Nectar reward and advertisement in hummingbird-pollinated *Silene virginica* (Caryophyllaceae)[1]

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We tested for an association between nectar and various floral traits and investigated their roles as primary and secondary pollinator attractants in hummingbird-pollinated *Silene virginica*. Our goal was to gain insight into the mechanisms of pollinator-mediated selection that underlies floral trait divergence within the genus. In a field population of *S. virginica*, we measured five floral and eight vegetative traits and quantified nectar volume, nectar sugar concentration, and total sugar reward (nectar volume × nectar sugar concentration). All three components of nectar reward were positively correlated to flower size, and nectar volume varied significantly among individuals within the population. To ascertain whether the correlation of specific floral traits with nectar reward influences the behavior of the primary pollinator of *S. virginica*, the ruby-throated hummingbird, *Archilochus colubris,* we investigated whether *A. colubris* preferred the expression of floral traits associated with high nectar volume and total sugar reward. We accomplished this by constructing floral arrays consisting of artificial flowers that had equal nectar quantity and total sugar reward but that differed in petal area and corolla tube diameter, which were positively correlated with nectar quantity and total sugar reward in our field study. In observations of visitation frequencies to the various floral-trait combinations, hummingbirds preferentially visited artificial floral phenotypes with larger petal displays, with the greatest preference for floral phenotypes with both larger petals and wider corolla-tube diameters. This association between primary and secondary floral attractants and hummingbird discrimination of floral features supports the concept that the floral traits of *S. virginica* reflect pollinator-mediated selection by the principal pollinator.

Key words: advertisement; Allegheny Mountains; Caryophyllaceae; hummingbird; nectar reward; pollination syndrome; pollinator-mediated selection.

Flowering plants differ markedly in floral traits associated with both the attraction and reward components of pollination (Fægri and van der Pijl, 1979). The attractive features of floral display are likely to have evolved to advertise rewards that are hidden or access-limited. Although the necessity to advertise reward appears to offer a mechanism for the evolution of floral attractive features, our knowledge of the links between pollinator discrimination, floral advertisement, and rewards is limited.

Pollination rewards differ in their composition, amount, and accessibility and are highly associated with specific functional groups of pollinators (Baker and Baker, 1983). A functional group describes pollinators that exert similar or convergent selection pressures on a plant because of similarities such as size and behavior within the flower (e.g., all hummingbird or all long-tongue bee species pollinating a flower, Fenster et al., 2004). Consequently, plants probably have diverged in response to selection by the varying nutritional demands or preferences of pollinators (Simpson and Neff, 1983). Reward systems often result in a highly restricted type of pollinator to visit a plant. For example, oil (Buchmann, 1987), fragrance (Dressler, 1982), floral resins (Armbruster, 1988, 1992), and even brood sites (Dufay and Anstett, 2002; Kephart et al., 2006) are among the many types of rewards that are associated with very specific pollinators. Phylogenetic analyses frequently indicate that shifts in pollinators occur in association with shifts in reward, as seen in the evolution of hummingbird pollination, which appears to reflect selection by hummingbirds for high volumes of dilute nectar relative to related taxa with alternative pollinators (reviewed in Fenster et al., 2004). At the population level, nectar is a major determinant of pollinator behavior, influencing the number of pollinator visits (e.g., Galen and Plowright, 1984; Mitchell and Waser, 1992; Mitchell, 1992) and the length of time a pollinator remains at a flower (Mitchell, 1992; Cresswell, 1999; Klinkhamer et al., 2001).

Accompanying the evolution of rewards is the evolution of floral traits that allow plants to advertise their rewards such as color, shape, or size of the flower and, when appropriate, fragrance (e.g., Fægri and van der Pijl, 1979; Fenster et al., 2004). Floral attractive features associated with different pollinators are often strikingly divergent, even among closely related species (e.g., *Stylidium,* Armbruster et al., 1994; *Lapeirousia,* Goldblatt and Manning, 1996; *Mimulus,* Schemske and Bradshaw, 1999; Beardsley et al., 2003), and may differ even at the intraspecific level (e.g., *Gilia splendens*, Grant and Grant, 1965, 1968; *Polemonium viscosum,* Galen, 1989; *Plantanthera ciliaris,* Robertson and Wyatt, 1990). One of the fundamental assumptions of pollination syndromes and the role that pollinators may play in mediating floral trait evolution in the context of reward evolution is that pollinators will discriminate among different individuals, offering different rewards based on the type of floral advertisement (Fægri and van der Pijl, 1979). Thus, pollinator-mediated selection on floral traits that are features of the flower's advertisement should be reflected in the ability of a pollinator to discriminate among individuals at the intrapopulation level. In support of this notion that pollinators select on floral advertising traits, there is limited evidence that pollinators utilize subtle floral

cues to discriminate among individuals within a population (reviewed in Cresswell, 1998; Raguso, 2004).

