

**EXPERIMENTAL FLORAL AND INFLORESCENCE TRAIT
MANIPULATIONS AFFECT POLLINATOR PREFERENCE AND
FUNCTION IN A HUMMINGBIRD-POLLINATED PLANT¹**

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- *Premise of the study:* Controversy is ongoing regarding the importance of pollinator-mediated selection as a source of observed patterns of floral diversity. Although increasing evidence exists of pollinator-mediated selection acting on female reproductive success, there is still limited understanding of pollinator-mediated selection on floral traits via male reproductive success. Here we quantify potential selection by the ruby-throated hummingbird, *Archilochus colubris*, on four floral traits of hermaphroditic *Silene* exerted through male floral function.
- *Methods:* In single trait manipulative experiments we quantified hummingbird visitation preference and/or fluorescent dye (a pollen analog) donation as a function of number of flowers displayed (inflorescence size), height of the floral display (inflorescence height), floral color, and corolla tube length.
- *Key results:* Hummingbirds preferred to visit larger floral displays and floral displays at greater height, likely representing a general pollinator preference for larger, more visible signals and/or greater rewards. In addition, hummingbirds preferred to visit red flowers, and male function was greater in flowers manipulated to have longer corolla tubes.
- *Conclusions:* Selection pressures exerted by hummingbirds on *S. virginica* floral and inflorescence design through male reproductive success are consistent with the contemporary expression of floral traits of *S. virginica* relative to related *Silene* species with different pollinators, and they are consistent with the hummingbird syndrome of traits expressed by *S. virginica*.

Key words: male fitness; male reproductive success; pollination syndromes; selection; *Silene virginica*; trait manipulation.

The long-standing interest in the role of pollinators as selective agents on floral design since Darwin (1862) continues to the present (e.g., Campbell, 2009; Harder and Johnson, 2009). It has evolved into a lively discussion on the relative role of pollinator-mediated selection vs. alternative mechanisms in their contribution to the evolution of floral and inflorescence design (Waser et al., 1996; Fenster et al., 2004; Ollerton et al., 2006; Strauss and Whittall, 2006; van Kleunen et al., 2008) and plant speciation (Whittall and Hodges, 2007; Kay and Sargent, 2009). Much of the data generated to date have focused on female reproductive success, seed and/or fruit production, owing to the relative ease in which these data may be collected (e.g., Peakall and Handel, 1993; Galen and Cuba, 2001; Caruso et al., 2003; Reynolds et al., 2010). However, recently greater attention has been paid to the role of selection on floral traits through male reproductive function, though it remains difficult to quantify success (Campbell et al., 1991; Snow and Lewis, 1993;

Arathi and Kelly, 2004; van Kleunen and Burczyk, 2008). Here we assess the potential role of pollinator-mediated selection on floral and inflorescence design through male reproductive success using experimental manipulations of floral and inflorescence traits representing biotic pollination broadly and the hummingbird pollination syndrome manifested by *Silene virginica* (Reynolds et al., 2009) specifically.

Studies of selection in the field have grown since the development of methodology to detect phenotypic selection with Lande and Arnold's (1983) seminal approach. Most of these field studies also have focused on how floral and inflorescence design influences female function and have virtually ignored male function owing to the difficulties in measuring pollen movement or conducting paternity analyses (e.g., Galen, 1989; Caruso et al., 2003; Reynolds et al., 2010). However, several studies have measured selection via male reproductive success, either by taking advantage of systems in which pollen can be easily tracked, for example, pollinia (e.g., O'Connell and Johnston, 1998; Maad, 2000), or by combining phenotypic selection approaches with molecular-based paternity analyses (e.g., Morgan and Conner, 2001; van Kleunen and Ritland, 2004; van Kleunen and Burczyk, 2008, and references therein). Although these molecular-based paternity studies of phenotypic selection are informative and provide an understanding of selection acting through male reproductive success, they suffer along with all phenotypic selection studies in that the selective agents are inferred and not directly measured (e.g., Mitchell-Olds and Shaw, 1987; Conner et al., 2009). Thus, in addition to phenotypic selection analyses, experimental manipulations are needed to directly demonstrate that it is the pollinators that are mediating

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the observed selection patterns (e.g., Mitchell-Olds and Shaw, 1987; Conner et al., 1996; Sandring and Agren, 2009).

