POLLINATOR-MEDIATED SELECTION ON THE NECTAR GUIDE PHENOTYPE IN THE ANDEAN MONKEY FLOWER, *MIMULUS LUTEUS*

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Abstract. *Mimulus luteus* (Scrophulariaceae) is a perennial herb occurring in the South American Andes that shows a wide variation in the size and shape of a red spot on the lower lobe of the yellow flower. We describe the preference of four insects (three bees and one butterfly) and one hummingbird species for floral characters, and estimated the strength, direction, and form of pollinator-mediated selection through female fitness. We applied geometric morphometrics to describe the preference of pollinator species for different guide shapes. Our results revealed striking differences in the floral phenotypes preferred by insects and hummingbirds. Insects visited flowers with corollas 1.25-fold larger and guides 1.72-fold larger than the hummingbird species did. While insects preferred flowers with nectar guides pointing toward the corolla tube, the hummingbird preferred flowers with heart-shaped nectar guides. Most of the floral preferences shown by pollinators translated into significant linear and nonlinear selection coefficients. When selection was analyzed on a per-flower basis and for female fitness, corolla size was under positive directional selection, and nectar guide size and shape were under disruptive selection. Because the insect and hummingbird pollinators showed a strong segregation in their daily activity time, we suggest that current disruptive selection on the nectar guide phenotype can result from the differential availability of the rewarding floral variants over a day. Our findings suggest that pollinator-mediated selection favoring extreme phenotypes in *M. luteus* may not only contribute to high nectar guide variation found in this species, but also can promote divergence of corolla and nectar guide traits.

Key words: *disruptive selection; divergence; geometric morphometrics; natural selection.*

INTRODUCTION

The extent to which floral characters evolve under pollinator-mediated selection is a central question in plant evolutionary ecology. Although this issue can be traced back to Darwin (1862), only in the last decade has this topic been evaluated using quantitative estimates of natural selection. This renewed emphasis has been stimulated, in part, by the development of statistical procedures designed to evaluate the importance of direct and indirect selection on suites of correlated characters in the field (e.g., Lande and Arnold 1983, Mitchell-Olds and Shaw 1987, Schluter 1988, Phillips and Arnold 1989, see review in Brodie et al. 1995). For instance, assessment of selection coefficients has provided convincing evidence that pollinators influence the evolution of a variety of floral traits, such as corolla size (e.g., Campbell 1989, Galen 1989, Campbell et al. 1991, 1996, 1997, Herrera 1993*a*, Conner et al. 1996*a*, Caruso 2000), stigma exsertion (e.g., Conner et al. 1996*b*), nectary–stigma distance (Johnston 1991), flower stalk length (e.g., O'Connell and Johnston 1998, Maad 2000), spur length (e.g., Herrera 1993*a*, Maad 2000), flowering date (e.g., Campbell 1989, Johnston 1991, Widén 1991, Kelly 1992, Gómez 1993), corolla

color (e.g., Nagy 1997, Campbell et al. 1997, Gómez 2000), and corolla shape (e.g., Herrera 1993*a*, *b*, Nagy 1997, Galen and Cuba 2001).

Many plant species attract pollinators through visual guides such as spots, lines, and blotches of contrasting color on petals that signal pollen or nectar reward (see reviews in Waser 1983, Waser and Price 1985). Even though floral guides are good candidate traits on which to assess pollinator-mediated selection, there is a lack of studies designed to evaluate the functional role of nectar guides in natural populations. This omission is unfortunate because the size and shape of nectar guides may be especially important for insects that rely on visual cues for short-distance orientation and landing on flowers (e.g., Lehrer et al. 1995, Lehrer 1997, Johnson and Dafni 1998). Most evidence indicating that visual nectar guides influence pollinator foraging behavior comes from ethological experimental studies performed on artificial flowers in the laboratory (e.g., Bolwig 1954, Manning 1956, Scora 1964, Free 1970, Lunau 1992, 1996, Dafni and Kevan 1996, Menzel et al. 1997). Implicit in most studies is the assumption that pollinators are able to perceive and discriminate among floral variants within populations, which in turn translate into plant fitness variation and phenotypic evolution. There is, however, a subtle but important distinction between the role of a floral trait in attracting pollinators, and the potential impact that pollinators