Hummingbird-pollinated *Silene virginica* (Fenster and Dudash, 2001) is closely related to two other species of *Silene* (Burleigh and Holtsford, 2003; B. Oxelman, Uppsala University, personal communication): the nocturnally moth-pollinated *S. stellata* and the bee- and diurnally moth-pollinated *S. caroliniana* (R. Reynolds, C. Fenster, and M. Dudash, unpublished data). All three species are highly divergent for floral traits that a priori could be assigned reward and advertising functions. In particular, compared with its sister taxa, the red-flowered *S. virginica* produces much more nectar; has larger, longer flowers; and has a relatively wider corolla tube opening (Gleason and Cronquist, 1963; R. Reynolds, C. Fenster, and M. Dudash, unpublished data). In this study we first investigated the pattern of correlation of nectar production and nectar concentration with secondary attractant floral traits in a natural population of *S. virginica* to determine which trait(s) may act to advertise nectar reward. Second, we performed an artificial-selection experiment, altering floral traits, singly and in combination, that are correlated to nectar reward in nature to determine which floral signals the hummingbirds use to discriminate among individual plants differing in nectar reward in artificial arrays.

MATERIALS AND METHODS

Study plant—*Silene virginica* is a short-lived perennial with red, semipendant flowers that is found in eastern North America. Its flowers are protandrous, with the male phase typically lasting 2 days, when five anthers emerge from the corolla tube on each day for a total of 10 anthers that are located at the center of the corolla-tube opening (day 1 male and day 2 male, respectively). On approximately the third day, three styles emerge from the corolla tube, and the anthers are displaced from their central location (neuter phase). On day 4 (usually), the styles hook and become receptive to pollen (female phase). The aforementioned floral ontogeny (Dudash and Fenster, 2001) is somewhat weather dependent; intervals of cold, inclement, or hot weather can delay or accelerate transitions between floral stages. Although the petals are not physically fused, the arrangement of the sepals around the petals forms a functional floral tube, which is primarily green except where the petals extend beyond the sepals. Flowering occurs from late May to early July near the Mountain Lake Biological Station (Allegheny Mountains) in Giles County, Virginia (Dudash and Fenster, 2001).

Field census data collection—To identify floral and plant traits that correspond to increased nectar reward, we performed a within-population census of *S. virginica* in a meadow site that has grown without canopy cover near the Mountain Lake Biological Station between early June and early July 2002. We randomly identified and staked 27 plants, tagged 2–14 buds on each plant, covered the plants with cages constructed of chicken wire and fiberglass screening material to exclude all pollinators, and monitored the flowers throughout their phenology. Flowers from all four sexual phases (day 1 male, day 2 male, neuter, and female phase) were harvested from 25 of the 27 caged plants across the 2-week duration of flowering. The remaining two plants produced only two flowers. All flowers were collected before 1130 hours to minimize evaporation of nectar. Flowers were placed in plastic scintillation vials and were stored on ice until floral traits could be measured at the laboratory (no later than 1500 hours on the same day).

We used dial calipers to measure (1) the length of the longest petal from the point of reflex (corona) to the tip of the petal, (2) the width of the longest petal at the widest point of the petal, (3) the length of the corolla tube from the base of the tube to the point of reflex of the petals, and (4) the width of the corolla tube opening from inside the petal edge to inside the opposite petal edge. We used 20-µL microcapillary tubes to probe the base of the corolla tube until no more nectar could be removed, and then dial calipers were used to measure the distance nectar had migrated up the tubes. Nectar volume was determined by converting the distance measures to volumes (µL). Nectar sugar concentration

was recorded by spotting these tubes on a temperature-controlled, hand-held refractometer (Sugar/Brix Refractometer, 0–32% w/ATC, Sper Scientific, Scottsdale, Arizona, USA). Total sugar reward was quantified as the product of nectar volume and sugar concentration.

After all flowers had been collected for the study, we measured the following vegetative traits for each of the 27 plants in the study: number of reproductive stems, total stem length, total number of leaves per plant, length and width of longest leaf. We also measured total reproductive effort as the total number of reproductive units per plant (the number of buds, fruits, and flowers plus the number of removed flowers). We measured these vegetative and reproductive traits as potential correlates of plant vigor to be used as covariates in the analyses to quantify the relationship (if any) between nectar production and floral traits.