Manipulation of the floral phenotype has long been a part of studies quantifying pollinators as potential selective agents since Clements and Long (1923), and recently has been reviewed by Campbell (2009). These studies have successfully demonstrated that pollinators can mediate selection on floral design associated with attraction (e.g., Clements and Long, 1923; Cresswell and Galen, 1991; Campbell et al., 1996; Fulton and Hodges, 1999; Schemske and Bradshaw, 1999; Temeles and Rankin, 2000; Fenster et al., 2006; Gomez et al., 2006), pollen transfer efficiency (Nilsson, 1988; Campbell et al., 1996; Fenster et al., 2009) and nectar reward (e.g., Melendez-Ackerman and Campbell, 1998; Schemske and Bradshaw, 1999; Fenster et al., 2006), as well as inflorescence design (Klinkhamer and De Jong, 1993; O'Connell and Johnston, 1998; Maad, 2000; Reynolds et al., 2010). However, to our knowledge very few phenotypic manipulative studies have quantified the functional significance of trait variation in terms of male reproductive success (Temeles and Rankin, 2000; Castellanos et al., 2004, 2006; Conner et al., 2009; Ren and Tang, 2010). Furthermore, Harder and Johnson (2009) have emphasized that when conducting experimental manipulations, it is preferable to manipulate trait variation to reflect the variation found in nature, to assess this variation on fitness (or fitness proxies) in both directions of the mean, and to have appropriate controls to facilitate the interpretation of the results, experimental conditions that often are not met in studies.

We previously demonstrated that the ruby-throated hummingbird (*Archilochus colubris*) is the most important pollinator of *Silene virginica* (Fenster and Dudash, 2001; Reynolds et al., 2009) and that the pattern of selection on floral traits via female reproductive success is consistent with the hummingbird pollination syndrome manifested by *S. virginica* (Reynolds et al., 2010). Additionally, we have shown that fluorescent dye is a good pollen analog for *S. virginica* because it enables us to use fluorescent dye particle transport as a proxy for the donation of pollen by different morphologies, an important component of male reproductive fitness (Fenster et al., 1996). Here we examine the relation between floral trait and inflorescence design variation in *S. virginica* and male function as quantified by hummingbird visitation preference and pollen transfer, using fluorescent dye as a pollen analog for the latter. We focus on four traits, two general and broadly related to pollinator attraction (floral display size and height) and two potentially more closely associated with the attraction of hummingbirds (red color) and efficient pollen transport via hummingbirds due to pollinator fit (Nattero et al., 2010) with flowers of *S. virginica* (relatively long corolla tube length). These four traits also were chosen because they differ among *S. virginica* and two related and co-occurring *Silene* species (Burleigh and Holtsford, 2003; Popp and Oxelman, 2007), large bee- and hawk moth-pollinated *S. caroliniana* and nocturnally moth-pollinated *S. stellata* (Reynolds et al., 2009). Consequently, we quantify the role of hummingbird-mediated selection in either the origin or maintenance of trait differentiation of *S. virginica* with two closely related *Silene* species, within the experimental framework suggested by Harder and Johnson (2009).

MATERIALS AND METHODS

Study organism and study site—*Silene virginica* (Caryophyllaceae) is a common plant of eastern North America that exhibits floral traits, including red

tubular flowers, large quantities of deeply held nectar, and lack of landing pads, nectar guides, or floral odor (Fenster and Dudash, 2001), consistent with the hummingbird pollination syndrome (Faegri and van der Pijl, 1979; Reynolds et al., 2009). This short-lived hermaphroditic perennial (Dudash and Fenster, 1997) is protandrous and highly outcrossing, though geitonogamy may occur (Dudash and Fenster, 2001). Flowering occurs from mid-late May through June at the study site near Mountain Lake Biological Station in Virginia, USA. *Silene virginica* is primarily pollinated by the ruby-throated hummingbird (*Archilochus colubris*) (Dudash and Fenster, 1997; Reynolds et al., 2009), which transports the pollen mainly at the base of its beak, on its forehead (Fenster et al., 1996).

