ORIGINAL ARTICLE

MULTIYEAR STUDY OF MULTIVARIATE LINEAR AND NONLINEAR PHENOTYPIC SELECTION ON FLORAL TRAITS OF HUMMINGBIRD-POLLINATED *SILENE VIRGINICA*

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Pollination syndromes suggest that convergent evolution of floral traits and trait combinations reflects similar selection pressures. Accordingly, a pattern of selection on floral traits is expected to be consistent with increasing the attraction and pollen transfer of the important pollinator. We measured individual variation in six floral traits and yearly and lifetime total plant seed and fruit production of 758 plants across nine years of study in natural populations of Ruby-Throated Hummingbird-pollinated *Silene virginica*. The type, strength, and direction of selection gradients were observed by year, and for two cohorts selection was estimated through lifetime maternal fitness. Positive directional selection was detected on floral display height in all years of study and stigma exsertion in all years but one. Significant quadratic and correlational selection gradients were rare. However, a canonical analysis of the gamma matrix indicated nonlinear selection was common; if significant curvature was detected it was convex with one exception. Our analyses demonstrated selection favored trait combinations and the integration of floral features of attraction and pollen transfer efficiency that were consistent with the hummingbird pollination syndrome.

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

Darwin (1862) championed the study of floral evolution because examples of floral features that increase the fit of flower and pollinator to maximize a plant's reproductive success are common in nature. For example, Darwin reasoned that the very long nectar spurs, 30 cm on average, "in Madagascar *Angracum sesquipedale* must depend on some gigantic moth" (Darwin 1862). The giant moth was later identified as *Xanthopan morganii praedicta*

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(Rothschild and Jordan 1903), the subspecies epithet given to note the predictive power of natural selection 40 years earlier by Darwin, whereas evidence of the plant–pollinator interaction was not gathered for another 90 plus years (Wasserthal 1997). However, Darwin's insight on the dynamic interplay between phenotype and natural selection was not confined to the univariate case. Darwin and other early workers recognized flowers to be complex adaptations with selection acting on suites of traits, that is, "all the parts of a flower are coordinated" (Darwin 1862) and "flowers are harmonious units" (Stebbins 1951). Consequently, pollination syndromes (the convergent expression of floral traits due to

parallel selection exerted by similar pollinators) may be viewed as complex multivariate structures that consist of suites of correlated characters increasing the attraction and pollen transfer efficiency of their major pollinators, i.e., important mediators of selection (Stebbins 1951; Vogel 1954; Faegri and van der Pijl 1979; Fenster et al. 2004). In the parlance of phenotypic selection theory (Lande and Arnold 1983), we expect correlational selection or selection on trait combinations to be the main signature of selection on floral traits comprising pollination syndromes.

Despite the expectation that pollinator-mediated selection should be prevalent, there is a deficit of case studies of phenotypic selection on floral traits comprising pollination syndromes. Past studies have found evidence of directional selection influencing the efficiency of pollen transfer of major pollinators on floral traits such as tube length (Maad 2000), corolla width (Campbell 1989), and nectary-stigma distance (Caruso et al. 2003), and features of attraction such as display height (Johnston 1991). However, recent studies also document evidence of phenotypic selection on floral traits that is attributable to nonpollinator sources of selection. Selection is often found to be context-dependent operating only in some years or populations (e.g., Caruso 2000; Caruso et al. 2003), and correlated with abiotic factors such as drought (Maad 2000). Predispersal larval seed predators have also been shown to exert selection on floral traits such as flowering phenology (Wright and Meagher 2003; Parachnowitsch and Caruso 2008). In total, many studies suggest the possibility that with respect to pollinators, the selection surface describing covariance of fitness with traits may be flat with occasional association induced by ecological context. However, analysis of selection gradients focused on detecting selection on combinations of traits comprising pollination syndromes is key to detecting signals of pollinator-mediated selection, and these types of studies are rare. Furthermore, with some notable exceptions (e.g., Armbruster 1990; Brodie 1992), there has been a resounding lack of evidence for the role of correlational selection in the evolution of complex adaptations (Kingsolver et al. 2001).