Artificial floral arrays—In prior work we have observed a close correspondence between visitation frequency by hummingbirds on *S. virginica* and male reproductive success as assessed through the use of fluorescent dyes as pollen analogs (Fenster et al., 1996; C. Fenster and M. Dudash, unpublished data). Furthermore, the populations of *S. virginica* that we have studied do experience pollen limitation of fruit set (Dudash and Fenster, 1997). Thus hummingbird preference as measured by visitation parameters is likely a reasonable proxy for measurements of both male and female reproductive success.

Therefore to determine whether pollinators prefer traits indicative of increased reward quality, we presented *A. colubris* with artificial floral arrays. Floral arrays were presented to pollinators in three separate locations at Mountain Lake Biological Station, each approximately 300 m apart. Two of these sites were power-line cuts that had been mowed prior to the trials, and the third was a caged garden plot. Hummingbirds were attracted to each of the three sites by one feeder placed at each site filled with 25% sucrose solution (approximate field nectar concentration). The position of the feeder within the site was assigned to a random position after each array to avoid biasing the direction of hummingbird entry into each array.

Floral arrays consisted of artificial plants (Fig. 1) with one flower per stem in which the two floral attractive traits found to be correlated with nectar reward volume, area of petals and diameter of the corolla-tube opening, were manipulated. Petal area was divided into Large and Small categories for which petal areas were approximately ±2 SD from the mean found in the natural population (130.25 ± 27.3 mm²). Corolla-tube opening diameter was divided into Wide and Narrow categories for which corolla-tube opening diameters were also approximately ±2 SD from the mean found in the population (3.33 ± 0.47 mm²). Thus, each array included flowers of four distinct floral phenotypes Large/Wide, Large/Narrow, Small/Wide, and Small/Narrow. Each array consisted of 12 plants: three within-array replicates for each of the four floral-trait combinations.

In each array, plants were arranged in a 3 × 2 m configuration with each plant spaced approximately 1 m from the next (i.e., 4 × 3 plants). The metal stem of each plant was inserted into the ground so that the bottom pair of leaves rested on top of the ground. The feeder then was removed from the site; covered with an opaque, black garbage bag; and placed next to the observer who was approximately 5 m from the array at the base of a tree or fencepost to minimize visibility to the hummingbirds. Before each array was conducted, all 12 plants were filled with 20 µL of 25% sucrose solution (25 g per 100 mL water). This volume represents a uniform but elevated reward based on the 14.6-µL mean volume and 22.6% mean sugar concentration found in the natural population. It was essential to control for nectar reward quality so that visitation by *A. colubris* would not be based on differential nectar volumes but on variation among floral trait presentation. After the completion of each array, excess nectar was purged with a glass Pasteur pipette. Twenty-five 30-min arrays were conducted over 3 days across the three sites. The duration of the interval was lengthened in two cases because of low levels of hummingbird visitation. Arrays were rotated among the three sites listed, beginning each day with a different site and moving to the other two sites before the first site was used again on a given day. All floral combinations were randomized with respect to position in the construction of each of the 25 arrays. An audiocassette recorder was used to note hummingbird entries to the array and the sequence of flowers that each hummingbird visited.

Statistical analyses—*Field census*—All statistical procedures were performed using SAS version 9.1 (SAS Institute, 2004). Pearson linear correlation coefficients were computed (Proc Corr) between nectar volume, nectar concentration, petal length, petal width, corolla-tube length, and corolla tube

Fig. 1. Female ruby-throated hummingbird visiting an artificial flower made to simulate *Silene virginica*. Artificial flowers were made to represent the extremes of petal areas, corolla-tube diameters, and their combinations found in a natural population (see section on artificial floral arrays in Materials and Methods).

width at the plant level ($N = 27$). Given the strong correlations among some floral trait variables, petal length and petal width were combined into a single variable: petal area (petal length $\times$ petal width). To preserve the dimensionality of the variables, all analyses were conducted on the cube root of nectar volume and the square root of petal area. All length measurements were natural log transformed. Because of the multiple-comparison nature of the correlation analysis, we adjusted the experiment-wise type 1 error rate using a sequential Bonferroni correction.

In addition, a principal components analysis (PROC PRINCOMP) was performed on the floral traits (petal length, petal width, corolla tube length, and corolla width) to reduce the dimensionality of these correlated variables. To determine the relation between the reward characters and the general flower size variables, the reward characters (nectar volume, sugar concentration, and total sugar reward) were correlated with the first principal component scores.