This study was conducted at an elevation of 1330 m at Mountain Lake Biological Station in Giles county, Virginia, USA (Salt Pond Mountain, 37°22'32"N, 80°31'20"W). We maintained approximately 150 potted plants grown from field-collected seed to construct the arrays in three open "arenas" (no tree canopy directly overhead) to examine the role of floral display size, floral presentation height, floral color, and corolla tube length on male reproductive success. In a previous study (Fenster et al., 1996), we demonstrated that pollen and fluorescent dye movement were significantly correlated with one another in *S. virginica* such that dye movement is a reasonable proxy for pollen movement in this system. Here we quantified male reproductive success using fluorescent dye as a pollen analog for the three traits: floral display size, floral display height, and corolla tube length. The amount of dye powder was deposited onto the anthers in a consistent manner to standardize the load to the best of our ability while introducing a consistent error across experimental treatments. Hummingbird visitation preference also was quantified for its relation to trait expression for floral display size, floral display height, and floral color. Because of the positive relation between hummingbird visitation and dye transfer (see below under Results, Floral Display Size and Height), we used hummingbird visitation as a surrogate for male fitness for our floral color experiment. Plants and flowers were artificially manipulated to examine the association of floral trait expression with hummingbird attraction and pollen transfer efficiency. All treatments spanned the range of variation for that trait found in a nearby *S. virginica* population (Reynolds et al., 2009, 2010), with the extremes representing approximately the mean \pm 2 SD. The effect of the following treatments on hummingbird visitation and pollen movement was based on a minimum of 12 different hummingbirds for each experiment (twelve or more hummingbirds, nearly always females, were observed competing for access to the arrays throughout the sum of all trials for each experiment). All observations were made until hummingbird behavior became erratic owing to intraspecific interactions, or feeding was terminated, or until 15 min after the last visit to the array.

All dye particles transferred from pollen donors onto stigmas of each flower were counted with an epifluorescent microscope. Fluorescent dye particles within each experiment were counted in a standardized manner by only one investigator to reduce human error. To avoid pseudo-replication, a plant average for dye particles received was used for all analyses. All statistical procedures were performed using SAS version 9.1 (SAS Institute, 2004).

Floral display size—The effect of floral display size (one vs. three flowers) on male reproductive success was examined by quantifying the frequency of hummingbird visits and the transfer of fluorescent dye powder from male-phase donor plants to recipient female-phase plants. In each array, two donor plants were surrounded by six recipient female-phase plants with all plants spatially separated by 1 m; this spatial distance arrangement also was used for all the following experiments. All plants were placed on inverted 6-inch plastic plant pots to enhance the attractiveness of the overall floral display and to facilitate observation of hummingbird visits to male-phase donor plants. One donor plant had one flower in male phase, and the other donor plant had three flowers, with at least one in male phase. We randomly chose male donor plants that had approximately equivalent floral display heights and flower sizes. Each of the six recipient female function plants surrounding the male donor plants was manipulated to have two flowers in female phase, and each plant was used only once as a recipient during the trials. To the single experimental flower on each male donor plant, orange or green fluorescent dye powder was applied to two (of five) dehiscing anthers with a toothpick. All combinations of donor plants, their position within the array, dye color, and number of flowers were used once over the four trials with each trial occurring between 10:00 AM and 1:00 PM on a different day. No significant differences were found among runs; therefore, all data were pooled into one analysis that used a paired *t* test in which dye particle deposition associated with display size was compared on each female-phase recipient plant as the replicate for the analysis (six female recipients per day \times 4 d = 24 replicates). Data were square-root transformed to meet the assumptions of the *t* test.

Hummingbirds were observed from the beginning of each trial until hummingbirds had not visited the array for at least 15 min, a period ranging from

30 to 90 min. The observer position was about 3 m from the array. We analyzed the effect of display size on hummingbird visitation by summing all visits ($N = 40$) to the one- and three-flower donor plants across the 4 d and tested hummingbird preference with a two-tailed χ^2 test based on a 2×2 contingency of two treatments and whether the treatment was visited or not visited.

