length (TL)	0.0522	-0.0250					
Petal length (PL)	0.0201	0.0122	0.0352				

	β	γ					
		TL	PL	PW	TD	SE	DHT
Petal width (PW)	-0.00237	0.0603	0.0299	0.00309			
Corolla tube diameter (TD)	0.0411	-0.0343	-0.0382	0.0242	-0.0234		
Stigma exsertion (SE)	0.0869	-0.0637	0.0428	-0.0154	-0.0414	-0.0877	
Display height (DHT)	0.132	-0.109	0.0750	-0.0501	0.192	0.0894	0.0395
1994							
Corolla tube length (TL)	0.163	-0.127					
Petal length (PL)	-0.191	-0.145	0.167				
Petal width (PW)	0.0576	-0.0636	-0.124	-0.106			
Corolla tube diameter (TD)	0.0179	0.257	-0.111	-0.0159	-0.0117		
Stigma exsertion (SE)	0.0931	0.0736	0.0313	-0.156	-0.0279	-0.0786	
Display height (DHT)	0.0334	-0.120	0.0320	0.00439	0.261*	0.0295	-0.152
1995							
Corolla tube length (TL)	0.0653	0.0401					

	β	γ					
		TL	PL	PW	TD	SE	DHT
Petal length (PL)	-0.0510	0.195	0.0121				
Petal width (PW)	0.195	-0.209	0.139	0.0299			
Corolla tube diameter (TD)	-0.0256	0.00593	-0.0673	-0.160	0.0663		
Stigma exsertion (SE)	0.124	-0.128	-0.108	-0.0391	0.154	-0.0176	
Display height (DHT)	**0.276*** **	-0.00073	-0.170	0.179	0.108	0.142	0.0120
2003							
Corolla tube length (TL)	0.102	-0.00934					
Petal length (PL)	0.0817	0.0441	0.0220				
Petal width (PW)	0.00431	-0.00831	-0.368*	0.235*			
Corolla tube diameter (TD)	0.0139	0.0137	0.111	-0.0955	-0.106		
Stigma exsertion (SE)	0.101	-0.0840	0.0851	-0.0142	-0.0200	-0.154*	
Display height (DHT)	0.0593	0.00836	0.127	-0.172	0.200	0.130	-0.0731
2004							

	β	γ					
		TL	PL	PW	TD	SE	DHT
Corolla tube length (TL)	0.00953	-0.0327					
Petal length (PL)	0.0463	0.00428	0.0063				
Petal width (PW)	0.0366	0.0986	-0.0234	-0.0973			
Corolla tube diameter (TD)	0.0990	-0.0940	0.0584	0.0129	0.0235		
Stigma exsertion (SE)	0.0877	-0.102	-0.0850	0.111	-0.0222	0.0345	
Display height (DHT)	0.173*	0.0407	-0.120	0.0449	0.0318	-0.0437	-0.0200
2005							
Corolla tube length (TL)	0.0229	0.0110					
Petal length (PL)	-0.0735	0.0387	-0.0600				
Petal width (PW)	0.102*	-0.0365	0.0906	-0.0703			
Corolla tube diameter (TD)	-0.0113	0.0696	-0.0128	-0.0344	-0.0023		
Stigma exsertion (SE)	0.0376	-0.0585	0.0968*	-0.0135	0.0405	-0.0130	
Display height (DHT)	0.0593	-0.00827	0.00807	0.0579	0.0776	-0.0782*	-0.00162

	β	γ					
		TL	PL	PW	TD	SE	DHT
2006							
Corolla tube length (TL)	-0.0420	0.187					
Petal length (PL)	0.119	-0.331	0.0826				
Petal width (PW)	-0.111	-0.191	0.326	-0.272			
Corolla tube diameter (TD)	0.0497	0.162	-0.296	0.484*	-0.249*		
Stigma exsertion (SE)	-0.209*	0.215	-0.0886	-0.120	0.0896	-0.0778	
Display height (DHT)	0.118	-0.0162	0.228	-0.261	0.0429	-0.221*	-0.0296

Appendix 4. The M matrix of eigenvectors from the canonical rotation of γ containing the quadratic and correlational estimates of selection using fruit production as the fitness component for *Silene virginica*. In the last two columns are the estimates of linear (θ) and nonlinear selection (λ) on the new latent axes described by the eigenvectors. The nonlinear selection gradient is the eigenvalue of each eigenvector. * P < 0.05; ** P < 0.01; *** P < 0.001.

	TL	PL	PW	TD	SE	DHT	λ	θ
1992								
M1	-0.174	0.544	0.709	--	-0.238	-0.339	-0.108 *	-0.00467
M2	0.358	0.288	0.335	--	0.616	0.545	-0.0220	0.0968*
M3	0.662	0.431	-0.292	--	-0.527	0.113	0.00742	-0.130*
M4	0.539	-0.144	0.0233	--	0.389	-0.732	0.0828	-0.0993
M5	0.336	-0.644	0.548	--	-0.366	0.197	0.112	-0.00305
1993								
M1	0.798	-0.206	-0.167	0.270	0.453	0.118	-0.151 *	0.217 *
M2	-0.337	0.179	0.0342	0.506	0.533	-0.560	-0.117 *	0.108
M3	0.352	0.450	-0.181	0.379	-0.623	-0.330	-0.00933	-0.0787

	TL	PL	PW	TD	SE	DHT	λ	θ
M4	0.299	0.518	0.699	-0.331	0.188	-0.0918	0.0178	-0.0510
M5	-0.0918	-0.188	0.585	0.633	-0.194	0.418	0.0332	-0.00995
M6	-0.163	0.648	-0.327	0.131	0.223	0.617	0.122	0.105
1994								
M1	0.474	0.0573	-0.00044	-0.496	-0.116	0.715	-0.298 **	0.138
M2	0.482	0.242	0.707	-0.0109	0.355	-0.288	-0.169 *	0.0451
M3	-0.206	-0.225	-0.0782	0.0812	0.877	0.353	-0.0845	0.107
M4	0.687	-0.0804	-0.604	0.290	0.150	-0.224	-0.0221	0.0514
M5	0.0433	0.256	0.178	0.801	-0.175	0.478	0.0398	-0.0208
M6	-0.162	0.903	-0.313	-0.146	0.194	-0.0354	0.251	-0.254*
1995								
M1	0.425	-0.410	0.513	0.0753	0.222	-0.578	-0.231*	-0.0413
M2	0.00494	0.441	-0.128	-0.301	0.822	-0.147	-0.105	0.0208
M3	0.639	-0.374	-0.298	-0.386	0.0900	0.454	-0.0307	0.178

	TL	PL	PW	TD	SE	DHT	λ	θ
M4	0.423	0.516	0.512	0.317	-0.00517	0.438	0.0119	0.159**
M5	0.175	-0.106	-0.499	0.799	0.264	-0.0341	0.134	0.00129
M6	-0.450	-0.469	0.346	0.127	0.443	0.495	0.334 ***	0.335 **
2003								
M1	0.200	-0.191	-0.194	0.287	0.685	-0.578	-0.305 *	0.126
M2	0.326	0.701	0.283	0.472	-0.213	-0.232	-0.114	-0.0318
M3	0.145	0.500	0.102	-0.745	0.401	-0.0444	-0.0999	0.0336
M4	-0.737	0.210	0.360	0.263	0.424	0.188	0.0167	-0.00605
M5	0.539	-0.211	0.387	0.163	0.341	0.611	0.116	0.235 *
M6	0.0149	0.364	-0.770	0.208	0.171	0.449	0.297 **	0.222
2004								
M1	-0.544	0.480	0.439	-0.363	-0.234	0.308	-0.191 *	0.0186
M2	-0.0791	0.442	-0.517	0.357	0.303	0.559	-0.128	0.148
M3	0.323	-0.0732	0.484	0.589	-0.396	0.390	-0.0523	0.0823

	TL	PL	PW	TD	SE	DHT	λ	θ
M4	0.767	0.374	0.0853	-0.483	0.0917	0.150	-0.0228	0.0289
M5	-0.00797	0.396	0.454	0.350	0.574	-0.429	0.0297	0.0581
M6	-0.0683	-0.522	0.304	-0.196	0.599	0.484	0.148 *	0.168*
2005								
M1	0.120	-0.606	0.700	-0.0129	0.228	-0.274	-0.122 **	0.135 **
M2	0.195	-0.113	-0.335	-0.464	0.775	0.146	-0.0304	0.0960 *
M3	0.507	0.409	0.0821	0.626	0.377	-0.188	0.00128	0.00407
M4	-0.274	0.342	0.538	-0.00015	0.222	0.685	0.00688	0.0467
M5	-0.262	-0.538	-0.317	0.621	0.154	0.363	0.0164	0.0307
M6	0.739	-0.217	0.00569	-0.0847	-0.363	0.517	0.0461	0.0821 *
2006								
M1	0.189	-0.317	0.697	-0.587	0.0623	0.171	-0.526 **	-0.156
M2	-0.390	-0.267	0.0809	0.345	0.552	0.588	-0.137	-0.0633
M3	0.132	-0.0962	0.312	0.498	-0.702	0.368	-0.0364	0.118

	TL	PL	PW	TD	SE	DHT	λ	θ
M4	0.574	0.647	0.221	0.193	0.357	0.196	0.00474	-0.00981
M5	-0.124	-0.116	0.518	0.464	0.203	-0.668	0.0889	-0.0204
M6	0.670	-0.622	-0.305	0.189	0.173	-0.0691	0.317	-0.179

Appendix 5. The M matrix of eigenvectors from the canonical rotation of γ containing the quadratic and correlational estimates of selection using seed production as the fitness component for *Silene virginica*. In the last two columns are the estimates of linear (θ) and nonlinear selection (λ) on the new latent axes described by the eigenvectors. The nonlinear selection gradient is the eigenvalue of each eigenvector. * P < 0.05; ** P < 0.01; *** P < 0.001.

	TL	PL	PW	TD	SE	DHT	λ	θ
1992								
M1	-0.267	0.634	0.547	--	-0.200	-0.432	-0.138 *	0.0177
M2	0.343	0.260	0.446	--	0.657	0.429	-0.0312	0.1078 *
M3	0.731	0.334	-0.378	--	0.0415	-0.458	0.00423	-0.177 **
M4	0.460	0.00149	0.296	--	-0.720	0.427	0.0313	0.0213
M5	-0.255	0.647	-0.521	--	-0.0873	0.487	0.128	0.0744
1993								
M1	0.0596	0.0965	-0.123	0.564	0.646	-0.487	-0.138 *	0.1230
M2	0.535	-0.308	0.0740	-0.339	0.595	0.381	-0.0879	0.0978
M3	0.677	0.169	-0.554	0.264	-0.358	0.0878	-0.0451	0.110

	TL	PL	PW	TD	SE	DHT	λ	θ
M4	0.309	-0.156	0.742	0.515	-0.229	0.112	0.0195	0.0284
M5	0.176	0.899	0.306	-0.226	0.124	0.0251	0.0503	-0.0213
M6	-0.354	0.1870	-0.168	0.426	0.184	0.772	0.143	0.181*
1994								
M1	0.575	0.0128	0.00732	-0.510	-0.155	0.621	-0.318 **	0.117
M2	0.222	0.172	0.800	0.0223	0.525	-0.0688	-0.179 *	0.142
M3	-0.598	-0.244	-0.0312	-0.0912	0.463	0.599	-0.107	0.0881
M4	-0.305	0.168	0.501	0.282	-0.657	0.341	-0.00767	-0.0357
M5	0.294	0.370	-0.297	0.711	0.225	0.363	0.0781	-0.0639
M6	-0.289	0.863	-0.138	-0.383	0.0600	-0.0482	0.226	-0.249
1995								
M1	0.445	-0.468	0.550	0.135	0.186	-0.476	-0.217 *	0.0523
M2	0.147	0.231	-0.137	-0.440	0.843	-0.0428	-0.0842	0.0342
M3	-0.396	0.535	0.179	0.521	0.200	-0.462	-0.0162	-0.152