have on the adaptive evolution of the trait. While the first aspect deals with the proximal mechanisms involved in pollinator attraction (e.g., Anderson 1977, Gould 1985, 1986, see reviews in Waser 1983, Kevan 1983, Dafni and Kevan 1997, Menzel et al. 1997), the second category deals with the ultimate fitness impact that pollinators have on plants (see reviews in Herrera 1996, Kingsolver et al. 2001). Clearly both approaches are necessary for a more complete evaluation of pollinator-mediated selection in the field. This study was designed to examine the proximate and ultimate factors that influence the evolution of the nectar guide phenotype in the Andean monkey flower *Mimulus luteus*, a perennial herb that inhabits the South American Andes. The aims of this study are: (1) to examine the importance of the visual nectar guide on pollinator attraction, and, (2) to evaluate the extent to which the nectar guide phenotype is under pollinator-mediated selection. More specifically, in this study we asked the following questions. (1) Is nectar guide a truthful advertisement of nectar availability? (2) What is the functional importance of the nectar guide in attracting pollinators? (3) Does variation in the size and shape of the nectar guide phenotype translate into correlated variation in seed production? The answers to these questions lead to a more general question: (4) What is the potential of the nectar guide phenotype to evolve under pollinator-mediated selection?

MATERIALS AND METHODS

Natural history and study site

Mimulus luteus (Scrophulariaceae) is a hydrophilic species that inhabits streamsides and boggy areas through the South American Andes from 29° S to 45° S (von Bohlen 1995). In Chile, flowering and fruiting occur from December to February. Plants are 53 cm tall on the average and most individuals produce 1–4 zygomorphic flowers that last 3.6 and 5.9 d on average if pollinated and unpollinated, respectively. The fruit set in open-pollinated flowers is 86.7%, and mean seed production per fruit is 335.2 (1 SE = 15.9, $N = 300$). Automatic self-pollination with pollinators excluded occurs in 10% of flowers, and mean seed production per fruit is 22.6 (1 SE = 13.2, $N = 100$). Flowers have inserted anthers and a bilobed stigma that closes upon contact. The five-lobed yellow corolla has a mean area of 960.2 mm² (measured from scaled frontal-viewed digital photographs, range = 487–1458 mm², CV = 14.8%), and presents a conspicuous red nectar guide spot on the lower lobe (mean area = 56.9 mm², range = 0–206.6 mm², CV = 76.4%, Fig. 1). Guide size is more variable between than within plants (one-way ANOVA: $F_{46,72} = 20.34$, $P < 0.001$).

This study was conducted during the summer seasons of 1999 and 2000 in a protected area of the mining company Los Pelambres (31°45′40.8″ S, 70°30′33.1″ W, 2730 m above sea level), located 55 km east of Salamanca, IV Región, Chile. In the study site, *M. luteus* is distributed in conspicuous monospecific patches, surrounded by a vegetation matrix that includes *Montiopsis sericea* (Portulacaceae), *Calceolaria biflora* (Scrophulariaceae), *Cerastium montioides* (Caryophyllaceae), *Chuquiraga oppositifolia* (Compositae), *Vicia graminea* (Fabaceae), *Senecio polygaloides* (Compositae), and *Astragalus looseri* (Fabaceae) as the most common species. Pollinator species consist of the bees *Centris nigerrima* (body length = 16.0 mm, Anthophoridae), *Megachile semirufa* (body length = 13.0 mm, Megachilidae), and *Melissoptila* sp. (body length = 9.0 mm, Anthophoridae), the butterfly *Tatochila mercedis* (wing span = 32–50 mm, Pierinae), and the Andean hummingbird *Oreotrochilus leucopleurus* (body length = 13 cm, Trochilidae). Nighttime observations indicate that nocturnal pollination is absent in this species (R. Medel, *personal observation*).