Long-term selection on floral trait combinations may lead to the evolution of positive or negative genetic and phenotypic covariance of pairs of floral traits (Phillips and Arnold 1989). However, correlational selection is often not estimated, regardless of taxa and trait (Kingsolver et al. 2001; Blows and Brooks 2003). Lack of evidence for correlational selection may reflect the situation that when many traits are analyzed, correlational selection between a given pair of traits can be difficult to interpret in the context of the set of all possible correlational selection estimates. However, methods exist from response surface methodology to simplify the situation. Phillips and Arnold (1989), and later elaborated on by Blows and Brooks (2003) and Blows (2007), have indicated that an efficient way to detect curvature in the selection surface (Phillips and Arnold 1989), is to conduct a canonical trans-

formation of the matrix (gamma) containing the correlational and quadratic (stabilizing and disruptive) selection gradients. Canonical analysis of gamma is a powerful tool in studies of phenotypic selection 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 selection jointly on the original floral traits. If the transformation yields estimates similar to the original quadratic estimates then one can interpret that correlational selection is minimal. Otherwise, correlational selection is prevalent even if any single correlational selection gradient is not significant. We apply this underutilized approach here in our investigations of selection on *Silene virginica* (Caryophyllaceae) floral traits. As far as we are aware, no other study has used this multivariate approach to quantify phenotypic selection on traits that define pollination syndromes.

Here, we present approximate estimates of lifetime fitness by integrating the combined effects of selection across multiple flowering seasons in addition to a long-term phenotypic selection study on floral traits of hummingbird-pollinated *S. virginica.* It is reasonable to assume that hummingbirds are sources of selection on *S. virginica* floral traits. Pollination data and experimental manipulations demonstrate hummingbirds are the most important pollinators (Reynolds et al. 2009). Second, published (Fenster et al. 2006) and unpublished studies from our laboratory have demonstrated that hummingbirds preferentially visit some manipulated floral phenotypes and plant display attributes over others. Novel aspects of the study are that linear and nonlinear phenotypic selection on floral traits that explicitly contribute to a hummingbird pollination syndrome (Reynolds et al. 2009) are estimated from data collected across eight separate years, and additional estimates were made using lifetime seed and fruit production of two cohorts. Furthermore, canonical analysis was used to document the extent of correlational selection on floral traits and to characterize curvature in the selection surface. To assess the significance of hummingbird pollinator-mediated selection on the evolution of *S. virginica* floral traits, we aim to: (1) fully describe the type, direction, and magnitude of phenotypic selection across years and across a complete generation; (2) completely characterize the slope and curvature of the selection surface; (3) discuss how the selection estimates are consistent with the hummingbird pollination syndrome.

Materials and Methods
STUDY SYSTEM

Silene virginica is a short-lived herbaceous perennial wildflower of eastern North America. In natural populations, seeds germinate in the early spring, and plants overwinter as rosettes of basal leaves, growing a minimum of 2 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 nonsexual phase with elongating style, and ending in a female phase with receptive stigmas exserted well beyond the corolla tube opening and lasting one to three days. Male and female phase 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 the unfused petals held together by fused sepals, to form a functional long tube, with a dilute and copius nectar reward (Fenster et al. 2006; Reynolds et al. 2009). 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. 2009). Flowers do not autonomously set seed and vegetative reproduction is very limited. A study of the demography of the population is ongoing, 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 nonreproductive, nonreproductives may become reproductive, reproductives may become nonreproductive, or reproduce again. Multiple reproductive episodes are possible, although two or fewer reproductive bouts before death are most common in our study population.

STUDY SITE AND DESIGN

The study was performed near Mountain Lake Biological Station (Giles County, Virginia) at one site (elev~1100 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 (design 1) consisted of naturally occurring flowering plants found within two adjacent transects. The second and third (design 2 and 3, respectively) consisted of maternal sibships or paternal half siblings, which have also been used for estimating floral trait genetic variance and covariance (R. Reynolds, M. Dudash, and C. Fenster, unpubl. ms.).