	TL	PL	PW	TD	SE	DHT	λ	θ
M4	0.496	0.427	0.373	0.307	0.0464	0.580	0.0407	0.236 ***
M5	0.470	-0.0561	-0.712	0.487	0.0636	-0.166	0.182	-0.128
M6	-0.396	-0.506	0.0107	0.430	0.456	0.441	0.238 *	0.274 *
2003								
M1	0.126	-0.159	-0.102	0.494	0.633	-0.550	-0.236	0.119
M2	0.257	-0.249	-0.103	-0.588	0.629	0.346	-0.142	0.101
M3	-0.0298	0.793	0.324	-0.330	0.242	-0.313	-0.0892	-0.0261
M4	0.223	0.204	0.476	0.514	0.212	0.610	-0.0107	0.148
M5	0.931	0.0490	-0.0324	-0.0451	-0.305	-0.187	0.00457	0.0877
M6	0.0337	0.490	-0.804	0.186	0.0751	0.268	0.388**	0.148
2004								
M1	0.486	0.0502	-0.763	0.156	0.377	0.114	-0.160	0.0642
M2	-0.314	0.561	-0.0500	-0.334	0.165	0.667	-0.095	0.107
M3	0.573	0.394	0.596	0.279	0.284	0.0530	-0.0263	0.0913

	TL	PL	PW	TD	SE	DHT	λ	θ
M4	-0.0944	-0.327	0.0416	0.669	-0.217	0.623	0.0346	0.209
M5	-0.5651	0.283	-0.0691	0.5311	0.436	-0.351	0.0685	0.0289
M6	-0.0948	-0.584	0.232	-0.237	0.716	0.168	0.0925	0.0284
2005								
M1	0.268	-0.676	0.539	-0.114	0.404	0.0638	-0.134 *	0.143 **
M2	0.180	-0.0294	-0.575	-0.464	0.384	0.524	-0.0981 **	-0.0129
M3	0.476	0.574	0.460	-0.470	-0.108	0.0363	-0.0113	0.0222
M4	-0.425	0.356	0.364	0.313	0.394	0.554	0.0173	0.0679
M5	0.449	0.273	-0.184	0.443	0.590	-0.381	0.0297	-0.00588
M6	0.536	-0.107	-0.0423	0.507	-0.417	0.518	0.0603	0.0364
2006								
M1	0.0630	-0.299	0.681	-0.599	0.138	0.254	-0.626 ***	-0.266
M2	-0.322	-0.0895	-0.00868	0.378	0.697	0.509	-0.177	-0.146
M3	0.0685	-0.224	0.414	0.600	-0.555	0.326	-0.0480	0.142

	TL	PL	PW	TD	SE	DHT	λ	θ
M4	0.524	0.687	0.384	0.192	0.259	-0.0381	-0.0169	0.0157
M5	0.365	0.158	-0.435	-0.271	-0.205	0.733	0.0915	0.0413
M6	0.692	-0.596	-0.169	0.168	0.279	-0.176	0.417 *	-0.266

Chapter 4: Evaluating spatial and temporal variation in the interaction of the nursery pollinator, *Hadena ectypa* (Lepidoptera: Noctuidae) and its host, *Silene stellata* (Caryophyllaceae): ecological and evolutionary implications.

Nursery pollination of *Silene* (Caryophyllaceae) by *Hadena* moths (Lepidoptera: Noctuidae), in which moths pollinate, lay eggs and their larval offspring subsequently consume the plant's reproductive tissue, is generally considered to be an antagonistic but non-obligate association. However, key ecological parameters have rarely been measured in the *Silene-Hadena* interaction. For example, the sign of the interaction in nursery pollination is known to be affected by the relative density of copollinators (pollinators that do not consume plant flowers seed or fruit) and nursery pollinators, but spatio-temporal variation in the relative density of copollinators and nursery pollinators has not been previously measured in the *Silene-Hadena* system. Furthermore, *Hadena* larvae are potential sources of selection on the mating system and flowering phenology, but their potential to exert phenotypic selection on floral traits and whether the pattern of selection may disrupt or reinforce a *Silene* species pollination syndrome has never been addressed. To determine the sign of the interaction between *H. ectypa* and *S. stellata*, we investigated spatial and temporal variation in *H. ectypa* density and *S. stellata* fruit and seed production at three sites in two years. Additionally, the community context of pollination was investigated by censusing adult moths across two consecutive flowering seasons. Phenotypic selection on *S. stellata* floral traits was measured using three

maternal fitness components in a single population in each of four years. Female *H. ectypa* oviposition preference was measured in relation to floral trait expression in one field season. Overall we found that the *Hadena-Silene* interaction is antagonistic, because copollinator service sufficiently compensates for the absence of *Hadena* moths. However, flowering time is a major predictor of the relative density of *H. ectypa* and copollinators, thus, the sign of the interaction may change as a function of flower phenology. *Hadena ectypa* fruit predation rates were variable by population and year of study, and phenotypic selection in floral traits attributable to larval fruit predation also varied depending on the intensity of fruit predation. Selection on floral traits changed when the effects of the fruit predators were included. Negative directional selection was generated by the fruit predators on corolla tube length in 2003 and 2006, which was in the direction of interspecific variation in corolla tube length relative to the long-tongued, bumble bee, carpenter bee and hawkmoth-pollinated *S. caroliniana* and hummingbird-pollinated *S. virginica*. Furthermore, significant stabilizing and disruptive selection on latent axes representing floral trait combinations was detected in 2003 and 2006, respectively. Therefore, fluctuating selection pressures on floral traits due to *H. ectypa* larvae and moth pollinators appears to be a feature of *S. stellata* floral evolution.

Keywords: Mutualism, nursery pollination, phenotypic selection, pollinator-mediated selection

Introduction

While floral trait evolution clearly involves pollinator-mediated selection, plants and their pollinators do not exist in isolation from other selection pressures (Fenster et al. 2004, Strauss and Irwin 2004, Bronstein 2006). Nursery pollinators, which provide pollination service to plants but also consume the plant's reproductive tissues for larval development, are exemplary systems for characterizing the potentially conflicting selection pressures exerted by pollinators and herbivores. Nursery pollination, ranging from the obligate interactions of yuccas and yucca moths (reviewed in Pellmyr 2003) to the non-obligate facultative interactions of *Silene* (Caryophyllaceae)-*Hadena* (Lepidoptera: Noctuidae), spans the spectrum from mutualism to antagonism (Dufay and Antsett 2003, Kephart et al. 2006). Traditionally, nursery pollination systems have been studied from the perspective of mutualism biology. Recent work in non-obligate nursery pollinator systems has focused on the variable nature of the sign of the interaction between the nursery pollinator and its host while characterizing the ecological factors responsible for the sign switch. In non-obligate nursery pollination such as the *Greya* moth-*Lithophragma* system, the relative abundance of copollinators (pollinators not using the plants as hosts) and nursery pollinators affects the sign of the interaction (Thompson and Pellmyr 1992). In populations or years in which the copollinators provide the majority of pollination service the interaction is negative but could switch to positive if the nursery pollinators become dominant (Thompson and Cunningham 2002, Thompson and Fernandez 2006). Datasets of spatial and temporal variation in the relative densities of copollinators and nursery pollinators are rare for other non-obligate nursery pollination

systems, and how the density variation affects the sign of the interaction has not been evaluated in other model systems.

Ecological conditions other than the relative composition of the pollinator community have been found to be highly variable and to affect plant reproduction directly in *Silene-Hadena* interactions. For example, *H. bicruris* pollinator egg density varies within a season for *Silene dioica*, but not for *S. latifolia*, its primary host from the study population in Germany (Bopp and Gottsberger 2004). *Hadena* moth seed predation can vary with flowering phenology and flower gender, and thus *Hadena* larvae have been implicated as selective agents in the evolution of flowering time traits and plant mating system (Biere and Honders 1996, Collin et al. 2002, Wright and Meagher 2003). Clearly the *Hadena* moths are antagonists for plant species they do not pollinate. In a survey of *Silene-Hadena* interactions Kephart et al. (2006) showed that fruit predation by *Hadena* larvae was highest in nocturnal moth pollinated species, but there was no appreciable difference in fruit predation rates of nocturnal species with or without *Hadena* as a major pollinator.

Differential selection pressures exerted by moth pollinators and *Hadena* moth larvae as seed and fruit predators may have important consequences for the evolution of pollination syndromes in *Silene*. Non-pollinating sources, such as herbivores and predispersal seed predators, are demonstrated selective agents on floral traits (Strauss and Irwin 2004, Kolb et al. 2007). It is possible that the evolution of diurnal pollination in part reflects a response to selection from *Hadena* fruit predation (Kephart et al. 2006). The cost of fruit predation does not appear to differ greatly between species of nocturnal moth syndromes with or without *Hadena* pollinators (Kephart et al. 2006). Thus, it may

be beneficial for *Silene* species to attract adult *Hadena* pollinators from an already established *Silene-Hadena* fruit predation interaction. In this scenario, the covariation of various floral traits with fitness may reflect the predilection of female *H. ectypa* to oviposit in flowers of particular phenotypes. Measuring the relative directions and magnitudes of selection exerted by *Hadena* larvae and moth pollinators is a first step for evaluating whether *Hadena* fruit predation is important for the evolution of *Silene* pollination syndromes. *Silene* are often subject to other selection pressures from non-pollinators such as anther smut disease (Antonovics et al. 2003, Giles et al. 2006,), in addition to *Hadena* fruit predation. Whether these sources of selection act in concert with pollinators or in opposition to pollinator-mediated selection on floral traits, thereby acting to disrupt a syndrome, is generally unknown.

Here, the previously unpublished interaction between *S. stellata* and its nursery pollinator, *H. ectypa* and its copollinators is described. *Silene stellata* exhibits floral traits corresponding to a nocturnal moth pollination syndrome and is specialized for nocturnal moth pollination (Reynolds et al. *in review*, chapter 2). Specifically we asked 1) How do *H. ectypa* adult moth and copollinator density change within the flowering season and what implications does this have for the sign of the interaction between *H. ectypa* and *S. stellata*? 2) Do copollinators provide ample pollination service in the absence of *H. ectypa* density? 3) Does the pattern of phenotypic selection exerted primarily by pollinators or *H. ectypa* larvae change, and if so, is the pattern acting to disrupt or reinforce *S. stellata*'s nocturnal moth pollination syndrome?

Materials and Methods

Study system

Populations of *Silene stellata* and its pollinators were studied near the University of Virginia's Mountain Lake Biological Station (MLBS) in the Southern Appalachian Mountains in Giles County, VA, during the 2003 to 2006 flowering seasons using plants at three sites: Meadow (37°20'53"N, 80°32'41"W, elevation ≈ 1,100-1,300 meters), Woodland (37°21'20"N, 80°33'14"W, elevation ≈ 1,100-1,300 meters), and Wind Rock (37°24'50"N, 80°31'10"W, elevation ≈ 1,300 meters). All three sites were located within 10 km of one another. The flowers of *Silene stellata* are white and funnel-form with fringed petals and are presented horizontally. Plants lack basal rosettes, but they produce one to many reproductive stems that emerge in early spring and reach up to 120 cm in length (R. Reynolds, personal observation). There are typically > 20 flowers per panicle inflorescence at the terminal ends of the reproductive stems with flowering occurring from early July through mid August. *Silene stellata* is specialized for nocturnal moth pollination, but it is pollinated secondarily by diurnal bees and small flies (Reynolds et al. *in review,* chapter 2). The nocturnal visitors of *S. stellata* include the noctuid moths *Hadena ectypa*, *Amphipoeaea americana*, *Feltia herelis*, *Autographa precationis*, and *Cucullia asteroids*, the arctiid *Halysidota tessellaris*, and the notodontid, *Lochmaeus manteo*. The diurnal visitors are primarily halictid bees (Hymenoptera: Halictidae), syrphid flies (Diptera: Syrphidae), and bumble bees (*Bombus spp*.), but when these diurnal visitors do pollinate, they are of minor importance relative to the nocturnal pollinators (Reynolds et al. *in review*, chapter 2). Population level outcrossing rate (73%)

was relatively high, and was measured in 2006 for plants of the Meadow site (Reynolds et al. unpublished).

In the course of our detailed study of the pollination of *S. stellata* (Reynolds et al. *in review,* chapter 2) we discovered that one of its moth visitors, *H. ectypa,* pollinates, lays eggs, and its larvae use *S. stellata* flowers and fruits as a host for feeding and development. Nectaring and oviposition behavior were observed directly and with digital camcorders using the night shot option (Sony Digital Handycams: model #TRV17), thus demonstrating that *H. ectypa* is a nursery pollinator. Larvae were collected from two sites (N = 52) and grown to pupation in the laboratory (10 adult *H. ectypa* emerged). Adult male and female moths may be found nectaring in the flowers of *S. stellata*. The egg laying behavior follows nectaring, as moths position the distal end of their abdomens inside the flower and oviposit on the surface of the nectaries or ovary wall. In the 2006 egg census of 418 flowers at the Meadow site (see below), the number of eggs per flower ranged between 0 and 24 with a median of 1 egg per flower and mean (SD) of 1.3 (2.2). Soon after the egg is laid, a larva hatches, makes a hole in the ovary and begins consuming immature seed or ovules. As development continues, larvae consume flower tissue or the immature seed in fruits from adjacent flowers on the same plants. We have never observed larvae consuming non-reproductive tissues such as leaves and stems. Larvae collected from plants in the field and reared in the laboratory required approximately 50 immature fruit to reach pupa stage. Larvae will not eat seed that have become sclerified. Therefore, larvae must move among flowers and fruits within plants and possibly may move between plants to complete development. Fruit that have been

consumed by *H. ectypa* larvae are noted by a conspicuous exit hole left in the hardened ovary wall, the presence of frass, and the complete absence of seed or ovules.