Floral display height—The effect of floral display height on male reproductive fitness was examined by using plant arrays consisting of four individual plants in a 2×2 square arrangement. Two of the plants were assigned a high floral display (tall) treatment in which they were placed on a large, inverted pot, attaining a height of 80 cm, and two plants were assigned a low floral display (short) treatment in which they were dug into the ground, with a maximum height of 30 cm. Each treatment alternated in the arrangement, and the plants were paired for number of open flowers and general appearance to control for display traits other than display height. Each plant also had at least one male-phase (pollen donor) and one female-phase (pollen recipient) flower. On each of the male-phase flowers, one of two colors of fluorescent dye was applied as described earlier. We ran one trial for each of 11 d with the dye treatments alternating throughout the 11 d along with the order of high and low plant arrangements. Hummingbird visitation preference to tall and short floral display plants was recorded. The stigmas also were collected from the female flowers on each plant, and the number of each color dye particles transferred to stigmas of the short and tall plants was quantified. We again found no significant trial day effect; therefore, we pooled the square-root transformed data across trials and conducted a paired t test. Because only 32 plants were visited across the 11 d, or 11 trials, we could examine dye deposition on only 32 plants, resulting in 32 replicates.

We analyzed the effect of display height on hummingbird visitation, using each day as a replicate and for each day summing the number of visits to short and tall displays, respectively, resulting in 11 paired comparisons that were used in a paired t test. Data were square-root transformed to meet the assumptions of the t test.

Floral color manipulation—Floral color was manipulated by excising natural petals and gluing paper petals of appropriate color, i.e., white, pink, and red. This was done without damaging the flower's reproductive structures, and previous results have shown no bias toward the artificial petals by hummingbirds (Fenster et al., 2006). We have only rarely observed flowers of *S. virginica* that vary in their red color, thus the other colors chosen represent the two closely related *Silene* species. Therefore, this floral color manipulation tested the ability of hummingbirds to discriminate red from pink and white, possibly accounting for the maintenance of color differences of *S. virginica* (red) from *S. caroliniana* (pink) and *S. stellata* (white). We reiterate that hummingbird visitation alone was used to assess the relation between floral color expression and potential male reproductive success because of the close association between pollinator visitation and fluorescent dye transfer found in both the floral display size and height manipulative experiments.

In each trial, a six-plant array was used to compare all three floral colors with two replicates of each color whose position was assigned by using a random-number table. Each plant had at least one male-phase flower and one female-phase flower, and plants again were paired for general appearance and inflorescence size to minimize the possible influence of flower sex and inflorescence size on hummingbird visitation. This experiment was conducted with different plants five times at each of three locations for a total of 15 trials. The number of visits to a particular color in a given trial was summed, resulting in 15 replicates for each color across the experiment. Different locations were used to increase the likelihood that different hummingbirds would visit the arrays. Array trials occurred between 9 AM and 5 PM and lasted between 30 and 60 min.

Observers noted frequency of first visitation and total visitation to each floral color morph during each trial. Frequency of first visitation was defined as the proportion of times each color morph was visited first in a visitation run relative to the total number of first visitations. Total visitation was defined as the proportion of times a floral color was visited relative to the total number of visits to all color morphs throughout the entire trial. Both relative frequency measures were arcsine square-root transformed to meet the assumptions of this analysis. A two-way ANOVA with floral color and date showed no trial date effect; therefore, we present the data pooled as a one-way ANOVA examining only floral color. Significant differences in mean visitation among all floral morphs were determined using a Tukey studentized range post hoc test.

Corolla tube length—To determine how corolla tube length may affect male fitness, artificial flowers were made with paper red petals and a green