To assess the relationship between whole plant traits and floral traits, we conducted a separate analysis. We examined whether the plant vegetative traits (number of reproductive stems, total stem length, number of reproductive units, length of longest leaf, and width of longest leaf) measured in this study were correlated with the floral reward traits. Mixed-model ANOVA (PROC MIXED) was used to model the response variables, nectar volume, nectar sugar concentration, or total sugar reward as a linear function of plant id (random) and flower gender (fixed), and their interaction (random). The model was analyzed with restricted maximum likelihood (REML) estimation of the residual error and random effects variance components. Hypothesis testing of the covariance parameters was carried out with likelihood ratio χ^2 tests. Models were run twice, with and without the random effect in question, and the corresponding difference in the -2*residual log-likelihood statistic was compared with $\chi^2_{\alpha=0.05}$ with 1 df for determining the significance of the covariance parameter. The REML estimates of the variance components were used to estimate fixed-effect parameters, and type III F tests were performed for the fixed effect. To test a priori the hypothesis that nectar accumulates in flowers as they progress from male phase to female phase, a contrast (statement = CONTRAST) was performed on the linear combination of parameters corresponding to the combined effect of day 1 and day 2 males vs. the combined effect of neuters and females. Least squares means and their standard errors were computed for each gender phase (statement = LSMEANS). PROC MIXED was used in the identical framework as described, except we used petal area as the response variable in place of nectar volume to model the effects of flower gender (fixed), plant id (random), and their interaction (random) on flower size variation in the natural population.

Artificial floral arrays—The array data were analyzed using the three response variables: total plant visits, number of first visits per foraging bout, and number of first visits per array. Number of first visits per foraging bout reflects the first choice of the hummingbird upon each entrance to the floral arrays. A number of foraging bouts occurred during any given array. However, for any array there was only one initial choice by the first hummingbird visiting the array, giving rise to the response variable of number of first visits per array.

We analyzed the array data with the three different response variables because hummingbirds were observed to sample most if not all flowers during the observational period of an array. Thus with an analysis of first visits, we could more specifically estimate the hummingbirds' preference for particular traits or trait combinations by minimizing the possibly confounding factors of experience with the plants' orientation, nectar depletion, or hummingbird territoriality. We estimate that 12–15 different hummingbird individuals visited the arrays, with most visits conducted by females.

Total plant visits, or the total count of visits by hummingbirds, summed across 25 arrays (trials) were modeled as a linear function of the floral-trait combinations with Poisson regression (PROC GENMOD). In this generalized linear modeling approach, the data were fit by maximum likelihood estimation of model parameters. The response variables' probability distribution was chosen as Poisson, and the link function was specified as log. This analysis allowed us to examine the significance of the type III main effects of petal area and corolla-tube width and their interaction by using results of likelihood ratio tests. We applied the identical model to the other response variables (number of first visits per foraging bout and the number of first visits per array).

RESULTS

***Field census**—Covariance of reward and floral traits in a natural population*—The degree of floral-trait expression appears to advertise the amount of nectar reward in the flowers of *S. virginica*. Petal area ($r = 0.59$; $P = 0.0011$; $N = 27$), corolla-tube width ($r = 0.56$; $P = 0.0021$; $N = 27$), and corolla-tube length ($r = 0.71$; $P < 0.0001$; $N = 27$) correlated positively with nectar volume even after Bonferroni adjustment of the type I experiment-wise error rate (Table 1). However, significant correlations were not found between these floral traits and nectar sugar concentration after sequential Bonferroni correction, nor did we find nectar volume to correlate significantly with nectar sugar concentration (Table 1). Total sugar reward (nectar sugar concentration $\times$ nectar volume) was correlated with corolla tube length ($r = 0.46$; $P = 0.0185$; $N = 26$) and corolla tube width ($r = 0.52$; $P = 0.0068$; $N = 26$) but not with petal area ($r = 0.30$; $P > 0.05$; $N = 26$) (Table 1). After sequential Bonferroni corrections, none of these correlations

TABLE 1. Correlation coefficients, P values, and sample sizes for floral traits, nectar volume, nectar sugar concentration, and total sugar reward in *Silene virginica*. Bolded correlation coefficients are significant after sequential Bonferroni adjustment of type I error. Data were transformed as described in Materials and Methods. Petal area = petal width × petal length.

Trait	Corolla tube width (mm)	Petal area (mm²)	Nectar volume (µL)	Nectar sugar concentration (mg sugar/mg solution)	Total sugar reward (mg sugar)
Corolla tube length	**0.560**	**0.602**	**0.715**	0.271	0.459
P value	0.0024	0.0009	<0.0001	0.1802	0.0185
N	27	27	27	26	26
Corolla tube width		**0.694**	**0.565**	0.361	0.517
P value		<0.0001	0.0021	0.07	0.0068
N		27	27	26	26
Petal area			**0.594**	0.355	0.302
P value			0.0011	0.0941	0.1336
N			27	26	26
Nectar volume				0.335	Not independent
P value				0.0941	
N				26	

were significant, although the correlation between corolla tube width and total sugar reward fell just short of the $P = 0.05$ significance level. All reward characters were positively correlated with the first principal axis (nectar sugar concentration: $r = 0.41$; $P = 0.0377$; $N = 26$; nectar volume: $r = 0.73$; $P < 0.0001$; $N = 27$; total sugar reward: $r = 0.53$; $P < 0.0056$; $N = 26$). In addition, plant vegetative or vigor traits were not found to correlate with nectar volume, nectar sugar concentration, or total sugar reward (no correlation was higher than that between length of longest leaf and nectar volume, $r = 0.261$; $P = 0.1893$; $N = 27$).