Temporal and spatial variability of moth pollinator and egg density

To investigate within and between year temporal variation in relative densities of *H. ectypa* and copollinators, adult moth densities were estimated in 2005 and 2006 across the flowering period of *S. stellata* at the meadow population. In addition, *H. ectypa* egg densities were estimated within and between years among the Meadow, Woodland and Wind Rock sites. To calculate adult moth densities, the number of moths observed contacting flowers during an approximate five minute interval was counted in patches of 10 plants on each night of sampling. Patches of plants were sampled after dusk while walking along predefined transects for a distance of up to 180 meters, and then returning to the starting point along a second, but parallel, transect. At each patch one of the 10 plants was randomly chosen, and the number of open flowers was counted. Patches were uniformly chosen along the transects, but the same patches were never sampled in consecutive nights. Headlamps with a red light were worn, which increased our visual acuity over white light and did not readily disturb nectaring moths. In 2005 on average (SE) 19.5 (1.4) patches were observed per night on 12 sample dates spanning the flowering period from 19 July to 11 August. In 2006 19 (1.2) patches were observed per night on 14 sampling dates from 17 July to 13 August. Observations of density were the sum of *H. ectypa* or copollinators observed each night divided by the number of patches and then divided by the average estimate of flowers per plant multiplied by 10 plants. Therefore, the *H. ectypa* and copollinator density estimates that we present are the

number of these pollinators per flower in a patch of 10 plants. Because scatter plots of within season change in the densities of copollinator and *H. ectypa* moths indicated nonlinearity in the relationships between density and date of sampling, non-parametric regression was used to fit the model, density = $f(x) + \varepsilon$, where x is number of days since 1 January, and f(x) is some unknown function that interpolates the values of x. The interpolation function is estimated by penalized least squares (Green and Silverman 1994) and the analyses were implemented with the TPSPLINE procedure (All statistical models were run with SAS (2004) version 9.1.2). Four separate models were estimated: one for each pollinator type and year.

To estimate the proportion of flowers with eggs, a single flower was collected from multiple plants distributed uniformly along transects at each of the sites and in each year. Presence or absence of eggs was determined by examining the flowers under the dissecting scope. Samples were taken on at least three occasions during the flowering seasons. The proportion of flowers with eggs was estimated as the number of flowers with eggs divided by the number of flowers sampled. During 2005 flowers were sampled 4, 3, and 3 dates across the flowering season at the Meadow, Woodland and Wind Rock sites. In 2006 flowers were sampled 21 times at the Meadow, and 4 times at the Woodland and Wind Rock sites, respectively. The average (SE) number of flowers sampled per day in 2005 was 84 (18), 106 (3.2) and 57 (14) for the Meadow, Woodland, and Wind Rock populations, respectively. In 2006, 32 (3.1), 59 (18), and 43 (7) flowers were sampled per day from the Meadow, Woodland, and Wind Rock populations, respectively. As with the adult density data, a nonparametric regression function was

estimated to determine the relationship between egg density and date of sampling for the 2006 Meadow sample.

Hadena ectypa *and copollinator effectiveness*

To determine whether pollinator effectiveness varies by type of pollinator, the amount of pollen deposited per visit was estimated by counting pollen grains on previously unvisited stigmas, which were collected after visitation by moths. Stigmas collected if no visit occurred were used as controls. The trials were conducted from 2004-2006 at the Meadow site. The previously unvisited flowers were observed with video cameras for at most two hours, and were visited by available pollinators at the site during the time interval. After the observation interval, flowers were collected and transported to the lab at MLBS, stigmas were removed, and fixed in fuschin glycerine jelly on microscope slides (Kearns and Inouye 1993). The video camera recordings were then viewed, and the visitor type, *H. ectypa* or copollinator, or if a moth failed to visit was noted. Moths were identified by looking for key distinguishing features such as the narrow white lateral line of the forewing and egg laying behavior for *H. ectypa* and generally larger body size for the copollinators. Additionally, nectaring behavior differences were noted as *H. ectypa* typically held their wings at rest and the copollinators fluttered. We noted these features based on our experience observing moths directly in the adult moth density sampling, and in non-sampling activities.

A general linear model (GLM) was used to model pollen grains per anther as the response and type of pollinator or control was the predictor. Significant differences among treatment means were analyzed by comparing all three treatments with one

another (option pdiff = all from the GLM). The *S. stellata* data were pooled across years as a GLM demonstrated year ($F = 0.29$, $DF = 2, 124$, $P = 0.7488$) was not a significant predictor of pollen grains on stigmas for the nocturnal pollinators.

Direction of Interaction: negative or positive.

To determine the sign of the interaction and whether copollinators could compensate in terms of fruit and seed production in the absence of *H. ectypa*, mature fruit were collected from *S. stellata* plants near the end of flowering in mid August from two additional sites, Woodland and Wind Rock. Plants (N = 30) were haphazardly chosen while walking transect lines at the two sites in 2005 and 2006. Fruit data were collected at the Meadow site using the same plants as were used for a multi-year phenotypic selection study (see below). All fruits with or without seed were collected from each plant. The fruits were scored in the lab for successful fruit set (fruit with mature seed), number of seeds and whether a fruit had been eaten by *H. ectypa* larvae. General linear models (GLM procedure) were used to model fruit set (number of fruits setting seed / total number flowers), seed set (number of seed per fruit), and proportion of flowers eaten as the response variables and site, year and year by site as the predictors. Pairwise differences among the all treatment level means (ls means option, pdiff = all) were compared and the familywise alpha level controlled via a Tukey adjustment.

Phenotypic selection analysis

To determine if the pattern of selection changes between pollinators and fruit predators, phenotypic selection analyses on floral traits were performed using three maternal fitness

components. Seventy-one plants were randomly selected along three transects in each of the years 2003 and 2005 for a total of 142 plants for use in a multiyear study of phenotypic selection on *S. stellata* floral traits. Three linear and parallel transects marked every 10 meters for 250 meters were used to select the study plants. Each transect was spaced by 10-15 meters. At each 10 meter interval the nearest flowering plant was located and marked for future study. The group from 2003 was studied each year from 2003-2006. The group from 2005 was monitored from 2005-2006. As these were mature plants with many flowers, a subsample of five flowers was selected from each of the plants to estimate average floral trait expression per plant. The measurement of five flowers per plant was sufficient to demonstrate high correlations between years in plant floral trait expression, and thus provide an accurate depiction of the floral phenotype for each plant. For example between 2003 and 2004 the correlation coefficients for all traits were significant and the range of correlation was between 0.3 and 0.8 with a median of 0.6. The traits measured were date of first flower, corolla tube length, corolla tube diameter, petal length and petal width, the distance between the nectaries and stigma, and the number of lobes on a single petal. The morphological floral traits were measured with dial calipers to the 0.1 mm. The number of lobes per petal was measured as the number of petal fringes per petal, and is a measure of the degree of petal dissection. Since stigmas continue to grow and the delicate petals become easily damaged, to minimize the potentially large measurement error all traits were measured on flowers in female phase within 24 hours of receptivity. If fewer than five flowers were available to measure at any one time, the plant was visited 2-3 days later to measure the remaining flowers at the appropriate stage. After flowering ended, the number of stems and the

heights of each stem were measured on all transect plants as a vegetative vigor covariate. About two to three weeks after each plant flowered one to two mature fruits were opened and the seed inside were inspected for the onset of sclerization. If browning was noted then all fruits that appeared to be at a similar developmental stage were removed and stored. At this time flowers failing to set seed were collected. The monitoring, inspection and removal of fruits from each transect plant was repeated 3-4 times until all reproductive units were removed from the transect plants.

Twenty four multiple regression models were used to analyze the phenotypic relationship between floral traits and the fitness components (N = 3) and to obtain estimates of linear and nonlinear selection for the yearly analyses (N = 4) of the pattern of phenotypic selection on floral traits (3 fitness components x 2 estimations x 4 years). Models were identical to those of the *S. virginica* study (Reynolds et al. *in prep.*, chapter 3) except for the additional fitness variable, initiated fruit, which represents the number of fruits set before *H. ectypa* began consuming immature seed. Initiated fruit was calculated as the sum of the number of fruits eaten and number of mature fruits per plant. The two additional fitness variables, mature fruit and seed production, were measured as all mature fruit and seed summed within plants. Floral trait values were averaged across the five flowers for phenotypic selection analyses at the individual plant level.

To obtain standardized selection gradients, floral trait values were z-transformed and fitness data were scaled by mean fitness of all plants in the year of analysis. Number of flowers per plant was used as a covariate in the analyses. As a maternal fitness component, flowers produced correlated strongly with fruit and seed production and it correlated strongly with plant vegetative vigor characters. Thus, flowers per plant

allowed us to analyze the direct effects of selection on floral traits holding plant vigor constant. We used the sequential Bonferroni control of type 1 errors at alpha = 0.05 for the four replicate measurements of selection on each fitness component performed for each year of study.

Linear (12 models: 4 years, 3 fitness components) and nonlinear (12 models: 4 years, 3 fitness components) selection gradients on *S. stellata* floral traits were estimated using the same approaches as were used for our previously described *S. virginica* analysis (Chapter 3). Briefly, general linear models of the relationship between initiated fruit production, seed production and mature fruit production per plant and floral trait expression were fit using the GLM procedure. New latent axes of the second order response surface were described by eigenvectors and eigenvalues, which were calculated by diagonalizing the matrix of standardized quadratic and correlational selection gradients using the RSREG procedure (Blows and Brooks 2003). Selection gradients were estimated for all years, but in 2004 the sample size was low (N = 51 plants), thus, only the directional selection gradient parameter estimates will be discussed from 2004.

Female moth oviposition preference

To study the relationship between the probability of egg deposition and floral trait expression and to determine whether the pattern of selection on flowers by female moths was in the same direction as the pattern of selection generated by the seed predators, flowers were measured for a series of floral traits in the field, removed from the plants, and then scored for the presence or absence of eggs in the laboratory. One female phase flower was sampled daily from thirty plants randomly chosen along the transects at the

meadow site on 16 days between 12 July and 31 July 2006. We measured corolla tube length (TL) and width (TD), petal length (PL)and width (PW), tube diameter (TD), stigma exsertion (SE), lobes per petal (Lobes), flower height above the ground, and the number of open flowers on each plant. All floral morphology traits were measured with dial calipers to 0.1 mm. Flowers were later scored for number of eggs under a dissecting scope.

A generalized linear model was used to model the binomial response, proportion of flowers with eggs, as a function of corolla tube length and width, petal length and width, tube diameter, stigma exsertion, lobes per petal, flower height above the ground, and the number of open flowers using the GENMOD procedure. An additional full second order polynomial regression model was used to model curvature in the surface describing the relationship between probability of egg deposition and floral traits. Canonical analysis was also used here, but with slight modification in that the models were not run using the RSREG procedure, since the response was a categorical variable with values of zero or one. Instead the matrix of quadratic and correlational partial regression coefficients estimated from the generalized linear model was diagonalized using the eigentools facility in Microsoft Excel. The IML procedure in SAS was used to transform the original data into the space of the eigenvectors produced from the matrix diagonalization and those data were run in the full second-order polynomial regression model using the GENMOD procedure to test the significance of the eigenvalues. To visualize the surface describing the probability of egg deposition as a function of the new latent axes realized by the canonical analyses, thin plate spline analyses were performed

using the TPSPLINE procedure. The response variable was probability of egg deposition and the predictors were M1 and M7, the axes with significant curvature (See below).

Results

Temporal and spatial variability of moth pollinator and egg density

Adult moth density

Within the 2005 season adult *H. ectypa* and copollinator moth density ranged between 0 - 0.00089 and 0 - 0.0040 moths per flower, respectively. Within the 2006 season *H. ectypa* and copollinator density ranged between 0 - 0.013 and 0 - 0.0098 moths per flower, respectively. Non-parametric regression analysis indicated that copollinator density increased across the flowering season in 2005 and *H. ectypa* was uniformly low and was unchanged across the flowering phenology (Figure 1). By contrast, in 2006 *H. ectypa* density decreased and copollinator density increased across the flowering season (Figure 2).