calyx to envelop the natural calyx and were held in place with glue to adjust the corolla tube length of the natural reproductive organs. The natural flowers had their floral petals excised but with their reproductive parts left intact. This manipulation directly affects how far the hummingbird needs to probe into the flower to acquire its nectar reward and affects its ability to contact the stamens to both transport pollen and deposit pollen on stigmatic surfaces. A nearby population of *S. virginica* had a mean corolla tube length of approximately 25 mm (Reynolds et al., 2009). We manipulated corolla tube length to represent the mean (control) and (\pm)2 SD from the mean, or 25, 21, and 29 mm, respectively. To determine male fitness, three plants with at least three male-phase flowers were used as fluorescent dye donors in each array. Each of these donors had one flower treatment representing a long corolla tube (29 mm), medium corolla tube (25 mm), and short corolla tube (21 mm). On each of these three treatments, a different color of fluorescent dye was applied with a wooden toothpick to two anthers of one male-phase flower. This experiment was replicated three times, and the three dye colors were rotated through the three treatments to control for the effects of dye color transfer on hummingbird visitation. The three donor plants were surrounded by 7 to 10 recipient plants, each with two female-phase flowers per individual, and hummingbirds were allowed to visit for a total of 1 or 2 d depending on weather conditions. The stigmas then were collected from each of the female flowers on the recipient plants, and the number of dye particles of each color was quantified by using an epifluorescent microscope. We assessed fluorescent dye transfer from the three corolla-length male donors (long, medium, short) at the plant level for our female recipient plants, averaging dye transferred per male donor to the female recipient plant to reduce the number of zeros for the analysis. We were unable to quantify hummingbird visitation to the long, medium, and short treatments directly because doing so would have required us to sit so close that hummingbird visitation would have been affected. No significant date effect was observed; therefore, we pooled the data from across the three trials. Thus, the unit of replication was the female recipient, with $N = 26$ female recipients across the three trials. Analyses were conducted on square-root transformed data to meet the assumptions of ANOVA, with treatment and maternal plant recipient as main effects.

RESULTS

Floral display size—Hummingbirds visited three-flowered plants approximately 1.7 times more frequently than one-flowered plants (mean number of visits per trial \pm 1 SE: three flowers, 3.5 ± 0.45 , compared with one flower, 2.0 ± 1.12 ; Table 1; $\chi^2 = 5.58$, $df = 1$, $P < .02$). We also observed a positive relation between fluorescent dye transfer and hummingbird visitation, with three-flowered donor plants transferring significantly more fluorescent dye than one-flowered plants to the surrounding recipient female plants (mean \pm 1 SE: three flowers, 54 ± 4.2 , vs. one flower, 42 ± 3.6 ; $t = 2.5$, $df = 23$, $P < .0202$).

Floral display height—Tall plants donated significantly more fluorescent dye particles to female-phase flowers of recipient plants than did short pollen donors (mean \pm 1 SE: 60 \pm 13 and 25 \pm 4, respectively; $t = 3.34$, $df = 31$, $P < .0022$). Hummingbirds preferred to visit flowers on tall plants approximately three times more frequently than flowers on short plants (mean \pm 1 SE: 1.98 ± 0.067 and 0.68 ± 0.146 , respectively; Table 1; paired t test: $t = 3.96$; $df = 10$, $P < .0027$).

In this experiment, the female-phase flower recipients were on the same plants as the male-phase pollen donor flowers. Thus we also could access the relation between hummingbird visitation frequency and pollen receipt. We observed a strong association between hummingbird visitation and deposition of fluorescent dye particles onto virgin stigmas (a measure of female reproductive success) in these artificial plant arrays (sign test: 9 of 11 trials in which stigmas with greater visitation averaged across a trial had greater dye deposition, $P < .0019$).

Floral color manipulation—Hummingbirds significantly preferred to visit red flower morphs first during the trials (mean

TABLE 1. Means and one SE of hummingbird visitation or fluorescent dye particle donation to stigmas of *Silene virginica* (number of replicates is in parentheses) while experimentally manipulating floral display size, floral display height, floral color, and corolla tube length. Means followed by different letters are significantly different from one another (see text for details).

| Trait | Experimental Manipulation | | |
|---------------------------------|----------------------------|----------------------------|----------------------------|
| Floral display size | Single flower | Three flowers | |
| Fluorescent dye donated (24) | 42 ± 3.6 ^a | 54 ± 4.2 ^b | |
| Total number of visits (40) | 2.0 ± 1.12 ^a | 3.5 ± 0.45 ^b | |
| Floral display height | Short | Tall | |
| Fluorescent dye donated (32) | 25 ± 4 ^a | 60 ± 13 ^b | |
| Number of visits per trial (11) | 0.68 ± 0.146 ^a | 1.98 ± 0.067 ^b | |
| Floral color | White | Pink | Red |
| % First visits per trial (15) | 0.283 ± 0.010 ^a | 0.295 ± 0.022 ^a | 0.421 ± 0.016 ^b |
| % Total visits per trial (15) | 0.207 ± 0.042 ^a | 0.215 ± 0.030 ^a | 0.577 ± 0.046 ^b |
| Corolla tube length | Short | Medium | Long |
| Fluorescent dye donated (26) | 9.2 ± 1.3 ^a | 13.2 ± 1.9 ^a | 24.0 ± 3.2 ^b |

percentage of first visits ± 1 SE: red, 0.421 ± 0.016; pink, 0.295 ± 0.022; white, 0.283 ± 0.010; $F_{2,42} = 17.44$, $P < .001$) and made significantly more visits overall to red flowers (0.577 ± 0.046; $F_{2,42} = 14.24$, $P < .001$), compared with results for white (0.207 ± 0.042) and pink (0.215 ± 0.030) flowers, which were visited at similar frequencies (Table 1).