From our ANOVA, flower gender was a significant predictor of nectar volume ($P < 0.0001$; df = 3, 64; $F = 12.01$), and to a lesser degree, so was the individual plant. The variance-component parameter of the random effect of plant id was significant ($P = 0.032$; df = 1; $\chi^2 = 4.6$). The contrast showed mean nectar volume for neuters and females combined was significantly greater than day 1 and day 2 male flowers combined ($F = 35.73$; $P < 0.0001$; Fig. 2). The among-plant variance component accounted for 19% of the total variation in nectar volume. A significant flower gender by plant interaction effect on nectar volume was not detected ($P = 0.3173$; df = 1; $\chi^2 = 1.0$). There were no significant effects of flower gender ($P = 0.1036$; df = 3, 60; $F = 2.15$) or individual plant (covariance parameter estimate = 0) on nectar sugar concentration, nor was the gender × plant covariance effect significant ($P = 0.0693$; df = 1; $\chi^2 = 3.3$). There was a significant flower gender effect on total sugar reward ($P < 0.001$; df = 3, 60; $F = 20.9$). There was no significant effect of individual plant on total sugar reward ($P = 0.1213$; df = 1; $\chi^2 = 2.4$), nor was the interaction between gender and plant significant because the model fit was identical with and without the interaction effect. In examining petal area as the response variable, we found that individual plant was a significant predictor of petal area ($P < 0.0001$; df = 1; $\chi^2 = 34.2$). The among-plant variance component accounted for 79% of the total variation in petal area.

Artificial floral arrays—*Covariance of hummingbird visitation and floral traits in an array experiment*—When presented with choice, hummingbirds consistently prefer to visit plants with large flowers. Modeling the total number of plant visits ($N = 776$) as a linear function of the predictors (the various floral-trait combinations) resulted in a strong effect of flower area ($P < 0.0001$; $\chi^2 = 17.36$) and no significant effect of tube width ($P = 0.7109$) or the tube width × petal area interaction ($P = 0.7109$; Fig. 3A). Because hummingbirds visited the same plants many times per replicate, the effect of hummingbird preference for large flowers may have been diluted if birds visited large flowered plants first and then visited plants with smaller flowers. In addition, the high visitation frequency may have overwhelmed our ability to detect the more subtle effects tube width may have had on hummingbird visitation preference. Therefore, the same predictor variables were modeled using different response variables: first visits per foraging bout ($N = 241$) and first plant visited per array ($N = 25$).

Using first visits per foraging bout as the response variable magnified the effect due to hummingbird preference for larger flowers (Fig. 3B). As before, flower size affected hummingbird visitation ($\chi^2 = 29.14$; $P < 0.0001$; df = 1), and again there was
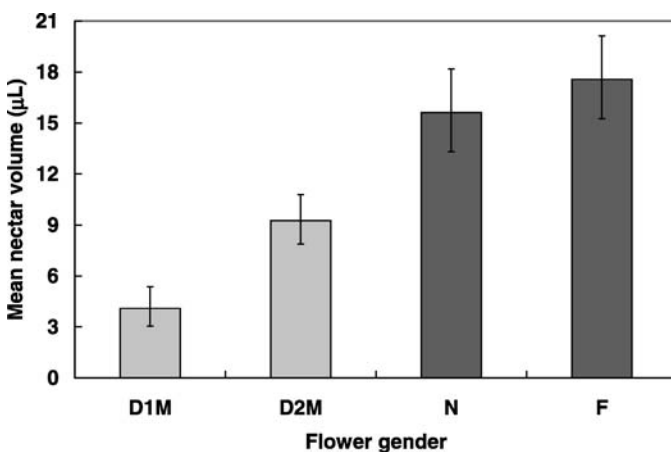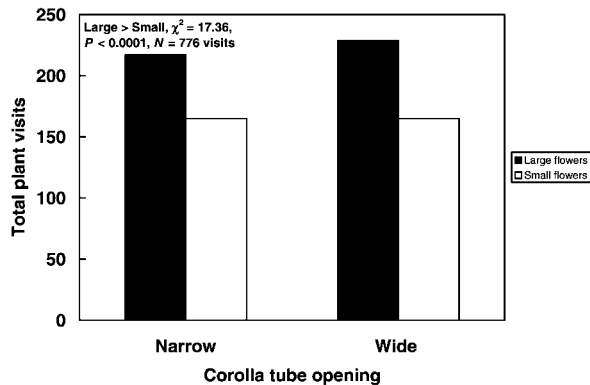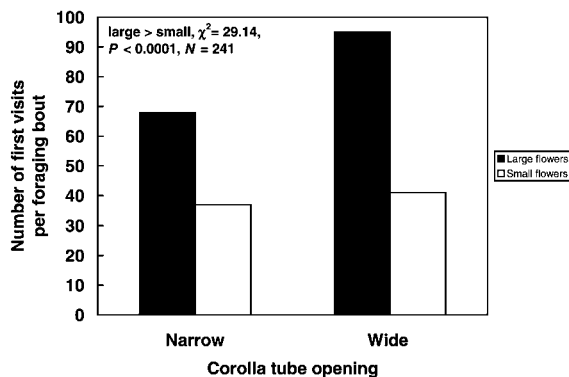