Field and laboratory procedures

To evaluate the importance of nectar guide as an advertisement of nectar availability, on 25 January, 1999 at 2000 hours, we randomly bagged 113 flowers on the first day that they opened (hereafter, ''first-day flowers''). Flowers were removed the next day at 0900–1000 ($N = 41$), 1300–1400 ($N = 41$), and 1700–1800 hours ($N = 31$). The nectar volume of each flower was calculated immediately after removal by measuring the column length in 1-μL capillary tubes. A digital caliper (precision 0.01 mm) was used for length measures. Flowers were recorded as digital pictures using a scaled FD-7 Sony digital Mavica (Sony, Tokyo, Japan), taking into account the perspective of approaching pollinators, in a plane 90° to the long axis of the corolla. Distance from the objective to the flower was kept constant by using a rigid support. To examine the functional relationship of guide size with nectar volume throughout the day, we performed regression analysis for the three time periods separately. Because corolla size is an important trait influencing pollinator attraction (e.g., Galen et al. 1987, Eckhart 1993, Robertson et al. 1994, Campbell 1996, Conner et al. 1996a, b, Galen 1999, 2000), we also measured corolla size from pictures and included this trait as covariate in a multiple regression model. To evaluate the relationship of nectar guide with nectar concentration, we measured the sugar concentration in five groups of 10 small-guided and 10 large-guided flowers, always between 930 and 1100 hours, using a Reichert temperature-compensated hand refractometer (Reichert, Depew, New York, USA). Because nectar volumes were not large enough to measure concentration accurately on a per-flower basis, we measured nectar concentration on groups of 10 flowers. To assure that our visual classification of the nectar guide size was correct, we took photographs of flowers as described earlier in this paragraph, and performed image analysis in the laboratory. The small-guided and large-guided samples differed significantly as expected

FIG. 1. Variation in the nectar guide phenotype of *Mimulus luteus* in the study site (31°45′40.8″ S, 70°30′33.1″ W).

from our a priori classification (mean $\pm$ 1 SE, 3.17 $\pm$ 0.28 and 142.37 $\pm$ 4.42 mm², respectively; one-way ANOVA, $F_{1,96} = 1952.93$, $P < 0.001$).

We assessed the functional importance of guides in attracting pollinators, during six consecutive days, by recording pollinator visits on a 7.45 m² patch. The patch had a mean ''first-day flower'' density of 530 flowers/m² ($N = 10$ randomly set 30 × 30 cm quadrants), providing an estimate of 3947 first-day flowers. To avoid disturbance, we censused flowers only in the first day of observation. Consequently, we monitored a total of 3947 flowers opened during all six days of the observation period. We made observations during two periods, morning (1000–1500 hours) and afternoon (1500–2000 hours). Each sampling period was repeated three times and randomly assigned to six consecutive days, rendering a total observation time of 1807 min. We scanned the patch surface from a height of 1.5 m observation point ~5 m away from the center of the patch. Once a pollinator entered the patch, we kept track of it and took a photograph of the first flower it visited as described earlier in this paragraph. Only visitors that entered the corolla tube were considered in analyses; insects that used the lower lobule of the flower only for landing purposes were not used for analysis. To assure that pictures represented truly independent floral phenotypic measures rather than repeated measures of the same flower, we removed the visited flowers after recording them. Although this procedure assumes that visited flowers are not visited again, it has the advantage of avoiding lack of statistical independence due to multiple records of the same flower. Because pollinators visited more than one flower per approach, we followed individual pollinators and counted the number of flowers probed while the pollinator was foraging in the patch (flowers probed per approach in Table 1). We took digital pictures of 339 flowers, each corresponding to the first flower visited in independent pollinator approaches, and captured the details of the nectar guide shape with an additional close-up on the lower lobe, taking care to minimize potential damage

to flowers due to handling. Only pictures with sufficient resolution to perform unbiased digitization were included in analyses ($N = 203$). Images were analyzed with UTHSCSA ImageTool for Windows, version 2.0 (University of Texas Health Science Center, San Antonio, Texas, USA). To compare pollinator preference, we grouped the flowers visited by each pollinator species and performed a one-way ANOVA for corolla and guide size among digitized floral data. Corolla and guide size data were log transformed before analyses.