Corolla tube length—Flowers with the longest manipulated corolla tube lengths donated significantly more fluorescent dye particles to flowers of the recipient plants ($F_{2,51} = 18.12$, $P < .0001$). The average number of fluorescent dye particles donated from long corolla tube flowers to the surrounding recipient plants was 24.0 ± 3.2 (mean ± 1 SE), which was significantly greater than from medium corolla tube donors (13.2 ± 1.9) and short corolla tube donors (9.2 ± 1.3). No significant difference existed between the donations of fluorescent dye from either medium or short corolla tube donor flowers (Table 1).

DISCUSSION

In this series of single-trait manipulative experiments, we demonstrate that potential male reproductive success can be determined by trait variation associated with attraction and efficient pollen transport. Specifically, we observed congruence between hummingbird visitation frequency and the ability to disperse dye (a pollen analog) and also found a significant positive relation between dye receipt on stigmas and hummingbird visitation. Others, e.g., Galen and Stanton (1989) in bee-pollinated *Polemonium viscosum*, also have found that increased visitation is associated with relatively higher amounts of pollen dispersal (see also references that follow). Thus, the increased male reproductive success associated with three of the four trait manipulations can be readily explained by increased attractiveness to hummingbirds. Hummingbird preference for larger floral displays presented higher from the ground is consistent with the preferences exhibited by other pollinator groups discussed later. Thus, these preferences are unlikely to contribute to the floral trait differences found between *S. virginica* and its close relatives. However, preference of the hummingbirds for red floral displays, vs. white or pink, is consistent with the pollinator attraction signal differences of *S. virginica* with its closest *Silene* relatives. Furthermore, the greater pollen donation associated with relatively longer corolla tubes is consistent with *S. virginica* diverging from related *Silene* species, which have pollinators with significantly shorter probing parts: mean (SE)

corolla tube length of *S. stellata* is 9.8 (0.9) and of *S. caroliniana* is 21.2 (1.6), compared with *S. virginica* 24.1 (2.1) (Reynolds et al., 2009). In the following, we compare our findings to studies with other systems as well as with our long-term studies on *S. virginica* to quantify natural selection acting through female reproductive success (Reynolds et al., 2010).

Because of the general preference of many groups of pollinators for larger floral display sizes and increased height of the display, it is not surprising that many examples exist in the literature for these preferences. For example, visitation of syrphid flies to *Raphanus raphanistrum* increased in plants that had more flowers open on a given day (Conner and Rush, 1996), syrphid flies and andrenid bees were more likely to visit larger inflorescences of the andromonoecious lily *Zigadenus paniculatus* (Emms et al., 1997), and more bumble bees visited *Salvia napponica* plants with larger inflorescences (Miyake and Sakai, 2005). Hummingbirds are more likely to visit *Ipomopsis aggregata* plants that present their flowers higher off the ground (Engel and Irwin, 2003; and this study). However, Peakall and Handel (1993) observed that in the deceptive orchid *Chiloglottis trilabra*, the preferred visitation height by male wasps was intermediate, corresponding to the cruising altitude of female wasps. Nevertheless, this latter example demonstrates that floral display height mediates pollinator visitation frequency. We also have observed hummingbird preference for flowers manipulated to have larger petals, which is consistent with larger signals of attraction resulting in higher pollinator visitation (Fenster et al., 2006). In *S. virginica*, as well as in many other plants, larger flowers are associated with greater nectar rewards (Fenster et al., 2006, and citations within). Thus, the increased attractiveness of larger displays, either through increased floral size or flower number or through higher (taller) displays, may be associated with greater visibility and reward and subsequent detection by pollinators.