For design 1, all flowering plants in each year were marked within 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 six flowered every year. The study was primarily cross-sectional because many new plants were added each year, but there was a longitudinal component because 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 germinated in spring 2002 on standard greenhouse soil (Sunshine HCI, Sun Gro Horticulture, Bellevue, WA) at the University of Maryland, College Park greenhouse. 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 sibling design were planted with 1 m spacing into three blocks of 60 plants with each block consisting of six rows and 10 columns. The plants originated from 43 maternal source plants. The maternal siblings from each of the 43 maternal families were randomly assigned to the three blocks and then they were randomized to a 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 four half siblings, with each half sibling 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 five rows and eight columns separated by 1 m. Single seedlings 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. The spacing of the plantings reflected the density of naturally occurring plants in the population.

DATA COLLECTION

Because we know that hummingbirds are the most important pollinator of *S. virginica* (Fenster and Dudash 2001; Reynolds et al.

2009) 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, and 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 just prior to natural fruit dehiscence, 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 (1992–1995 and 2003–2006), and each year two maternal fitness components (total fruit-set, total seed production) were measured. Because there were far fewer plants surviving and flowering in 2007, selection in this year was not quantified in terms of yearly selection pressures (however data from 2007 were used in the lifetime fitness assays, see below). 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-sibling 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. Although 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 were 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 were concerned that applying the repeated selection models may increase the type 1 error of finding a significant selection gradient in any given year. To account for the multiple testing the most significant selection gradient for each trait had to meet a false discovery rate (FDR) adjusted level of 0.0065 to ensure the proportion of type 1 errors at $Q = 0.05$ (Benjamini and Hochberg 1995). 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. 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 correlational selection to be strong as pollination syndromes are hypothesized to be integrated sets of floral trait combinations reflecting selection for attraction of pollinators and efficient pollen transfer resulting in successful fruit and seed production. Therefore, it seems appropriate to investigate nonlinear selection on linear combinations of floral traits.

The models we used were the following and were analyzed using the GLM and RSREG procedures in SAS version 9.1.2 (SAS 2004). We first fit models 1 and 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),

$$w_i/\bar{w} = \alpha + \eta f + \sum_{j=1}^{6} z_{ij}\beta_j + \varepsilon, \tag{1}$$

$$
\begin{aligned}
w_i/\bar{w} = \alpha + \eta f + \sum_{j=1}^{6} z_{ij}\beta_j + 1/2\sum_{j=1}^{6} z_{ij}^2\gamma_j \\
+ \sum_{j=1}^{6}\sum_{k=1}^{6} \gamma_{jk}z_j z_k(j \neq k) + \varepsilon,
\end{aligned}
\tag{2}
$$

where w is the measured fitness component, $\bar{w}$ the mean fitness, α the intercept, f the covariate flower number, 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,

$$w = \alpha + \eta + z'\beta + z'\gamma z + \varepsilon, \tag{3}$$

and then following Phillips and Arnold (1989) the matrix of quadratic and correlational estimates, γ, was diagonalized,

$$M\gamma M' = \Lambda, \tag{4}$$

where M is the 6×6 matrix of eigenvectors and Λ 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,

$$y = zM', \tag{5}$$

and then the transformed data were analyzed with a similar model as equation (3) (Simms 1990; Bisgaard and Ankenman 1996). The new model may be written as,

$$w = \alpha + y'\theta + y'\Lambda y. \tag{6}$$

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. The linear canonical coefficients were estimated but not reported, as we were chiefly concerned with curvature along the transformed axes. Quadratic selection gradients and the eigenvalues from the canonical rotation were doubled as indicated by equation (2) and by Stinchcombe et al. (2008). Because it is unclear that a negative (positive) quadratic selection gradient is truly stabilizing (disruptive), we will use the lexicon suggested by Phillips and Arnold (1989) in relation to describing nonlinear selection with negative (positive) coefficients indicating selection as convex (concave). A permutation test was used to calculate empirical P-values for hypothesis tests of the eigenvalues as there is evidence that the distribution of the eigenvalues under the null hypothesis of no nonlinear selection is nonzero (R. Reynolds et al., in press).