The shape of the nectar guide was described using elliptic Fourier analysis (EFA) for bidimensional outlines in D. E. Slice's Morpheus et al. (software package available online).[2] Because ellipses can be defined by four coefficients for each harmonic, corresponding to Fourier series of sine and cosine curves of decreasing amplitude and period, they can fit and describe any object outline provided an adequate number of harmonics (Kuhl and Giardina 1982). The first few Fourier coefficients are expected to define overall shape, whereas finer details may be described with an increasing number of harmonics (see examples in Rohlf and Archie 1984, Ferson et al. 1985, McLellan and Endler 1998). We digitized the photos using F. J. Rohlf's tpsDig (program available online)[3] and stored each outline as a complete set of *x* and *y* coordinates to be used in calculations of elliptic Fourier coefficients. To decide the number of harmonics needed for an adequate description of guide shape, we computed the four elliptic Fourier coefficients for an increasing number of harmonics, and assessed the change in the percentage of shape variation that was accounted by each harmonic (see Lestrel 1997). We used the 16th harmonic because the error was <5% on average for all outlines. After saving the 4 × 16 shape coefficients for every flower, we reconstructed the mean nectar guide shape of the flowers visited by each pollinator species and performed contrasts using canonical variates analysis

[2] URL: ⟨http://life.bio.sunysb.edu/morph/morpheus/⟩
[3] URL: ⟨http://life.bio.sunysb.edu/morph/soft-dataacq.html⟩

TABLE 1. Pollination process and floral choice by each pollinator species.

	Pollinator visits			Floral trait		
Pollinator species	Visitation rate (visits·flower^{-1}·h^{-2})	Flowers probed per approach	Percentage of visits	Corolla size (mm^2)	Guide size (mm^2)	N
Bees						
Centris nigerrima	4.63 (3.55)[a]	15.77 (4.66)[a]	52.8	889.35 (73.69)[a]	55.20 (7.86)[ab]	11
Megachile semirufa	1.53 (1.13)[a]	4.31 (2.05)[b]	19.3	829.67 (47.21)[a]	88.57 (7.36)[a]	37
Melissoptila sp.	1.27 (0.59)[a]	6.33 (2.30)[ab]	2.6	859.11 (27.26)[a]	74.28 (5.28)[a]	80
Butterflies						
Tatochila mercedis	0.83 (0.41)[a]	2.60 (0.82)[b]	6.0	905.42 (67.89)[a]	79.42 (12.81)[a]	15
Birds						
Oreotrochilus leucopleurus	2.13 (0.83)[a]	4.27 (0.93)[b]	19.3	685.72 (18.91)[b]	41.83 (3.66)[b]	60

Notes: Values in parentheses are 1 SE of the respective mean. Within columns, values that do not share letters are significantly different ($P < 0.05$, one-way ANOVA followed by Tukey's hsd test). Total visits = 339.

(CVA) in NTSYSpc (Numerical Taxonomy System, version 2.1; Exeter Software, Applied Biostatistics, Setauket, New York, USA). CVA is a commonly applied method to reveal differences in discriminatory power among morphometric descriptors. It is a generalization of discriminant function analysis that separates the samples into groups on the basis of the principal components. We performed single-classification MANOVA to test the probability of the null hypothesis that all pollinator species visit flowers with similar guide shape. A second MANOVA was made to evaluate if insects as a whole differed from the hummingbird in their guide shape preference. All 64 elliptic Fourier coefficients per flower were used as dependent variables in multivariate analyses.

To evaluate a potential time segregation among pollinator species, we recorded their activity throughout the six days of observation, pooled data for one-hour intervals, and calculated the time overlap by the symmetric Czekanowski's overlap index (Feinsinger et al. 1981). Graphically, this index corresponds to the area of intersection of the utilization histograms of the two species. The index is calculated as, $O_{12} = O_{21} = 1 - 0.5 \Sigma \mid p_{1i} - p_{2i} \mid$ where p_{1i} and p_{2i} are the frequency of occurrence of species one and two at the one-hour time interval i. The index ranges from 0 to 1, indicating nil and total time overlap, respectively. The statistical significance of the observed overlap was calculated by bootstrapping 1000 times the activity of pollinator species and assessing the tail probability of obtaining pseudovalues less than or equal to the observed overlap value. We used the randomization algorithm three in EcoSim statistical package, version 7.0 (EcoSim: null models software for ecology; Acquired Intelligence and Kesey-Bear, Burlington, Vermont, USA). This algorithm retains the time specialization of species, in other words, the number of time intervals showing pollinator activity, but randomizes which hours are utilized.