Fig. 2. Nectar volume increases with flower age in *Silene virginica*. D1M, day 1 male ($N = 26$ plants); D2M, day 2 male ($N = 26$ plants); N, neither male nor female ($N = 25$ plants); F, female ($N = 25$ plants). Flowers typically take 4 days to move from D1M to F. Data are mean nectar volume ± 1 SE. Orthogonal contrast showed mean nectar volume for neuters and females (dark bars) combined is significantly greater than that of male flowers (light bars) combined ($F = 35.73$, $P < 0.0001$).
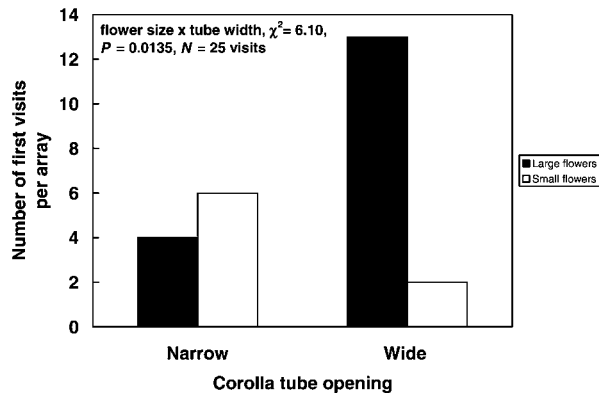
A.

B.

C.

Fig. 3. Visitation response of hummingbirds to artificial flowers expressing one of four floral phenotypes: large flower with narrow tube opening, small flower with narrow tube opening, large flower with wide tube opening, and small flower with narrow tube opening. Filled bars represent large flowers, and open bars represent small flowers. (A) Total visits by hummingbirds summed across all arrays and foraging bouts. Modeling the number of visits as a linear function of the floral trait combinations showed that only flower size was a significant predictor of hummingbird visitation. (B) Total first visits per foraging bout by hummingbirds summed across all arrays. The dependent variable is the number of times a hummingbird visited a floral trait combination first in a

no effect of tube diameter ($P = 0.1139$) or the interaction ($P = 0.4029$). Moreover, the effect of flower size increased over the previous analysis (previously: $\chi^2 = 17.36$), indicating that using plant visits rather than first visit per run as the response variable may dilute the effect of flower size.

Using first plant visited per array ($N = 25$) as the response variable resulted in the observation that hummingbirds may prefer flowers with wide corolla tubes but only when combined with large petals (Fig. 3C). There was a significant flower area $\times$ tube width interaction effect ($P = 0.0135$; df $= 1$; $\chi^2 = 6.10$); however, the main effect of flower area ($P = 0.1283$) and of tube width ($P = 0.9361$) was not significant, probably because of the smaller number of replicates in this analysis (25 replicates $= 25$ arrays).

DISCUSSION

We identified three floral traits correlated with nectar volume (one component of nectar reward): corolla tube length, corolla tube opening diameter, and petal area. Of these floral traits, petal area and corolla tube opening diameter likely contribute directly to attraction. The corolla tube is enclosed by sepals, thus it is less likely to play a large role in pollinator attraction. In addition, our multivariate analysis indicates that overall flower size is positively correlated to the flower's total sugar reward and to both of its individual components, nectar sugar concentration and nectar volume. In our floral arrays, we disassociated all nectar rewards from floral size or attractive traits and found that hummingbirds uniformly prefer artificial flowers that present greater petal area. When the metric of preference is first visit to an array, the combination of larger petal area with wider corolla tube was most successful at attracting hummingbird visitors to our experimental arrays. Next we discuss how these findings provide insight into the role of pollinator preference as a selective agent underlying floral diversification.