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. Furthermore, the pattern of selection did not vary appreciably when analyzed on fruit or seed production. It seems reasonable that selection via seed production, which is closer to fitness, should only be reported. However, phenotypic selection through fruit production does reveal important information in species with substantial seed predators such as the relative *S. stellata* (Kephart et al. 2006), and because phenotypic selection via fruit set is often reported in other systems, we report it here for comparison.

Finally a sign test was used (Wilcoxon 1945) to test the consistency of the signs of the directional, quadratic, and correlational selection gradients. This approach allows us to maximize the information from the estimated selection gradients across years. It may be the case that while in any single year significant
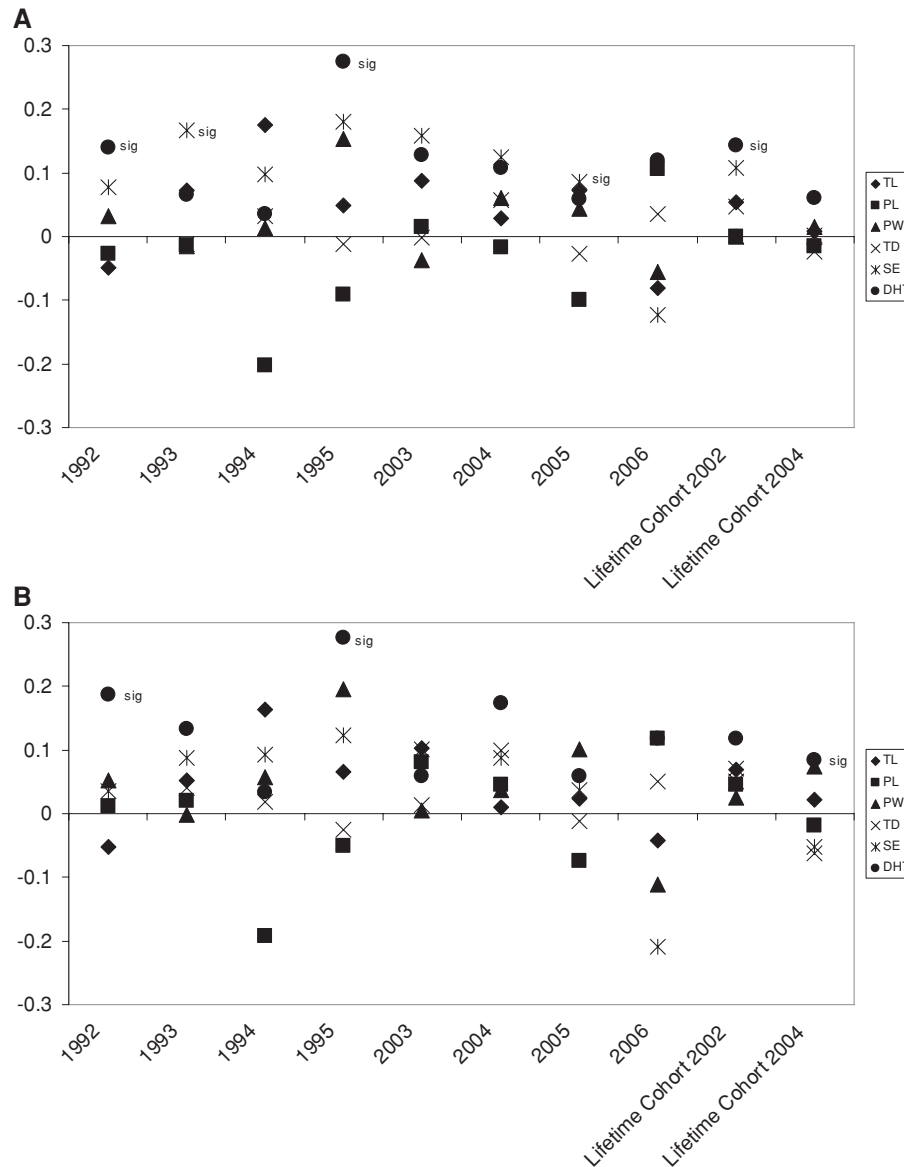
Figure 1. Variation among years in directional selection gradients through (A) fruit production (B) seed production. Trait codes: TL, Corolla tube length; PL, Petal length; PW, Petal width; TD, Corolla Tube diameter; SE, Stigma exsertion; DHT, Display height. Sig = Significant at the FDR adjusted type 1 error rate of $Q = 0.05$.