Hadena *eggs in flowers*

Within the 2005 season, the probability that *S. stellata* flowers contained eggs at the Meadow, Woodland, and Wind Rock sites ranged between 0 - 0.20, 0.059 – 0.23, and 0 – 0. Within the 2006 flowering season, the probability that *S. stellata* flowers contained eggs at the Meadow, Woodland, and Wind Rock sites ranged between 0.098 – 0.67, 0.042 – 0.47, and 0 – 0.29. Non-parametric regression analysis indicated that the probability *S. stellata* flowers contained *H. ectypa* eggs declined across the flowering

season in 2005 (Figure 3) and 2006 (Figure 4). The probability that flowers contained eggs also declined in the Woodland site in 2005 and 2006. No eggs were found in flowers at Wind Rock in 2005 and it appeared that the probability of flowers with eggs was highest at the Wind Rock site in the middle of the flowering period in 2006.

Hadena ectypa *and copollinator effectiveness*

Results from the general linear model demonstrate the pollinator type or control (no visits) were significant predictors of pollen grain deposition on stigmas ($F = 13.94$, $DF = 2, 265$, $P < 0.0001$). There was no significant difference between *H. ectypa* and copollinators ($P = 0.7274$), but *H. ectypa* ($P = 0.001$) and copollinators ($P < 0.0001$) were significantly different from the control (Figure 5).

Direction of Interaction: negative or positive.

Site ($F = 18.85$, $DF = 2, 341$, $P < 0.0001$), year ($F = 6.89$, $DF = 1, 314$, $P = 0.009$) and the year by site interaction ($F = 11.74$, $DF = 2, 314$, $P < 0.0001$) were significant predictors of fruit predation. Figure 6 shows the results of all pairwise contrasts between treatment levels. Notably, fruits eaten by seed predators and flowers with eggs were not sampled from Wind Rock in 2005 indicating the absence of *H. ectypa*. However, fruit predation was observed at Wind Rock in 2006. Fruit predation and the presence of eggs were also observed at the Woodland and Meadow populations in 2005 and 2006.

Year ($F = 29.96$, $DF = 1, 341$, $P < 0.0001$), year by site ($F = 3.32$, $DF = 2, 341$, $P = 0.0374$), but not site ($F = 1.26$, $DF = 1, 314$, $P = 0.2853$) were significant predictors of

fruit set. Figure 6 shows the results of all pairwise contrasts between treatment levels. Notably, fruit set was not significantly different among sites in 2005 or 2006.

Year ($F = 17.63$, $DF = 1, 314$, $P < 0.0001$), site ($F = 19.28$, $DF = 2, 314$, $P < 0.0001$), but not the site by year interaction ($F = 1.24$, $DF = 2, 314$, $P = 0.2906$) were significant predictors of seed set. Figure 6 shows the results of all pairwise contrasts between treatment levels. Notably seed set was significantly lower at Wind Rock in 2005 than the Woodland or Meadow sites, and in 2006 was significantly lower than the Meadow site but not the Woodland site.

Phenotypic selection analysis

Linear selection.

Seed predation (percent fruits eaten of total) by *H. ectypa* in from 2003-2006 was 27%, 13%, 10%, and 29%, respectively (Table 1). No directional selection was detected on floral traits when initiated fruit was used as the maternal fitness component (Table 2). However, when mature fruit or seed production were the fitness components negative directional selection was detected in 2003 and 2006 on corolla tube length (Tables 3 & 4), the years with the highest seed predation.

Nonlinear selection:

One nonlinear selection gradient was significant after sequential Bonferroni correction. In 2003, significant selection was detected on the positive correlation between corolla tube length and number of lobes per petal but only when mature fruit was the maternal fitness component (Table 4).

When initiated fruit was the maternal fitness component the canonical analysis demonstrated that convex (=stabilizing) selection was detected along two latent axes (M5 & M6) in 2005 (Table 5), but significant curvature was not present in 2003 or 2006. One axis of convex selection was most strongly associated with lobes per petal and corolla tube length, and the other axis was associated with corolla tube diameter and petal width. No significant curvature was detected on the selective surface when seed production was the maternal fitness component (Table 6). The canonical analysis demonstrated curvature in the selection surface in 2003 and 2006, but only when mature fruit was the fitness component. In 2003 concave (=disruptive) selection was detected on one latent axis, and in 2006 convex selection was detected along another single latent axis when the maternal fitness component was mature fruits (Table 7). The 2003 concave selection was most strongly associated with lobes and tube length. The 2006 convex selection was most strongly associated with stigma exsertion and corolla tube diameter.

Female moth oviposition preference

Hadena ectypa moth eggs were more likely to be found in flowers held relatively high above the ground and on plants with relatively fewer flowers, but egg presence was statistically unassociated with the other floral traits, notably corolla tube length (Table 8). However, the full 2^{nd} order polynomial regression model demonstrated moths preferred to lay eggs on that part of phenotypic space that reflects a negative correlation of corolla tube length and petal width and the positive correlation of petal length and petal width (Table 8). That is, more eggs were laid on longer tubed flowers with narrower petals, shorter tubed flowers with wider petals and flowers with long and wide petals or short

and narrow petals. The pattern of correlational preference was manifested as significant convex preference on one axis, concave preference on another and positive linear preference along a third axis (Table 9). The concave preference was most associated with lobes per petal, corolla tube length and petal length. The convex preference was most closely associated with corolla tube length, tube diameter, petal length and petal width, and the linear preference was highly associated with display height. Therefore, linear preference was minimal, but we found substantial nonlinear preference moderately associated with five of the seven measured floral traits.

Discussion

Silene stellata is specialized for pollination by nocturnal moths (six common moth copollinators, and the nursery pollinator *Hadena ectypa,* Reynolds et al. *in review,* chapter 2). In the current investigation, we have demonstrated temporal and spatial variability and within season variability in the densities of copollinators and *Hadena* moths. Here we explore the consequences of that variation for determining the sign of the interaction between *H. ectypa* and *S. stellata* and for context-specific selection pressures on floral trait variation.

Hadena ectypa and the other nocturnal copollinators are equally effective pollinators in terms of pollen grain deposition onto stigmas, and both are present in high densities at some times in the season in both years. The highest adult *H. ectypa* density was observed in 2006, and plants were at greater risk of high fruit predation rates by *H. ectypa* larvae (the maximum fruit predation rate was 29% measured in 2006), but if copollinators are absent and pollination service is attributable solely to *H. ectypa,* the net

direction of the interaction between the two partners could be positive. Evidence from sampling of plant reproductive effort at multiple sites suggests that the interaction is not positive because copollinators can compensate in terms of fruit and seed production when *H. ectypa* is absent or at reduced density. With the substantial temporal variation in fruit predation by *H. ectypa* it is not surprising a pattern of selection on floral traits through mature fruit and seed (includes fruits eaten by larvae) differed from initiated fruit. Therefore, *H. ectypa* pollinators and copollinators as indicated by the pattern of selection on initiated fruit and *H. ectypa* fruit predators as indicated by the pattern of selection on mature fruit are both potential sources of selection on floral traits.

Hadena-Silene interactions are rarely one to one in that multiple *Silene* may harbor a single *Hadena* species and multiple *Hadena* may utilize a single *Silene* species, which may or may not pollinate the host (Kephart et al. 2006). The non-obligate interactions between *Silene* and *Hadena* perhaps suggest selection to minimize the cost of maintaining the interaction for both partners is weak. Two reviews have indicated that overall the *Silene-Hadena* interaction is likely antagonistic, although more evidence is required (Dufay and Antsett 2003, Kephart et al. 2006). There is good evidence in other non-obligate systems that the relative densities and effectiveness of the nursery pollinators and copollinators affect the sign of the interaction, which can change depending on the site or year (Thompson and Pellmyr 1992, Thompson and Cunningham 2002, Thompson and Fernandez 2006). However, density and effectiveness data have rarely been documented in *Silene-Hadena* systems. *Hadena ectypa* is a more prominent member of the *S. stellata* moth pollinator community compared to the antagonistic *S. vulgaris-H. bicruris* interaction, which has 25 copollinator species that are more frequent

and effective than *H. bicruris* (Petterson 1991). Furthermore, at the meadow population from 2005-2006, based on egg or adult density data *H. ectypa* are most abundant early in the flowering period than later, and copollinators exhibit the opposite trend. *Silene stellata* plants across the entire flowering period may be subdivided into plants pollinated almost exclusively by *H. ectypa*, a mixture of *H. ectypa* and copollinators and then exclusive pollination by copollinators. Therefore, the question of whether the interaction is a mutualism or an antagonism for *S. stellata* and other *Silene-Hadena* interaction is complicated by the pollinator community context, which changes with site, year and strongly across the flowering season.

One way to quantify the sign of the interaction of *H. ectypa* on *S. stellata* is to compare *S. stellata* fitness between populations that vary in the presence or absence of the seed predator. In 2005 and 2006 copollinators at the Wind Rock site were able to compensate for the absence of *H. ectypa*, since fruit set was not different among the sites, indicating that overall the interaction is not positive. While the seed set data were in agreement with the fruit set data in 2005, in 2006 mean seed set at Wind Rock was significantly lower than the Meadow but not the Woodland. This result suggests a positive interaction of *H. ectypa* on *S. stellata* female reproductive success. However, copollinators and *H. ectypa* are equally effective pollinators. Thus, seedset at *Hadena* only, or mixed, or copollinator only sites should be similar all else being equal. Therefore, reductions in seed set at particular populations with or without *H. ectypa* may reflect variation in pollinator density (including copollinators) or resource limitation differences. Because plants at the Wind Rock site were large (R. Reynolds, personal observation), we attribute the reduced seed set to lower pollinator density rather than

resource allocation. Results from the site and year plant reproduction study should be treated with caution because it is currently known that *Hadena* pollinator density varies across the flowering season. It is possible that the plants sampled at the Woodland and Meadow sites received most of their pollination from copollinators and all of the seed predation from *H. ectypa* larvae, which could have biased our results toward a "not a positive interaction" conclusion. Perhaps a better future test would be to compare plant reproduction at different times of the flowering period when *H. ectypa* or copollinators are known to dominate the pollinator community. Moreover, testing the direction of the interaction by quantifying maternal fitness components is only half the equation, and a complete study would require investigating total reproductive effort through male and female reproductive success. Nonetheless, our study revealed that copollinators are certainly capable partners for *S. stellata* reproduction, and demonstrates that the *Silene-Hadena* interaction is non-obligate, which both argue against a net positive effect of *H. ectypa* on *S. stellata* female reproductive success.

The seasonality and site and year variability in the composition of *S. stellata*'s pollinator community is not an unusual finding (e.g., Ivey et al. 2003) but it does raise interesting points about which plants should receive the bulk of damage from the seed predators with implications for plant reproduction. Within-season changes in the pollinator community in the form of increasing visitation rates and/or changes in species composition has been shown to affect components of plant reproduction such as rate of pollen transfer (Ashman and Stanton 1991), and genetic variation of plant progeny (Hirao et al. 2006). We found for two populations and years that *H. ectypa* density decreases across the flowering period. By contrast *H. bicruris* egg density roughly followed the

whole flower phenology of *S. latifolia* from a population in Germany (Bopp and Gottsberger 2004) but was present on the alternate host, *S. dioica* only early in flowering. At a single site we found that copollinator density increased across the season. The higher densities at the beginning of *S. stellata* flowering suggest that adult *H. ectypa* moth emergence is synchronized to the onset of flowering. Because early flowering plants are more susceptible to *H. ectypa* visitation and oviposition, then the fruits of these plants should be more susceptible to seed predation. However, flower date or flower date squared was not a significant covariate in the phenotypic selection analysis (see discussion below) on floral traits through any fitness component (analysis not shown), which included plants flowering across the entire season. In addition, multiple regression models of number of fruit eaten per plant and floral trait variation demonstrated that flower date or flower date squared were not significant covariates (analysis not shown). In future study, seed set, fruit set and seed predation, and population outcrossing rates, could be estimated in plants at the beginning of flowering, pollinated primarily by *H. ectypa*, or at the end of flowering by co-pollinators, to measure accurately the effects of temporal variation in pollinator community composition on plant reproduction.