Increased pollinator visitation to larger inflorescences and flowers positioned higher off the ground may explain the direct positive selection on these traits found in several studies. Maad (2000) found that flower height (stalk length) in the hawk moth-pollinated orchid *Platanthera bifolia* is under positive directional selection through both male (pollinia removed) and female (pollinia receipt) reproductive success. In another orchid, the nonrewarding *Cypripedium acaule*, flower height also was shown to be under positive directional selection through both male and female reproductive success (O'Connell and Johnston, 1998). Johnston (1991) found positive directional selection through female reproductive success on flower height in

hummingbird-pollinated *L. cardinalis* but not in bee-pollinated *L. siphilitica*. In a population of *S. virginica* close to where our manipulation experiments occurred, we also observed sometimes strong but always consistent positive directional selection of inflorescence height through female reproductive success (Reynolds et al., 2010). Thus, in the examples in which both male and female reproductive success has been quantified, flower height is under positive directional selection through both sexual functions. To our knowledge, no one has quantified selection on inflorescence size independently from total female reproductive success, though many researchers have noted an increase in seed and/or fruit production with increasing number of flowers or inflorescence per individual (e.g., Caruso et al., 2003).

In contrast to inflorescence size and display height, floral color and corolla tube dimensions have traditionally been more closely associated with particular groups of pollinators (Faegri and van der Pijl, 1979) and thus have been presumed to reflect selection by those pollinators. However, this traditional view has been challenged recently. For example, Waser et al. (1996) observed little association of insect pollinator type with floral color, though experimental manipulation of floral color often is associated with changes in the visitation by contrasting pollinators. For example, in an F2 population from crosses between the red-flowered and hummingbird-pollinated *Mimulus cardinalis* with pink-flowered and bee-pollinated *M. lewisii*, hummingbirds favored F2s with more anthocyanin in their flowers (= more red), whereas bees favored flowers with less anthocyanin (= more pink) (Schemske and Bradshaw, 1999). Similarly, Campbell et al. (1997) and Melendez-Ackerman and Campbell (1998) demonstrated that hummingbirds favor red over pale-colored morphs in a hybrid zone between two *Ipomopsis* species exhibiting alternative hummingbird and moth pollination syndrome traits. Preferential visitation by hummingbirds and bees to red and blue flowers, respectively, also was observed in Louisiana iris hybrid zones (Wesselingh and Arnold, 2000). Pollinator-mediated selection on flower color has been difficult to document (Rauscher, 2008; but see Medel et al., 2003, 2007), possibly because in those cases in which color polymorphism exists within a population, by definition there is no pollinator preference, and the color polymorphism may reflect other sources of selection (e.g., Schemske and Bierzychudek, 2007) or drift. However, studies quantifying the association of color transitions with pollinator transitions often demonstrate color variation consistent with our notion of pollination syndromes, and thus consistent with pollinator-mediated selection (Fenster et al., 2004; Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., 2009; Martén-Rodríguez et al., 2010; but see Smith et al., 2008). Our findings are consistent with hummingbirds as the selective agents responsible for the origin or maintenance of red flowers in *S. virginica*, with selection acting through at least male reproductive success.

The notion that corolla tube length or analogous floral traits that force a pollinator to probe the flower for a nectar reward, e.g., nectar spurs, reflects directional selection that is pollinator mediated dates to the very origin of the role of natural selection in the evolutionary process (Darwin, 1862; Wallace, 1867). Experimental verification of long-tongue pollinators selecting for deeply held nectar was elegantly demonstrated by Nilsson (1988) in the hawk moth-pollinated boreal orchids, *Platanthera bifolia* and *P. chlorantha*, in which both male and female reproductive success was decreased when the moths were prevented from probing deeply into the flower. Similarly, Campbell et al. (1996) manipulated the ability of hummingbirds to insert their