selection gradients went undetected, however the signs of the selection gradients may remain constant among years. The sign test tests the null hypothesis that the sign of the selection gradients would fluctuate at random.

Results

YEARLY ANALYSES

Linear selection

The means, standard deviations, and sample sizes for *S. virginica* plants of the yearly studies are reported in Table S1. The parameter estimates for the yearly analyses are included as four tables in a digital Supporting information (Tables S2–S5) file. Significant

(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 (Fig. 1A; Table S2). 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 (Fig. 1B; Table S3), and after controlling the FDR at $Q = 0.10$, in 2004. The sign test indicated the probability was less than 0.05 that the positive signs of the selection gradients for stigma exsertion and display height fluctuated at random across years.

Table 1. Eigenvalues (λ_i) and eigenvectors (M_i) of the six latent axes with statistically significant nonlinear selection. *P*-values are from a permutation test.

Year	Fitness component	$\mathbf{M}_i$	TL	PL	PW	TD	SE	DHT	λ_i	*P*-value
1994	Fruit	M2	0.482	0.242	0.707	0.0109	0.355	−0.288	−0.338	0.046
1994	Seed	M3	−0.598	−0.244	−0.0312	−0.0912	0.463	0.599	−0.214	0.036
1995	Fruit	M6	−0.450	−0.469	0.346	0.127	0.443	0.495	0.668	0.021
2003	Fruit	M3	0.145	0.500	0.102	−0.745	0.401	−0.0444	−0.200	0.026
2005	Seed	M2	0.180	−0.0294	−0.575	−0.464	0.384	0.524	−0.196	0.007
2006	Seed	M1	0.063	−0.299	0.681	−0.599	0.138	0.254	−1.25	0.022

Thus, the direction of selection on these traits was constant among years.

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 uncorrected alpha $= 0.05$ level (Tables S2 and S3). The sign test indicated that quadratic selection on stigma exsertion through seed production was consistently negative (convex downward) across years. The sign test also indicated correlational selection through fruit production was consistently negative between corolla tube length and stigma exsertion, and through seed production consistently positive between tube diameter and display height.

Using canonical analysis, we found statistically significant nonlinear selection on at least one latent axis in 5 of the 8 years, suggesting selection occurred commonly on trait combinations, and with one exception the eigenvalues were negative. Table 1 contains the eigenvectors with statistical support for nonlinear selection ($P < 0.05$). All eigenvectors and eigenvalues are reported in Tables S4 and S5. Using fruit production as our fitness measure, two eigenvalues (1994, 2003) provide significant statistical support ($P < 0.05$), and four eigenvalues (1992, 1994, 1995, 2006) provide marginal evidence ($0.05 < P < 0.10$) for convex nonlinear selection. One eigenvalue had solid statistical support for concave upward nonlinear selection in 1995. Using seed production as our fitness measure, three eigenvalues (1994, 2005, 2006) provide significant statistical support ($P < 0.05$), and two eigenvalues (1994, 2003) provide marginal evidence for convex nonlinear selection ($0.05 < P < 0.10$). One eigenvalue had marginal statistical support for concave nonlinear selection (2003).