Hadena fruit predation has been documented to associate with flower gender (Collin et al. 2002) and flowering phenology (Biere and Honders 1996, Wright and Meagher 2003) indicating that *Hadena* seed predators are potential selective agents on pollination and plant mating system traits. Anther-smut fungus has also been implicated as a selective agent on floral traits in bumble bee-pollinated *S. dioica* (Giles et al. 2006). Non-pollinating selective agents on floral traits may interact to disrupt or act in concert influencing the fit of floral traits and their major pollinators. In a review of *Silene*-

Hadena interactions Kephart et al. (2006) determined that *Hadena spp.* were pollinators of two thirds of all *Silene* with nocturnal syndromes. How *Hadena* larval fruit predation may maintain or disrupt a nocturnal moth syndrome has as yet remained unexplored.

Significant linear selection gradients on *S. stellata* morphological floral traits varied by year of study and by fitness component, which either included the effects of *H. ectypa* larvae (mature fruit and seed production) or was due primarily to pollination (initiated fruit). Negative directional selection on corolla tube length was detected through mature fruit and seed production in 2003 and 2006 when seed predation levels were high, but linear selection was never detected through initiated fruit. Fruit predation was positively related to corolla tube length in those years, based on multiple regression models of fruits eaten per plant and floral trait variation (analysis not shown). However, the probability of female moth oviposition was only linearly and positively associated with flower display height and negatively associated with flower number per plant. Therefore, negative directional selection on corolla tube length appeared to be unrelated to female *H. ectypa* preference for floral traits. Another possibility is that *H. ectypa* larvae prefer larger flowers with longer corollas, which could have more seed for consumption. Kephart et al. (2006) found among a number of *Silene* and *Hadena* species that average fruit predation per species and ovule number per flower per species were marginally but positively associated. For *S. stellata*, seeds per fruit was not correlated with corolla tube length in any year of study, which argues against a resource-based explanation for the fruit predation - corolla tube length association (analysis not shown). The only floral characters consistently correlated with tube length were petal length and corolla tube diameter (both positive). Whatever the cause for the consistent *H. ectypa*

larvae-mediated negative directional selection on corolla tube length it is in the direction expected based on interspecific variation of this character in the related longer-tubed *S. caroliniana* and *S. virginica* (Reynolds et al. *in review,* chapter 2). Therefore, with respect to corolla tube length *H. ectypa* have at least not opposed the direction of divergence in pollination syndromes of the three *Silene* species.

Like linear selection, nonlinear selection operated in a context dependent manner. In two of the three years with large samples of plants, there was no curvature in the selection surface as revealed by the canonical analysis through initiated fruit. However, in 2005 when fruit predation rate was at the four-year low of 10%, there were two axes of significant stabilizing selection, which together were correlated with 4 of the 6 measured floral traits. Therefore, moth pollinators appear to exert no directional selection on floral traits (2003-2006) and stabilizing selection on floral trait combinations when *H. ectypa* density is low. Because *H. ectypa* was present at high density in 2003 (based on seed predation rates) and 2006 (based on adult censuses and seed predation rates), perhaps the combined influence of *H. ectypa* adult and copollinator visitation nullifies the signal of stabilizing selection found when *H. ectypa* are absent or at low density. Given the strong within-season differences in the density of copollinators and *H. ectypa*, subsets of plants flowering early and late in flowering may be experiencing differing pollinator-mediated selection pressures on floral traits.

Only when *H. ectypa* density was high did a pattern of nonlinear selection on floral trait combinations emerge through mature fruit production. Nonlinear selection was never observed through seed production. Overall these analyses indicated that the pattern of pollinator-mediated selection on morphological floral traits was changed by *H.*

ectypa larvae depending on fruit predation intensity in any given year. It is also possible that pollinator-mediated natural selection is acting via unmeasured floral traits such as a primary attractant, i.e., flower scent or nectar, and *Hadena* moths are known to be attracted to specific lilac aldehydes emitted by *Silene spp.* (Jurgens et al. 2002, Dotterl et al. 2006). Therefore, floral trait selection appears to be highly context dependent on the density of larval and adult *H. ectypa*. When the effects of the *H. ectypa* are included in the maternal fitness components, either a pattern of nonlinear selection revealed by adult pollinators is nullified (2005) or a pattern of linear and nonlinear selection is generated where none existed (2003 and 2006).

In summary, *H. ectypa* pollination and subsequent larval fruit predation of its host *S. stellata* has consequences for plant reproduction that varies by site and year and with floral traits. Our research showed a strong within flowering season decline in *H. ectypa* density and concomitant increase in copollinator density, which was replicated at two sites and years. The highest *H. ectypa* adult density and the onset of *S. stellata* flowering appear to be synchronized. This important and novel finding for a *Silene-Hadena* interaction should be studied in other systems known to have a significant *Hadena* pollination component. Therefore, the sign of the interaction may be a function of the within flowering season variability in the ratios of *H. ectypa* and copollinator. The consequences of this ecological variation on plant reproduction and plant population persistence remains to be explored. The strong within-season effect on the relative density of copollinators and *H. ectypa* and the high yearly variation in *H. ectypa* larval fruit predation also may induce varying selection pressures on floral traits and therefore

both *H.* ectypa larvae and adult pollinators may have been important for pollination syndrome evolution in *Silene*.

Table 1. Means (SD) of *Silene stellata* fitness data and floral traits (mm) in each year of study. Lobes is a count variable.

	Flowers	Mature Fruit	Seed	Fruits eaten	Corolla tube length	Petal length	Petal width	Corolla tube diameter	Stigma exsertion	Lobes per petal
2003 N = 70	31.1 (26.7)	11.9 (11)	212 (212)	8.43 (10.5)	10.8 (1.02)	9.10 (0.975)	11.1 (2.03)	7.02 (1.14)	9.32 (1.44)	11.6 (2.51)
2004 N = 51	26.8 (38.0)	17.3 (27.2)	250 (421)	3.37 (5.28)	9.71 (0.866)	8.91 (0.866)	11.3 (1.48)	7.99 (0.973)	10.4 (1.32)	11.9 (2.95)
2005 N = 120	47.9 (54.9)	24.3 (27.1)	450 (554)	4.96 (8.45)	9.96 (0.927)	9.15 (0.822)	11.3 (1.50)	7.68 (1.09)	9.74 (1.44)	11.4 (2.78)
2006 N = 109	72.0 (67.5)	30.0 (37.5)	504 (688)	20.6 (20.5)	9.84 (0.935)	8.71 (0.803)	10.8 (1.53)	7.71 (1.17)	10.1 (1.56)	11.5 (2.49)

Table 2. The vector of standardized selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) for *Silene stellata*, estimated initiated fruit production as the fitness component. Estimates in bold were significant at the sequential Bonferroni adjusted alpha level = 0.05 for the 4 models run for each year of study. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	β	γ					
		TL	PL	PW	TD	SE	Lobes
2003							
Corolla tube length (TL)	-0.0506	-0.00288					
Petal length (PL)	-0.0307	-0.00393	-0.00527				
Petal width (PW)	0.0205	-0.0491	-0.123	0.0238			
Corolla tube diameter (TD)	0.0347	0.00156	0.0669	0.0599	-0.0648		
Stigma exsertion (SE)	0.0177	-0.00568	0.109	-0.0568	-0.0762	0.00815	
Lobes per petal (Lobes)	0.0211	0.113*	0.0719	-0.0642	0.0304	0.0245	-0.0273
2004							
Corolla tube length (TL)	0.0150	0.0342					
Petal length (PL)	-0.0395	0.0005	-0.0995				

	β	γ					
		TL	PL	PW	TD	SE	Lobes
Petal width (PW)	0.0456	0.0376	0.251	-0.00578			
Corolla tube diameter (TD)	0.0661	0.188*	0.0136	-0.0379	-0.101		
Stigma exsertion (SE)	0.0429	-0.0626	0.145	0.0655	-0.124	0.0723	
Lobes per petal (Lobes)	-0.0474	0.00962	-0.0177	0.101	0.0349	0.0998	-0.118
2005							
Corolla tube length (TL)	0.0318	-0.0249					
Petal length (PL)	0.0348	-0.00629	0.0135				
Petal width (PW)	0.0534	0.0458	-0.0338	-0.0124			
Corolla tube diameter (TD)	0.00670	0.0344	-0.107	-0.0254	0.0215		
Stigma exsertion (SE)	0.0296	0.0434	-0.0109	-0.0075	0.0380	0.0167	
Lobes per petal (Lobes)	-0.00129	-0.0527	-0.0473	0.0437	-0.00174	0.0802	-0.0215
2006							
Corolla tube length (TL)	-0.0428	0.0203					

	β	γ					
		TL	PL	PW	TD	SE	Lobes
Petal length (PL)	0.0392	-0.0280	0.0446				
Petal width (PW)	0.0144	0.0154	-0.0824	0.0152			
Corolla tube diameter (TD)	0.0172	0.0142	0.000445	0.0247	-0.0300		
Stigma exsertion (SE)	-0.0102	-0.0226	0.00300	-0.00599	-0.00402	-0.00362	
Lobes per petal (Lobes)	0.0220	-0.00436	0.00611	0.00523	-0.00957	-0.00616	0.0181

Table 3. The vector of standardized selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) for *Silene stellata*, estimated using seed production as the fitness component. Estimates in bold were significant at the sequential Bonferroni adjusted alpha level = 0.05 for the 4 models run for each year of study. * P < 0.05; ** P < 0.01; *** P < 0.001.

	β	γ					
		TL	PL	PW	TD	SE	Lobes
2003							
Corolla tube length (TL)	**-0.305****	-0.119					
Petal length (PL)	-0.143	0.117	-0.0419				
Petal width (PW)	0.117	-0.201	-0.326	0.0414			
Corolla tube diameter (TD)	0.0769	0.114	0.0897	0.155	-0.0868		
Stigma exsertion (SE)	0.0903	0.0920	0.124	0.101	-0.275	0.0590	
Lobes per petal (Lobes)	0.0510	0.384*	0.233	-0.181	0.00562	0.132	-0.0269
2004							
Corolla tube length (TL)	-0.0282	0.0337					
Petal length (PL)	-0.0257	0.0361	-0.149				

	β	γ					
		TL	PL	PW	TD	SE	Lobes
Petal width (PW)	0.0802	-0.0684	0.317	0.00393			
Corolla tube diameter (TD)	-0.0270	0.0330	-0.144	0.0451	-0.0699		
Stigma exsertion (SE)	-0.0197	-0.0714	0.0159	0.0302	-0.135	0.0862	
Lobes per petal (Lobes)	-0.0113	0.0628	-0.245	0.144	0.0639	0.0731	-0.132
2005							
Corolla tube length (TL)	0.0476	0.00668					
Petal length (PL)	-0.0118	0.0591	0.00523				
Petal width (PW)	0.0522	-0.0310	0.00288	-0.0430			
Corolla tube diameter (TD)	-0.00506	-0.0248	-0.0770	0.0260	-0.00106		
Stigma exsertion (SE)	0.0652	0.0210	0.0356	-0.0823	-0.0825	0.0168	
Lobes per petal (Lobes)	-0.0503	0.0116	-0.00646	0.0312	0.00942	0.105	-0.0192
2006							
Corolla tube length (TL)	**-0.254****	0.0533					

	β	γ					
		TL	PL	PW	TD	SE	Lobes
Petal length (PL)	0.176	-0.164	0.123				
Petal width (PW)	-0.0124	0.127	-0.0960	0.000429			
Corolla tube diameter (TD)	0.0177	0.0190	-0.0496	0.115	-0.100		
Stigma exsertion (SE)	0.00824	0.105	-0.0903	-0.00169	0.184	-0.0744	
Lobes per petal (Lobes)	0.0497	-0.0352	0.153	-0.0140	-0.185	-0.0352	0.00896

Table 4. The vector of standardized selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) for *Silene stellata*, estimated using mature fruit production as the fitness component. Estimates in bold were significant at the sequential Bonferroni adjusted alpha level = 0.05 for the 4 models run for each year of study. * P < 0.05; ** P < 0.01; *** P < 0.001.