beaks into *Ipomopsis aggregata* flowers and found that increased insertion of the beak into the corolla increased pollen transfer efficiency. Hummingbirds will avoid flowers that do not allow them to effectively probe for nectar and will spend more time when visiting longer flowers (Grant and Temeles, 1992). Thus corolla tube length may reflect pollinator-mediated selection of two types: to ensure probing of the correct pollinator, increasing pollen transfer, and to deter probing by the wrong type of pollinator, reducing pollen wastage. The culmen length of ruby-throated hummingbirds (19–22 mm) and the dimensions of *S. virginica* flowers (approximate mean corolla tube length = 25 mm and tube diameter at opening = 3.6 mm) are consistent with positive directional selection exerted by the hummingbird (Smith et al., 1996). The evolution of corolla tube length or nectar spurs also is closely associated with transitions in pollinators, with less accessible nectar associated with transitions to pollinators with increased length of probing structures (reviewed in Fenster et al., 2004; for recent examples, see Whittall and Hodges, 2007; Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., 2009; Martén-Rodríguez et al., 2010). Furthermore, pollination ecotypes paralleling these types of transitions also have been quantified (Grant and Grant, 1965; Johnson and Steiner, 1997; Anderson and Johnson, 2008, 2009). Thus, we conjecture that the increased dye donated by flowers artificially manipulated to be longer in our experimental arrays reflects that hummingbirds probe more deeply and perhaps for a longer time, resulting in more dye (and thus we infer more pollen) transferred to the hummingbirds (see Muchhala and Thomson, 2009, for experiments addressing the functional significance of the evolution of deeper corolla tubes in bat-pollinated flowers).

Phenotypic selection studies also have demonstrated that pollinators can mediate selection on corolla tube length via female reproductive success (e.g., Schemske and Horvitz, 1984). In hummingbird-pollinated *I. aggregata* (Campbell, 1989, 1996) and in our own studies on *S. virginica* (Reynolds et al., 2010), positive directional selection has been consistently quantified on stigma exertion. Increasing stigma exertion brings the female reproductive parts closer to the hummingbird, which is analogous to corolla tube length bringing the pollinator closer to the reproductive parts. In 6 of 8 yr that we quantified natural selection on corolla tube length, we found selection to be positive, though never significant in any given year. A longer-term study may reveal that this pattern is consistent, though replication for 8 yr was insufficient (sign test, $P > .15$). However, here we provide strong evidence that hummingbird-mediated directional selection on corolla tube length through male reproductive success may be a significant factor in the evolution and/or maintenance of corolla tube length in *S. virginica*.

The experimental manipulations of *S. virginica* flowers and the outcome in terms of hummingbird visitation and donation of fluorescent dye to conspecific flowers are consistent with pollinator-mediated selection, and for some traits (floral color and corolla tube length) they are consistent with selection mediated by hummingbirds. We have shown that selection can occur through male reproductive success, parallel to our finding of selection on display height via female reproductive success but in contrast to our findings of limited directional selection on corolla tube length via female reproductive success (Reynolds et al., 2010). However, one does not always observe a concordance between male and female function selection pressures in hermaphroditic plants (Ellis and Johnson, 2010, and references within). Our results add to the growing body of literature (cited here previously) demonstrating that pollinator-mediated selection

on floral and inflorescence traits can occur through selection on male function of a hermaphroditic plant species. This is particularly notable in that we failed to observe consistent pollen limitation of female reproductive success in *S. virginica* (Dudash and Fenster, 1997), congruent with a large body of literature also demonstrating that pollen limitation is frequently not observed (Burd, 1994; Larson and Barrett, 2000; Ashman et al., 2004; Knight et al., 2005). Thus, although selection on floral traits via female reproductive success may be inconsistent, selection via male reproductive success may more likely result in floral evolution.

However, basing patterns of selection on individual components of pollination syndromes may lead to an incomplete picture (Campbell, 2009). The patterns of selection underlying pollination syndrome evolution, which by definition are suites of correlated traits, may not reflect selection acting on traits in isolation but rather on trait combinations and their resultant fitness effects. When we examined the fitness effects of floral variation on female reproductive success in *S. virginica*, we found evidence for consistent and negative correlational selection between corolla tube length and stigma exertion and a consistent and positive relation between tube diameter and display height (Reynolds et al., 2010). Furthermore, in a two-trait factorial experimental design, we detected hummingbird preference for wider corolla tubes only in combination with wider petals (Fenster et al., 2006). Thus, experimental manipulation to deconstruct the selective pressures responsible for floral form, which includes manipulation of many traits at a time in a fully factorial manner, is necessary for a fuller comprehension of the pattern of pollinator-mediated selection responsible for floral diversity (Campbell, 2009).

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