SELECTION THROUGH LIFETIME MATERNAL FITNESS COMPONENTS

There was a decline in survivorship and probability of flowering for plants in the 2002 and 2004 cohorts (Fig. 2). 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 S6.
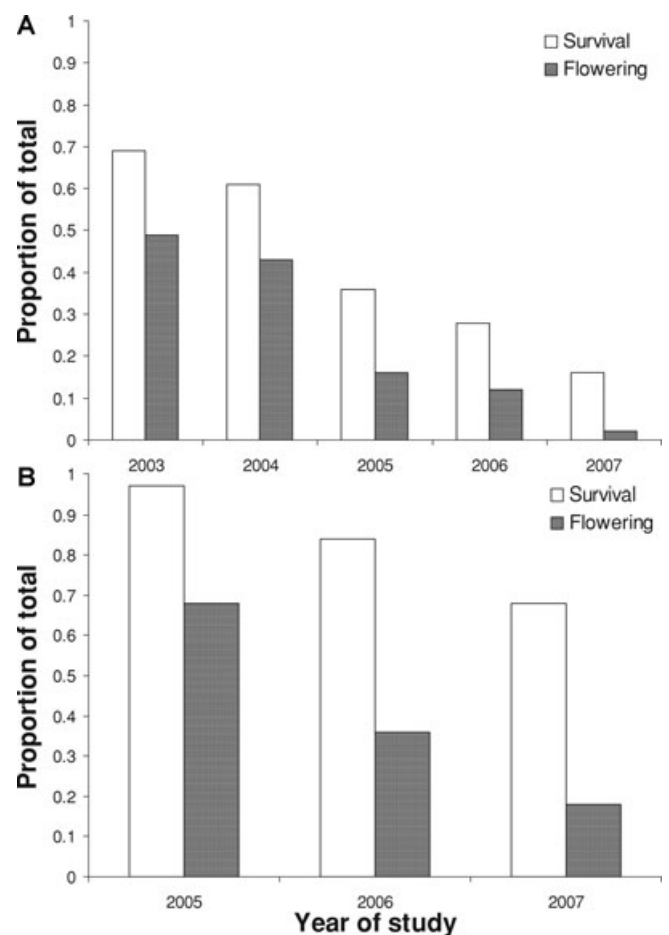

Figure 2. Probability of *Silene virginica* survival and flowering across the years of study for two cohorts. (A) The first cohort was planted in 2002 and (B) the second planted in 2004.

Significant positive linear selection was detected on display height through fruit production using the 2002 cohort (Table S7) and through seed production for the 2004 cohort (Table S8).

Nonlinear selection

Following Bonferroni correction, significant negative correlational selection was detected between petal length and petal width through fruit production in the 2002 cohort (Table S7), and positive correlational selection was detected between petal length and petal width through seed production in the 2004 cohort (Table S8). Viewing the results per table, at the alpha $= 0.05$ level, yielded significant convex selection on corolla tube diameter through both fruit and seed production in the 2002 cohort (Tables S7 and S8).

Canonical analysis demonstrated no statistical support for nonlinear selection through lifetime fruit or seed production (Tables S9 and S10, respectively).

Discussion

With estimates of linear and nonlinear phenotypic selection across eight years and separate estimates of selection across a full generation of *S. virginica*, we obtained a comprehensive picture of the type, strength, and direction of selection acting on floral traits in a single population. Positive directional selection was observed through fruit and seed production on display height (8/8 years) and stigma exsertion (7/8 years). However, selection on these traits was only of a large enough magnitude to be significant in two of the eight years for each case. Significant directional selection was not detected on the other floral traits when conservatively controlling for type 1 error. A notable advantage is the replicated nature of this study compared to a shorter-term analysis, which may have concluded that linear selection was altogether absent. Although it is true that most years selection on stigma exsertion and display height was not statistically significant, we reject the hypothesis that linear selection is equally likely to be positive or negative with the sign test.

Apparently nonlinear selection is weak and inconsistent on *S. virginica* floral traits and trait combinations. Using unadjusted alpha levels, there were numerous examples of significant correlational and quadratic selection gradients from the yearly analyses, but they appeared to be false positives after multiple testing correction. However, the sign test revealed that while weak, correlational selection was consistently negative between corolla tube length and stigma exsertion and consistently positive between corolla tube diameter and display height. Analysis of selection through lifetime fitness demonstrated a significant correlational selection gradient between petal length and width, but the sign changed depending on the cohort. The canonical analysis allowed us to verify that selection on trait combinations was a common feature in the data despite the few cases of significant correla-

tional selection from gamma. Correlational selection was clearly manifested as nonlinear selection on linear combinations of floral characters.