	β	γ					
		TL	PL	PW	TD	SE	Lobes
2003							
Corolla tube length (TL)	**-0.266****	-0.0667					
Petal length (PL)	-0.135	0.159	-0.01039				
Petal width (PW)	0.0475	-0.227	-0.117	0.00469			
Corolla tube diameter (TD)	0.0412	0.101	0.0767	0.172	-0.11074		
Stigma exsertion (SE)	0.0517	0.0886	0.135	0.0702	-0.224	0.0257	
Lobes per petal (Lobes)	0.0435	**0.444****	0.104	-0.242	0.0507	0.169	0.0212
2004							
Corolla tube length (TL)	-0.0360	0.0349					

	β	γ					
		TL	PL	PW	TD	SE	Lobes
Petal length (PL)	-0.0460	-0.0203	-0.131				
Petal width (PW)	-0.0103	0.0840	0.277*	-0.0403			
Corolla tube diameter (TD)	0.0778	0.152	-0.275	0.130	-0.0597		
Stigma exsertion (SE)	0.0269	-0.182*	0.139	0.0976	-0.110	0.0929	
Lobes per petal (Lobes)	-0.0641	0.0835	-0.235*	0.142	0.00697	0.111	-0.141*
2005							
Corolla tube length (TL)	0.0120	-0.0132					
Petal length (PL)	0.0571	0.00971	0.0140				
Petal width (PW)	0.0194	0.0104	-0.0324	-0.0123			
Corolla tube diameter (TD)	0.0116	0.0253	-0.0452	-0.0395	0.0190		
Stigma exsertion (SE)	0.0256	0.0427	-0.0541	0.00380	0.0517	0.0178	
Lobes per petal (Lobes)	-0.0357	-0.0302	-0.0127	0.0162	-0.0182	0.0457	-0.0206
2006							

	β	γ					
		TL	PL	PW	TD	SE	Lobes
Corolla tube length (TL)	-0.140*	-0.0284					
Petal length (PL)	0.0957	-0.0690	0.0635				
Petal width (PW)	-0.00734	0.119	-0.0785	0.00104			
Corolla tube diameter (TD)	0.0444	-0.0349	0.0707	0.0434	-0.0920		
Stigma exsertion (SE)	0.00212	0.131	-0.111	-0.0137	0.217*	-0.0853	
Lobes per petal (Lobes)	0.0423	-0.0633	0.0317	0.0279	-0.0916	-0.0444	0.0303

Table 5. The M matrix of eigenvectors from the canonical rotation of γ containing the quadratic and correlational estimates of selection on *Silene stellata*, using initiated fruit production as the fitness component. In the last two columns are the estimates of linear (θ) and nonlinear selection (λ) on the new latent axes described by the eigenvectors. The nonlinear selection gradient is the eigenvalue of each eigenvector. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	TL	PL	PW	TD	SE	Lobes	λ	θ
2003								
M1	0.290	0.544	-0.588	-0.0314	0.332	0.402	0.118	-0.0151
M2	0.596	-0.276	-0.103	0.174	-0.643	0.337	0.0375	-0.0203
M3	0.445	-0.0812	0.652	0.0354	0.518	0.317	-0.00146	-0.00322
M4	-0.249	0.531	0.308	0.693	-0.236	0.161	-0.00969	0.00956
M5	0.546	0.272	0.00241	0.176	0.0317	-0.772	-0.0838	-0.0383
M6	-0.0501	-0.515	-0.351	0.676	0.388	-0.0486	-0.129	0.0780
2004								
M1	-0.309	0.357	0.408	-0.289	0.723	0.0639	0.169	-0.0464
M2	0.720	0.334	0.515	0.322	-0.0215	0.0204	0.0908	0.0406

TL	PL	PW	TD	SE	Lobes	λ	θ	TL
M3	0.408	-0.387	-0.387	0.123	0.598	0.401	0.00333	-0.0681
M4	-0.191	-0.386	0.505	-0.0423	-0.255	0.702	-0.0539	-0.0275
M5	-0.424	0.202	-0.0799	0.862	0.0967	0.141	-0.148	-0.0460
M6	0.0614	0.648	-0.396	-0.228	-0.212	0.568	-0.280	-0.148
2005								
M1	0.150	-0.593	0.0944	0.630	0.397	0.252	0.0878	0.00209
M2	-0.0468	0.221	0.223	-0.438	0.658	0.524	0.00313	0.0538
M3	0.227	0.448	-0.588	0.270	0.487	-0.304	0.0119	0.00818
M4	0.751	0.110	0.557	-0.0591	0.0503	-0.329	0.00325	0.03804
M5	-0.197	0.619	0.377	0.578	-0.176	0.266	-0.0544*	0.0329
M6	0.567	0.0654	-0.378	-0.0316	-0.373	0.626	-0.0849**	0.00202
2006								
M1	0.282	-0.777	0.552	0.0817	-0.0734	-0.028	0.0792	0.00602
M2	0.794	0.203	-0.196	0.116	-0.281	-0.444	0.0211	-0.0373

TL	PL	PW	TD	SE	Lobes	λ	θ	TL
M3	0.359	0.137	0.0179	-0.0166	-0.291	0.876	0.0189	-0.00156
M4	0.324	-0.325	-0.486	-0.260	0.680	0.149	-0.00749	-0.0409
M5	0.210	0.431	0.531	0.343	0.607	0.0442	-0.0104	0.0222
M6	-0.109	-0.213	-0.371	0.891	0.00274	0.103	-0.0366	0.00700

Table 6. The M matrix of eigenvectors from the canonical rotation of γ containing the quadratic and correlational estimates of selection on *Silene stellata*, using seed production as the fitness component. In the last two columns are the estimates of linear (θ) and nonlinear selection (λ) on the new latent axes described by the eigenvectors. The nonlinear selection gradient is the eigenvalue of each eigenvector. * P < 0.05; ** P < 0.01; *** P < 0.001.

	TL	PL	PW	TD	SE	Lobes	λ	θ
2003								
M1	0.408	0.473	-0.525	-0.0599	0.227	0.528	0.344	-0.276
M2	-0.0700	-0.0955	0.291	-0.445	0.838	0.0187	0.140	0.125
M3	0.363	-0.0455	0.623	0.554	0.0940	0.402	0.0126	0.0308
M4	-0.336	0.753	0.117	0.368	0.220	-0.351	-0.108	-0.0536
M5	0.517	-0.260	-0.314	0.357	0.325	-0.578	-0.234	0.0316
M6	0.563	0.363	0.373	-0.478	-0.288	-0.319	-0.329	-0.259
2004								
M1	-0.350	0.316	0.457	-0.300	0.691	-0.00468	0.147	-0.00855
M2	0.0763	0.451	0.672	0.119	-0.561	-0.0976	0.0873	0.0284

TL	PL	PW	TD	SE	Lobes	λ	θ	TL
M3	0.869	0.0318	0.188	0.0477	0.324	0.319	0.0260	0.0452
M4	-0.315	-0.395	0.327	0.557	0.0507	0.570	0.0116	0.0570
M5	0.0514	0.203	-0.0905	0.748	0.314	-0.539	-0.142	-0.00179
M6	-0.122	0.706	-0.434	0.153	-0.0276	0.524	-0.357	-0.123
2005								
M1	0.319	0.404	-0.232	-0.475	0.633	0.236	0.0990	0.0315
M2	-0.400	-0.531	-0.0537	0.219	0.492	0.515	0.0302	0.0228
M3	0.771	-0.0832	0.0618	0.518	-0.0548	0.351	-0.00418	0.00897
M4	-0.247	0.497	0.660	0.0233	-0.117	0.491	-0.0167	-0.0123
M5	-0.264	0.540	-0.370	0.657	0.207	-0.164	-0.0492	-0.0397
M6	0.116	-0.0964	0.606	0.161	0.545	-0.536	-0.09361	0.0885
2006								
M1	0.454	-0.676	0.296	0.256	0.247	-0.351	0.265	-0.226
M2	0.624	0.0731	0.308	-0.311	-0.0888	0.637	0.0448	-0.129

TL	PL	PW	TD	SE	Lobes	λ	θ	TL
M3	0.178	0.681	0.430	0.457	0.266	-0.200	0.0146	0.0816
M4	0.471	0.170	-0.755	0.00558	0.420	-0.0551	-0.0288	-0.0674
M5	-0.367	-0.193	0.0433	0.272	0.641	0.584	-0.0681	0.0964

Table 7. The M matrix of eigenvectors from the canonical rotation of γ containing the quadratic and correlational estimates of selection on *Silene stellata*, using mature fruit production as the fitness component. In the last two columns are the estimates of linear (θ) and nonlinear selection (λ) on the new latent axes described by the eigenvectors. The nonlinear selection gradient is the eigenvalue of each eigenvector. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	TL	PL	PW	TD	SE	Lobes	λ	θ
2003								
M1	0.524	0.316	-0.418	-0.0234	0.259	0.618	0.353*	-0.188
M2	-0.176	0.0814	0.256	-0.420	0.844	-0.0884	0.0805	0.0248
M3	0.154	0.407	0.661	0.597	0.0994	0.0897	0.00885	-0.126
M4	0.186	-0.814	0.317	0.0735	0.103	0.433	-0.0454	0.168
M5	0.709	-0.182	-0.0913	0.109	0.181	-0.642	-0.255	-0.0702
M6	-0.365	-0.181	-0.463	0.670	0.409	-0.0568	-0.278	0.168
2004								
M1	-0.475	0.359	0.127	-0.398	0.684	-0.0451	0.230*	-0.0821
M2	0.500	0.189	0.698	0.265	0.291	0.270	0.0996	-0.0495

TL	PL	PW	TD	SE	Lobes	λ	θ	TL
M3	-0.189	-0.609	-0.206	0.362	0.467	0.450	0.0282	0.0232
M4	0.659	-0.0205	-0.452	-0.510	0.268	0.174	-0.0785	-0.0586
M5	0.221	0.113	-0.275	0.496	0.388	-0.683	-0.141*	0.149*
M6	-0.0740	0.672	-0.418	0.367	-0.0821	0.476	-0.383*	-0.128
2005								
M1	0.192	-0.504	0.00488	0.547	0.632	0.106	0.0721	0.00419
M2	0.225	0.380	-0.525	0.566	-0.182	-0.420	0.0256	0.0133
M3	0.561	0.572	0.0798	-0.282	0.509	0.119	0.00230	0.0476
M4	-0.536	0.273	-0.519	0.0387	0.260	0.547	-0.00644	0.0135
M5	-0.0909	0.382	0.612	0.547	-0.206	0.360	-0.0368	0.0177
M6	0.549	-0.234	-0.274	-0.00769	-0.446	0.608	-0.0521	-0.0340
2006								
M1	0.446	-0.651	0.345	0.138	0.377	-0.312	0.140	-0.117
M2	0.00116	0.428	-0.287	0.445	0.277	-0.678	0.0654	0.0480

TL	PL	PW	TD	SE	Lobes	λ	θ	TL
M3	0.336	0.556	0.670	0.239	0.0727	0.255	0.0105	0.0332
M4	-0.187	-0.113	-0.261	0.464	0.577	0.579	-0.0277	0.0703
M5	0.751	0.184	-0.495	-0.326	0.145	0.172	-0.0629	-0.0984
M6	0.298	-0.192	-0.190	0.636	-0.650	0.112	-0.236*	-0.0631

Table 8. The vector of linear parameter estimates (β) and the matrix of quadratic and correlational parameter estimates (γ) for *Silene stellata*, estimated from a multivariate logistic regression of floral traits on *H. ectypa* egg presence or absence. * P < 0.05; ** P < 0.01; *** P < 0.001.

	β	γ						
		TL	PL	PW	TD	SE	Lobes	DHT
2006								
Corolla tube length (TL)	0.150	0.0657						
Petal length (PL)	0.0652	0.187	-0.0415					
Petal width (PW)	0.100	-0.415**	0.321*	-0.0903				
Corolla tube diameter (TD)	-0.126	0.135	-0.131	0.135	-0.07			
Stigma exsertion (SE)	-0.0465	-0.0556	-0.109	0.0233	0.140	-0.136		
Lobes per petal (Lobes)	0.0569	-0.0125	0.0226	-0.256	0.0436	0.000352	0.131	
Display height (DHT)	0.485***	-0.0992	0.0876	0.122	-0.0222	-0.0371	-0.140	-0.11327

Table 9. The M matrix of eigenvectors from the canonical rotation of γ containing the quadratic and correlational parameter estimates for *Silene stellata*, using presence or absence of eggs as the response variable. In the last two columns are the estimates of linear (θ) and nonlinear curvature (λ) on the new latent axes of the response surface. * P < 0.05; ** P < 0.01; *** P < 0.001.