Nectar volume in flowers increased at a constant rate across their 4-day lifespan as the sexual function changed from male to female. At the midpoint when flowers changed from male to female, they had accumulated 50% of their total nectar volume production, and nectar production continued for 2 additional days. Because nectar volume at the end of a flower's lifespan should be associated with the rate of nectar production, we think it likely that floral traits can serve as accurate indicators of nectar production throughout the lifespan of the flower. In addition, total sugar reward increased with the age of the flower. The increase in total sugar reward across the gender phases likely reflects the concomitant increase in nectar volume because nectar sugar concentration did not vary by gender phase. We know from field observations that a *S. virginica* flower is visited about twice per day throughout its lifespan (R. Reynolds, C. Fenster, and M. Dudash, unpublished data.).

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foraging bout. Modeling the number of visits as a linear function of the floral trait combinations showed that only flower size was a significant predictor of hummingbird visitation. (C) Total first visits by hummingbirds per array. The dependent variable is the number of times hummingbirds visited a given floral trait combination first when entering the array. Modeling the number of visits as a linear function of the floral trait combinations showed that the flower size $\times$ tube width interaction was a significant predictor of hummingbird visitation.

Consequently, hummingbirds remove nectar through all floral gender phases of individual flowers. The constant production of nectar may encourage multiple visits to a flower across days, enhancing both male and female plant reproductive success and may also reduce pollen limitation of fruit set in *S. virginica* (Dudash and Fenster, 1997). Low hummingbird visitation rates to *S. virginica* may in part explain why male function is distributed across 2 days. Thus, constant nectar production may help ensure that hummingbirds visit the flowers at least once during both the male and female phases.

Because we measured a number of flowers per plant, the amount of between-plant variation relative to within-plant variation for nectar production was quantified. Individual plants varied significantly in their nectar production in the field, indicating a consistent production of nectar within flowers of the same plant. Similar observations have been made on hawkmoth-pollinated *Mirabilis multiflora* (Hodges, 1993). It is perhaps noteworthy that we could not detect any vegetative correlates with nectar volume. Because plant vigor is positively correlated with the number of flowers produced by a plant, there is no trade-off between nectar volume per flower and the number of flowers produced by a plant. Therefore, from the floral traits measured in our field census, it does not seem likely that overall plant vigor alone dictates nectar volume. It is possible that unknown microenvironmental factors contribute to between-plant variation in nectar volume (Carroll et al., 2001; Leiss and Klinkhamer, 2005a). However, this caveat aside, the consistency or repeatability of within-plant nectar production sets an upper limit on the heritable variation of 19% (Falconer, 1981). In contrast, our upper estimate of heritability of petal area using this approach is 79%, consistent with our expectation of greater environmental influence on nectar traits relative to petal size traits. There have been relatively few estimates of nectar heritability. Greenhouse estimates are generally two- to threefold higher than our repeatability-based estimate (reviewed in Mitchell, 2004; Leiss and Klinkhamer, 2005a), but field-based heritability estimates of nectar volume in *Echium vulgare* (Leiss et al., 2004) and *Ipomopsis aggregata* (Campbell, 1996a) are roughly similar to our upper-bound repeatability estimate. Taken together, these results suggest nectar volume is an evolutionarily labile trait and may, at least for North American *Silene*, have readily diverged because of the different nutritional demands of alternative pollinators.

If the evolution of nectar volume reflects selection by pollinators, then we would expect to observe similar selection on floral traits that advertise this reward. Thus, pollinators should discriminate among floral traits based on expected reward. Flower-size variation among individuals has been shown repeatedly to be correlated with the size of the reward, whether nectar or pollen (Thomson and Plowright, 1980; Harder et al., 1985; Cresswell and Galen, 1991; Kaczorowski et al., 2005; and reviewed in Cresswell, 1998; but see Klinkhamer and Veen-van Wijk, 1999) This association conforms to the notion that flower size may evolve as an advertisement for greater rewards. Hummingbird and social bee flight behaviors suggest they forage efficiently (reviewed in Cresswell, 1998), and thus it is not surprising that these same pollinators have been shown to be highly discriminating toward floral variants within a population (Galen and Newport, 1987; Cresswell and Robertson, 1994; Ashman et al., 2000). Cresswell and Galen (1991) suggest that learned preference for large flowers with large reward could be dependent on the

relative proportions of individuals with large or small flowers. Furthermore, environmental context has been shown to alter pollinator discrimination against plants with low nectar volume. In natural populations of *Echium vulgare*, bee pollinators preferentially visit denser patches of flowers, and plants producing less nectar may benefit from this patch attraction, thus negating any usual discrimination against them (Leiss and Klinkhamer, 2005b). However, if pollinator preference for greater expression of secondary floral features of reward is inherent, it is likely the evolutionarily stable combination is a large reward associated with expression of particular secondary floral attractive features (Cresswell and Galen, 1991).