The pattern of phenotypic selection observed on *S. virginica* floral traits was consistent with the hummingbird pollination syndrome. Thus, the pattern of phenotypic selection is consistent with the notion that hummingbirds are responsible for the origin and or maintenance of floral trait divergence between *S. virginica* and its nonhummingbird-pollinated relatives. For example, in *S. virginica*, exserted stigmas are associated with hummingbird pollination, nonexserted stigmas with its sister species, large bee-pollinated *S. caroliniana* (Reynolds et al. 2009), and we observe positive linear selection on long stigmas in a *S. virginica* population. We also found that selection appeared to be convex on stigma exsertion, implying that the increase in fitness attributable to increasingly exserted stigmas has its limits. Therefore, it seems plausible that the selection on stigma exsertion, and its functional significance in increased pollen transfer efficiency of large bodied hummingbirds could have driven the divergence of stigma morphology between the two sister taxa. *Silene stellata*, with exserted stigmas, is the putative ancestral species in the three species clade (Burleigh and Holtsford 2003; Popp and Oxelman 2007). Thus, an alternative explanation is that negative directional selection on stigma exsertion by large bees of *S. caroliniana* could be acting in place of or in tandem with selection observed in *S. virginica.*

Hummingbirds as selective agents maintaining or underlying floral divergences has been shown using comparative approaches, e.g., *Penstemon* (Castellanos et al. 2003; Wilson et al. 2007), *Costus* (Kay et al. 2005), and Antillean Gesneriaceae (Martén-Rodríguez et al. 2009), also reviewed in Fenster et al. (2004). In addition, following trait manipulation, hummingbirds are often attracted to classic "hummingbird" traits as was seen in the F2 of a cross between bee- and hummingbird-pollinated *Mimulus* species (Schemske and Bradshaw 1999). A definitive role of pollinator-mediated selection in the generation of floral diversity within *Silene* will require the mapping of trait evolution on a phylogeny and matching it with transitions to alternative pollinators.

Under the pollination syndrome concept, phenotypic selection is expected to occur on floral trait combinations. Therefore, we expect floral traits in natural populations should typically be under correlational selection. Ruby-throated hummingbirds are the most important pollinators of *S. virginica* (Fenster and Dudash 2001; Reynolds et al. 2009) and are known to prefer particular floral trait combinations in experimental trials (e.g., Fenster et al. 2006). Additional evidence from unpublished experimental manipulations also have revealed that floral traits are selected in a nonadditive way (C. Fenster, C. Williams, R. Reynolds, and M. Dudash, unpubl. ms.), and the results from the canonical analysis presented here also indicate that floral trait combinations are under selection. Berg (1960) hypothesized and found support for

the concept that traits composing flowers, and 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. The concept of 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. There is also evidence at the within species level that populations with more specialized pollination systems having greater phenotypic integration, reflecting pollinator-mediated selection (Pérez et. al. 2007; Pérez-Barrales et al. 2007; but see Herrera et al. 2002).

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 both the attraction and pollen transfer efficiency of queen bumblebees. Correlational selection was related to attracting bees to tall flowers, but the increased success was only seen when combined with flowers exhibiting smaller labellums, 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. To our knowledge, there are only four studies that have documented phenotypic correlational selection on floral traits (Ordano et al. 2008). However, the two trait correlations that have been documented to be under selection often represent general features of pollinator attraction that are not specific to pollination syndromes.