	TL	PL	PW	TD	SE	Lobes	DHT	λ	θ
2006									
M1	0.525	-0.154	-0.569	0.0595	-0.00854	0.550	-0.265	0.580*	-0.190
M2	0.702	0.384	-0.0460	-0.0294	-0.167	-0.566	0.0907	0.266	0.259
M3	-0.0373	0.698	0.213	-0.314	-0.285	0.526	0.0948	0.138	0.269
M4	0.172	0.237	0.381	0.787	0.330	0.199	-0.0351	-0.0136	-0.0896
M5	0.0561	-0.168	-0.164	0.185	-0.155	0.153	0.927	-0.292	0.469***
M6	0.0685	0.186	-0.124	-0.371	0.870	0.0191	0.223	-0.372	0.109
M7	0.439	-0.472	0.664	-0.325	0.00801	0.191	0.0402	-0.815**	0.159

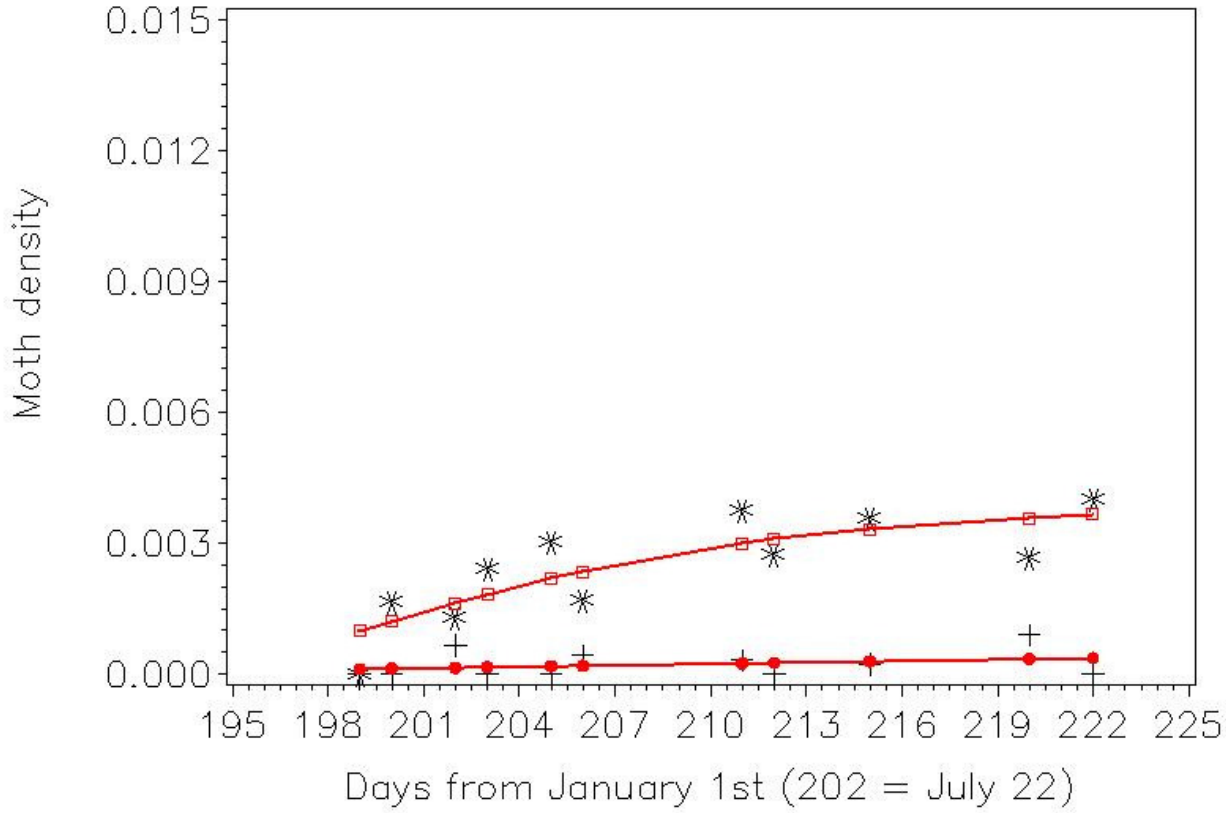
0.015
0.012
0.009
0.006
0.003
0.000
Moth density
195 198 201 204 207 210 213 216 219 222 225
Days from January 1st (202 = July 22)

Figure 1. Adult *Hadena. ectypa* and copollinator densities (number of moths observed per flower) across the *Silene stellata* flowering period at the meadow site in 2005. Black asterisks are copollinator raw density data and open squares are predicted values from the nonparametric regression. Black pluses are *H. ectypa* raw density data and closed circles are predicted values from the nonparametric regressions.

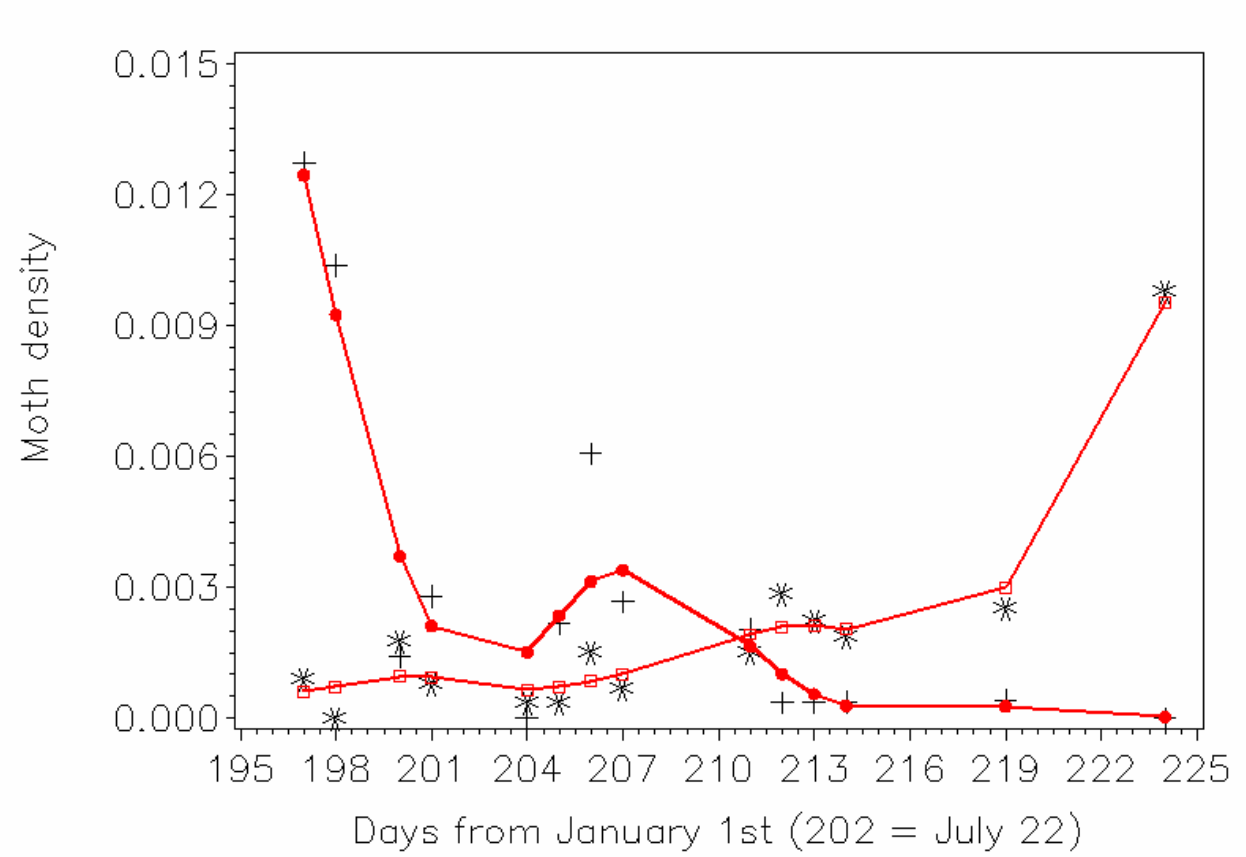
0.015
0.012
0.009
0.006
0.003
0.000
Moth density
195 198 201 204 207 210 213 216 219 222 225
Days from January 1st (202 = July 22)

Figure 2. Adult *Hadena. ectypa* and copollinator densities (number of moths observed per flower) across the *Silene stellata* flowering period at the meadow site in 2006. Black asterisks are copollinator raw density data and open squares are predicted values from the nonparametric regression. Black pluses are *H. ectypa* raw density data and closed circles are predicted values from the nonparametric regressions.

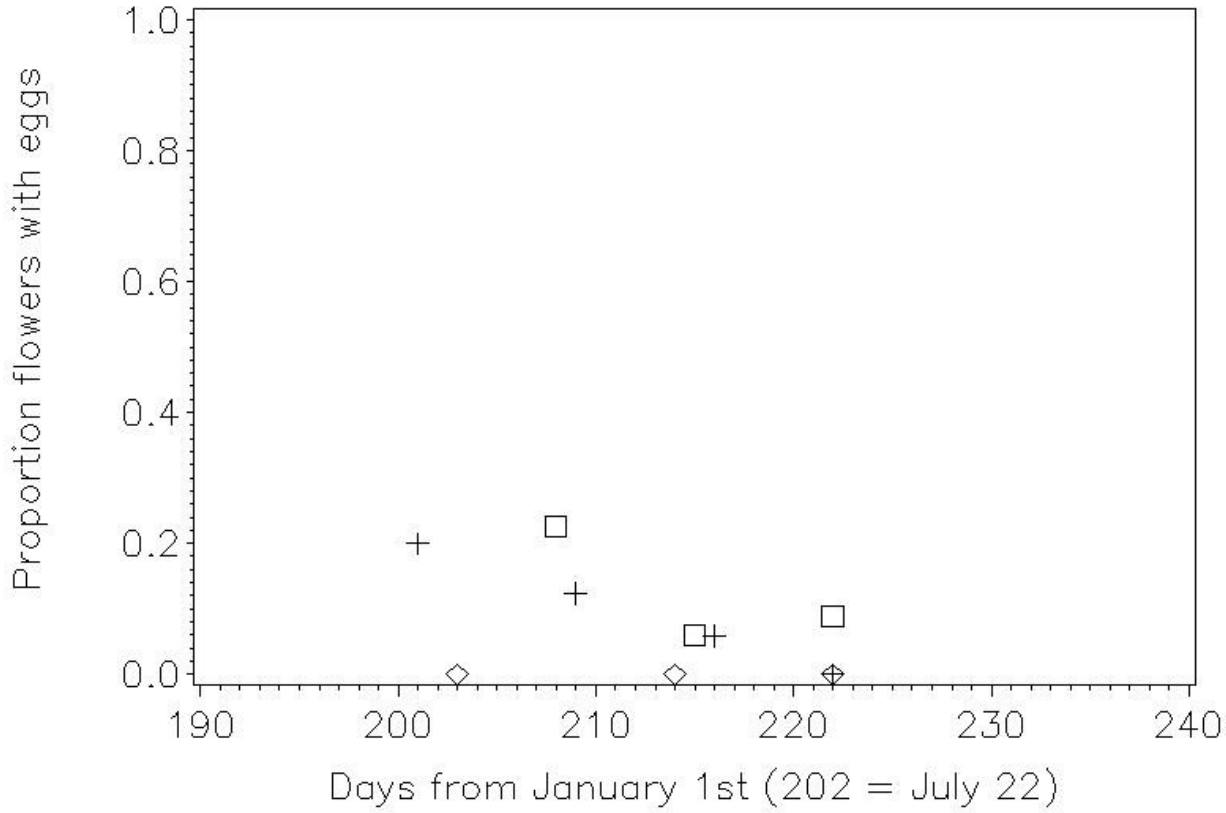

Figure 3. Proportion of *Silene stellata* flowers with *Hadena ectypa* eggs across the flowering period at three sites in 2005: Meadow (pluses), Woodland (squares), and Wind Rock (diamonds).