Data from our artificial arrays support the notion that hummingbirds have a preference for certain floral traits and combinations that are indicators of the amount of reward present in field populations of *S. virginica*. In our manipulative array work, each measurement of hummingbird preference is a proxy for fitness. All the response variables that were measured provided the same result; hummingbirds initially chose individuals on the basis of larger flower size. We did not detect discrimination by the hummingbirds with respect to corolla tube diameter alone but did note an association when petal area was manipulated simultaneously. At the level of first visits to an array, the lack of significance of petal area likely reflects insufficient sample size. A power analysis suggests that we could have detected a significant effect of petal area on the first visit to an array with 31 replicates instead of with the 25 replicates in this study. In hummingbird-pollinated *Ipomopsis aggregata*, male reproductive success was quantified using fluorescent dye particles as pollen analogs to demonstrate that hummingbirds prefer flowers with greater nectar reward (volume) and subsequently enhance male function of those flowers by visiting them more frequently (Mitchell, 1992; Mitchell and Waser, 1992). Higher nectar volume also increased hawkmoth visitation to *Mirabilis multiflora* (Hodges, 1995). We too have observed a close correspondence between visitation frequency by hummingbirds on *S. virginica* and male reproductive success as assessed through the use of fluorescent dyes as pollen analogs (Fenster et al., 1996, unpublished data). Male function via pollen transfer also is enhanced by length of time a pollinator stays at the flower, which in turn is often dictated by the amount of reward (Galen and Stanton, 1989; reviewed in Cresswell, 1998).

We do not know whether the correlation between the traits is shaped through underlying pleiotropy and/or by inherent or learned preference of the hummingbird pollinator for increased expression of both primary and secondary floral attractants. Hummingbirds are known to be able to discriminate among flowers that differ in reward (both volume and sugar concentration) as well as in spatial location between arrays such that hummingbirds first inspect flowers where a reward was present in a prior array (Bateson et al., 2002, 2003). Regardless of the basis for the association between reward and attraction, the higher visitation rates we have quantified to larger flowers or larger flowers combined with greater corolla tube diameter in our experimental arrays of artificial flowers suggest a basis for natural selection on these traits. Petal size and corolla tube diameter in populations of *S. virginica* may experience natural selection by hummingbirds because the expression of these traits is correlated with nectar availability. The preference by hummingbirds for artificial flowers in our arrays with wider corolla tube diameters, only in combination

with larger petals, suggests that hummingbirds are first attracted to larger flowers and then may make the decision to probe the flower based on the corolla tube diameter. Corolla tube diameter limits the ability of the hummingbird to probe the flower (Grant and Temeles, 1992; Campbell, 1996b; Temeles, 1996) to the extent that hummingbirds make many more insertion errors in narrower-tubed flowers (Smith et al., 1996) and thus could be viewed as a tertiary attractive feature indicating the accessibility of the nectar reward to the pollinator. From a functional perspective, it may be noteworthy that nectar volume is positively correlated to corolla tube length. Hummingbirds should forage more deeply in shorter corolla tube flowers if the nectar volume is relatively diminished compared with that of longer-tubed flowers. Thus the positive correlation between nectar volume and corolla tube length may be adaptive, reflecting an optimal insertion distance in terms of contact with the anthers and stigma of the flower.

Flowers are complex organs reflecting the coordination of many parts that function together to attract and reward pollinators while optimizing efficient pollen transport (Darwin, 1877; Stebbins, 1950; Armbruster et al., 2000; Fenster et al., 2004). As such, flowers are adaptations that can shed light on the dynamics underlying natural selection. Here, we add to a small but consistent literature linking pollinator preference to traits that advertise rewards. The work presented here is part of a larger body of research to assess the role of pollinator-mediated selection in the divergence of three closely related taxa of *Silene*. *Silene virginica*, red-flowered and pollinated by hummingbirds, *S. stellata,* white-flowered and pollinated by nocturnal moths, and *S. caroliniana*, pink-purple–flowered and pollinated by diurnal bees and hawkmoths, vary greatly in petal area, corolla tube diameter, and nectar reward (R. Reynolds, C. Fenster, and M. Dudash, unpublished data). This study has demonstrated the role of hummingbird preference in establishing a mechanism for natural selection to act and likely contribute to the divergence among these candidate sister *Silene* taxa.

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