The extent to which we were able to detect and interpret correlational selection on floral traits within a pollination syndrome explanatory framework relies less on correlational selection estimates from gamma and more from the canonical analysis. As the number of traits measured increases from three, as in O'Connell and Johnston's (1998) study, to six as in the present study, the number of correlational terms to estimate and interpret increases from 3 to 15. Trivial as it may seem to attempt the full second-order polynomial model in this case, it is necessary to estimate all these terms to get a complete picture of the slope and curvature in the selection surface. We were able to find indications of correlational selection on three pairs of floral traits, but considering the 18 additional second-order terms (the 12 pairs of floral traits on the off diagonal and the six quadratic terms on the diagonal), interpretation becomes nearly intractable. Rather, we conclude that the selection surface contains curvature in directions that are not parallel to the measured traits, instead of speculating on the adap-

tive significance of each correlational selection gradient (Blows and Brooks 2003). Canonical analysis of gamma enabled us to detect curvature on at least one latent axis in five of the eight years. Evidently, phenotypic selection on floral trait combinations was a common feature from our data, which is exactly what is expected under the pollination syndrome concept.

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). We can at least make a hypothesis of which trait combinations are under nonlinear selection by examining the trait loadings on the eigenvectors associated with nonlinear selection. Of the eigenvectors with solid statistical support for curvature (see Table 1), the largest trait loading corresponded to petal width in three of six cases. In those instances, other traits moderately associated with the nonlinear selection were tube diameter (2006), tube diameter and display height (2005), and tube length (1994: Fruit). In another case (2003), tube diameter was most strongly associated with nonlinear selection and petal length and stigma exsertion moderately so. In the remaining two cases (1994: Seed, 1995), the nonlinear selection was associated moderately with several floral characters. If curvature was detected on the selection surface, in four of six cases it was along canonical axes most associated with tube diameter and/or petal width. During 2006 (Table S3) correlational selection was detected on tube diameter × petal width, and stabilizing selection was detected on tube diameter through seed production. However, in both cases we could not exclude the possibility these results were false positives. Using an experiment approach, we did find a nonadditive preference of hummingbirds for combinations of large-sized flowers and wide tubes (Fenster et al. 2006). A canonical analysis, and an experimental approach, and to a lesser degree selection gradient estimates of gamma all suggest that in some years selection acts on floral trait combinations of petal width and tube diameter. The approaches we have deployed to quantify nonlinear selection on *S. virginica* floral traits have demonstrated at the very least that hummingbirds select for syndrome characters of attraction (e.g., petal width, display height) and pollination transfer efficiency (e.g., tube diameter, tube length) in a nonadditive way.

There are several limitations of the present analysis. First, we were only able to estimate selection on female fitness components. It is possible that selection on floral traits may be acting via more efficient pollen export as it has been shown in *Ipomopsis* (Campbell 1989). Furthermore, a lack of pollen limitation for the measured maternal fitness components (Dudash and Fenster 1997) might suggest selection to be more through male reproductive success (Wilson et al. 1994) instead of female. However, a lack of pollen limitation does not preclude the detection of maternal fitness and trait covariation. In the pollen limitation experiment (Dudash and Fenster 1997), control or pollen supplementation

treatments were not controlled according to floral trait variation. Thus, the pollen limitation experiment does not provide information on the potential for fitness and trait covariation at the individual level. Although pollen limitation of seed set was not detected for the 1992–1995 selection studies, it may have been operating during the 2003–2007 studies. Finally, natural selection on floral traits in *S. virginica* could also come from nonpollinator sources such as herbivores (Strauss and Irwin 2004) and abiotic factors (Galen 1999). 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).

Notwithstanding the above limitations, we still detect phenotypic selection that is interpretable through pollinator-mediated selection. The consistent finding of directional and correlational selection on floral features with a functional relationship for pollen transfer and pollinator attraction supports the hypothesis that floral character evolution in *S. virginica* reflects hummingbird-mediated selection.

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Supporting Information

The following supporting information is available for this article:

Table S1: Means (SD) of the fitness data and floral traits (millimeters) for *Silene virginica* taken across all plants in each year of study.

Table S2: 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*.

Table S3: 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*.

Table S4: 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*.

Table S5: 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*.

Table S6: Means (SD) of the fitness data and floral traits (millimeters) across years of study for two cohorts of *Silene virginica* plants, the first planted in 2002 and flowering from 2003 to 2007 and for the other planted in 2004 and flowering from 2005 to 2007.

Table S7: 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*.

Table S8: 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*.

Table S9: 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*.

Table S10: 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*.

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