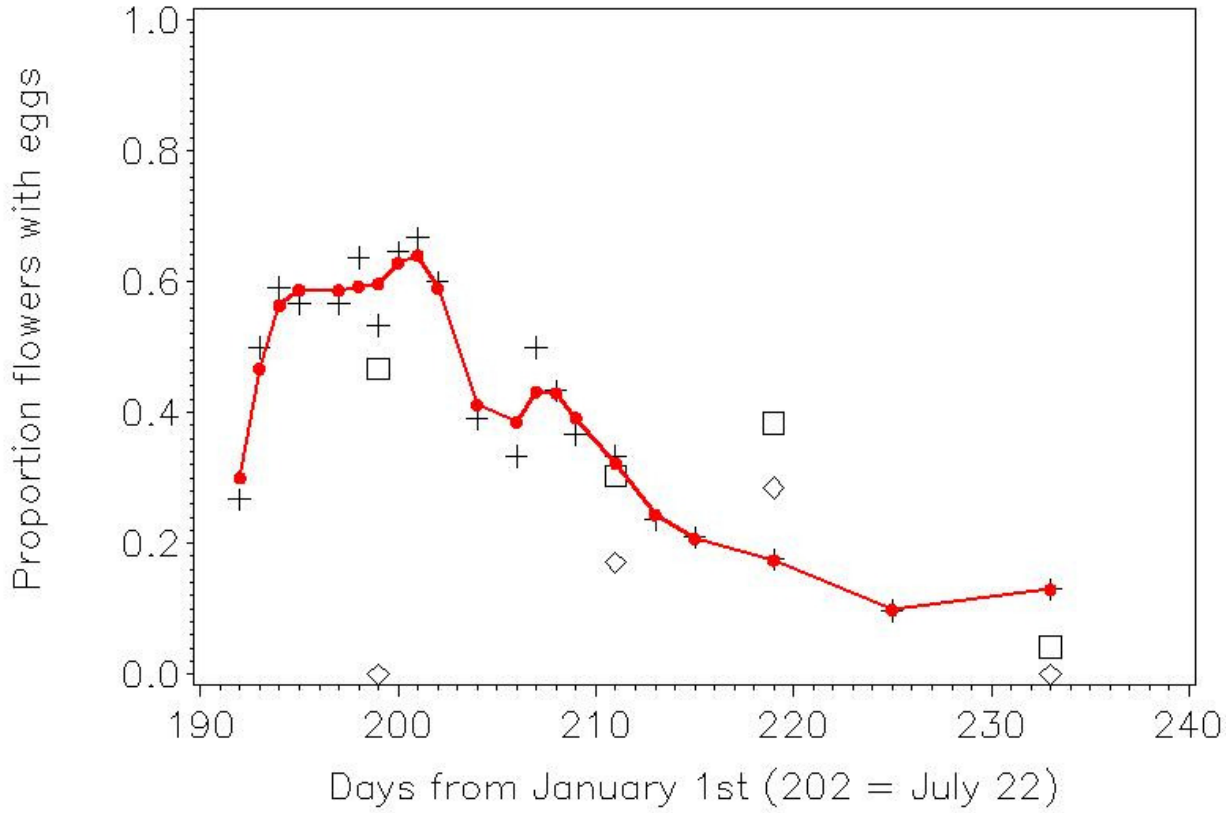

Figure 4. Proportion of *Silene stelalla* flowers with *Hadena ectypa* eggs across the flowering period at three sites in 2006: Meadow (pluses), Woodland (squares), and Wind Rock (diamonds). Closed circles are predicted values of egg density at the meadow site from the non-parametric regression.

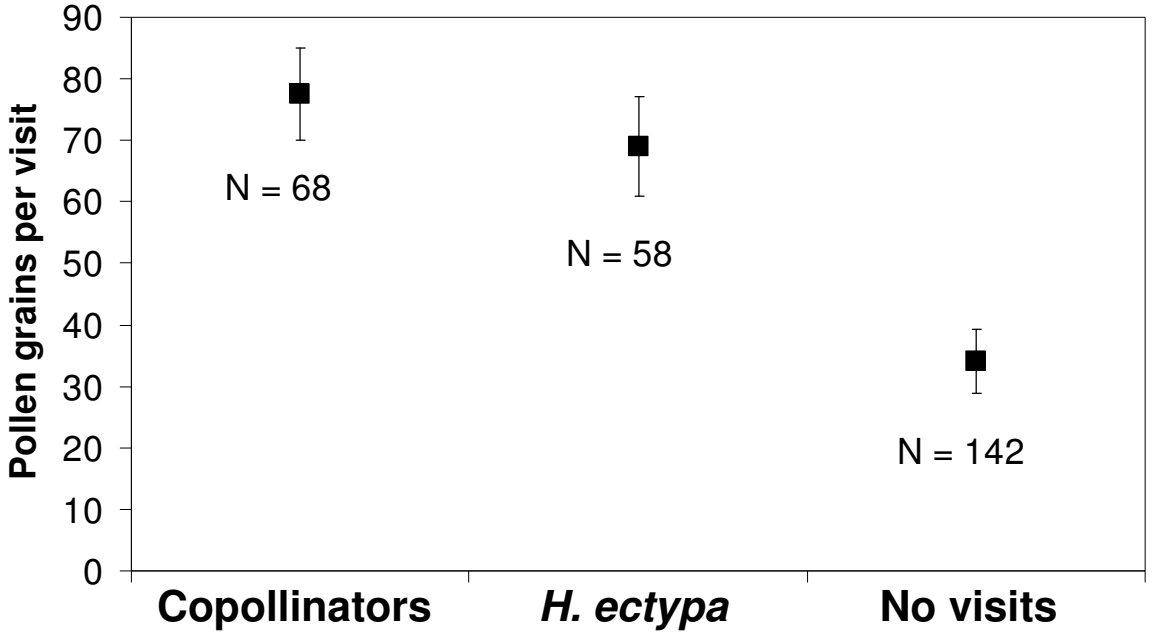

Figure 5. Average number of pollen grains deposited per visit on stigmas by copollinators, *Hadena ectypa* or unvisited stigmas. Pollen grain deposition between copollinators and *H. ectypa* were not significantly different, but both were significantly greater than pollen grains on unvisited stigma

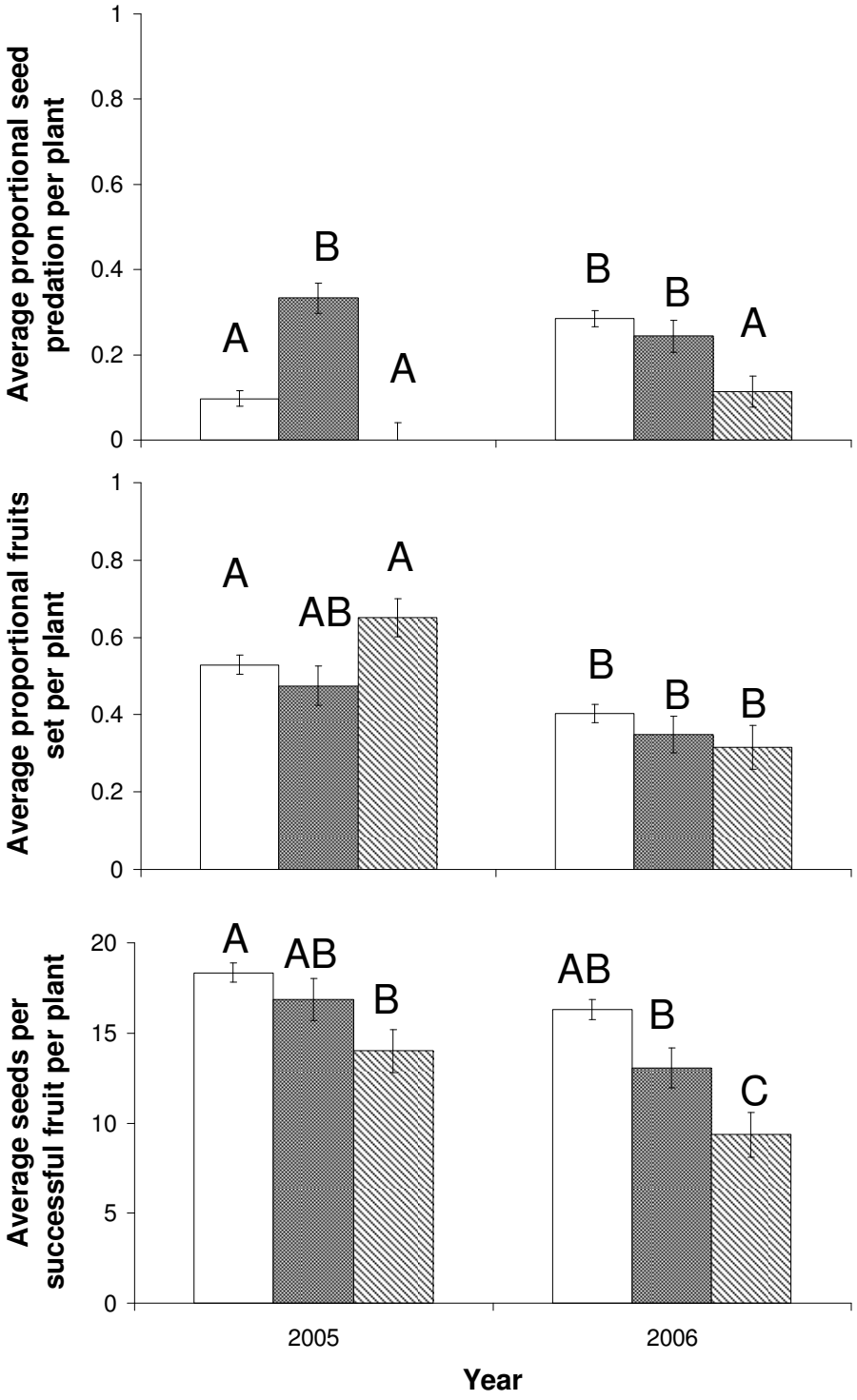
Average proportional seed predation per plant
1
0.8
0.6
0.4
0.2
0
A
B
A
B
B
A
Average proportional fruits set per plant
1
0.8
0.6
0.4
0.2
0
A
AB
A
B
B
B
Average seeds per successful fruit per plant
20
15
10
5
0
A
AB
B
AB
B
C
2005
2006
Year

Figure 6. Average fruit predation, fruit set, and seed set of *Silene stellata* at the Meadow (open bar), Woodland (shaded bar), and Wind Rock (cross – hatched bar) sites in 2005 and 2006. Treatment levels sharing the same letters are not significantly different.

Appendices

Appendix A. Example of Visual Basic code for simulating the nonparametric 95% confidence intervals of pollinator importance.

```
Sub bombusimp()
Dim vis(1 To 46) As Double
Dim dep(1 To 64) As Double

For i = 1 To 46
   vis(i) = ThisWorkbook.Sheets("Raw Data").Cells(i + 1, 3)
Next i

For j = 1 To 64
   dep(j) = ThisWorkbook.Sheets("Raw Data").Cells(j + 1, 4)
Next j

Set myrangedep = ThisWorkbook.Sheets("Sheet2").Range("A1:A64")
Set myrangevis = ThisWorkbook.Sheets("Sheet2").Range("B1:B46")
Set mstab = ThisWorkbook.Sheets("Sheet2").Range("E1:E10000")
Set vstab = ThisWorkbook.Sheets("Sheet2").Range("F1:F10000")

Dim meanstab(1 To 50) As Double
Dim deposition(1 To 64) As Double
```

```
Dim visitation(1 To 46) As Double
Dim meandep(1 To 10000)
Dim meanvis(1 To 10000)
Dim importance(1 To 10000) As Double
Dim y As Integer
Dim x As Integer
'the m index is the check of stability
For m = 1 To 50
'the n index is the number of iterations
For n = 1 To 10000
'now I need to randomly sample the original data sets o times and take their means
   For o = 1 To 64
      Randomize
      y = Int(Rnd * 63 + 1)
      deposition(o) = dep(y)
      ThisWorkbook.Sheets("Sheet2").Cells(o, 1) = deposition(o)
   Next o
   For p = 1 To 46
      Randomize
      x = Int(Rnd * 45 + 1)
      visitation(p) = vis(x)
      ThisWorkbook.Sheets("Sheet2").Cells(p, 2) = visitation(p)
   Next p
```

```
    meandep(n) = Application.WorksheetFunction.Average(myrangedep)
    ThisWorkbook.Sheets("Sheet2").Cells(n, 3) = meandep(n)
    meanvis(n) = Application.WorksheetFunction.Average(myrangevis)
    ThisWorkbook.Sheets("Sheet2").Cells(n, 4) = meanvis(n)
    importance(n) = meandep(n) * meanvis(n)
    ThisWorkbook.Sheets("Sheet2").Cells(n, 5) = importance(n)
Next n
meanstab(m) = Application.WorksheetFunction.Average(mstab)
ThisWorkbook.Sheets("Sheet2").Range("E1:E10000").Sort
Key1:=Worksheets("Sheet2").Range("E1")
ThisWorkbook.Sheets("Sheet2").Cells(m, 7) =
ThisWorkbook.Sheets("Sheet2").Cells(250, 5)
ThisWorkbook.Sheets("Sheet2").Cells(m, 8) =
ThisWorkbook.Sheets("Sheet2").Cells(9750, 5)
ThisWorkbook.Sheets("Sheet2").Cells(m, 9) = meanstab(m)
Next m
End Sub
```

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