Assessment of phenotypic selection

To evaluate the potential of traits to evolve under pollinator-mediated selection, we estimated the linear and nonlinear selection coefficients by following the methodology of Lande and Arnold (1983). Selection coefficients are useful descriptors of natural selection. They provide information of the strength, direction, and form of the within-generation selection surface, and give insight into the expected distribution of phenotypes in subsequent generations, provided some genetic assumptions are fulfilled (Lynch and Walsh 1998). In this paper we focused on the within-generation description of pollinator-mediated selection, postponing between-generation genetic analyses (the evolutionary response to selection) for future studies. We performed phenotypic selection analysis on corolla size, guide size and shape, and plant height from a set of flowers different from those used in the nectar and pollinator preference analyses. We tagged 300 first-day flowers, each from a different individual, and digitally recorded their corolla and guide as above. Guide shape was described as in the pollinator preference analysis. We used the scores for the first two dimensions in CVA (accounting for 49.3 and 48.8%, respectively, of the total variance in the Fourier coefficients) as raw shape data in phenotypic selection analyses. Because plant size is an important trait affecting pollinator attraction and reproductive success through female function (Wolf and Hainsworth 1990, Johnston 1991, Widén 1991, Andersson and Widén 1993, Mitchell 1994, Maad 2000), we included this trait as covariate in phenotypic selection analysis. Plant height was measured as the linear distance (precision 5 mm) from the ground to the top of the highest flower. The capsules of recorded flowers were collected after 22 d, when no evidence of additional fruit formation was observed. Seeds were counted in the laboratory under a binocular microscope.

The selection differential, S_i, is the amount by which selection shifts the mean of character i between the actual and potential parents. This coefficient was calculated as follows:

$$S_i = \text{COV}(z_i, w) \qquad (1)$$

where z_i is the character i, and w is the relative fitness (absolute fitness divided by mean absolute fitness). This

coefficient is an estimate of the total selection acting upon the focal trait, including both direct and indirect selection through correlated characters. The statistical significance of the S_i values was tested by Pearson's product-moment correlation of relative fitness with characters. To describe the direct force of selection acting on the phenotypic character i, excluding indirect selection, we applied the multivariate model of Lande and Arnold (1983):

$$w = \alpha + \sum_i^n \beta_i z_i + \varepsilon \qquad (2)$$

where, α is a constant, β_i represents the average slope of the selection surface in the plane of the character i, and ε is an error term. In this equation, the linear gradient of selection, β, provides information of the direction and magnitude of change expected after current selection. The nonlinear selection gradients, γ, describe the nature of selection on quadratic deviations from the mean for both single and pairwise combinations of characters. Nonlinear gradients were estimated from the second order coefficients of the following model:

$$w = \alpha + \sum_{i=1}^n \beta_i z_i + \frac{1}{2} \sum_{i=1}^n \gamma_{ii} z_i^2 + \sum_{i=1}^n \sum_{i \neq j}^n \gamma_{ij} z_i z_j + \varepsilon \quad (3)$$

where γ_{ii} represents the curvature of the relationship between the character i and relative fitness. When $\gamma_{ii} < 0$, it implies downward concavity and stabilizing selection acting upon the character. When $\gamma_{ii} > 0$, it reflects upward concavity and disruptive selection. The correlational selection gradient, γ_{ij}, reflect the extent to which selection acts upon the correlation of traits i and j. The directional selection gradients (β) were estimated only from Eq. 2 because if characters are multivariate nonnormally distributed, z_i and z_i^2 in Eq. 3 are intercorrelated (Lande and Arnold 1983). The significance of β and γ in Eqs. 2 and 3, respectively, was determined directly from the regression coefficients after the effects of all other parameters had been removed. All characters were standardized to zero mean and unit variance (z-transformation) before analysis by substracting the mean of the trait from each value and dividing by the standard deviation. This transformation is equivalent to expressing the original individual trait in units of standard deviation and allows comparison of the strength of selection among populations or species (Lande and Arnold 1983). Standardized differentials are written as S' and standardized linear and quadratic gradients are written as β' and γ', respectively. All phenotypic selection analyses were made with the SAS 6.12 package for Windows (SAS Institute 1996). Because stabilizing and disruptive selection may represent ideal rather than real selection surfaces (see Mitchell-Olds and Shaw 1987, Phillips and Arnold 1989), we estimated the form of selection on the characters showing statistical significance in the nonlinear

analysis. We used the univariate cubic spline routine provided by Schluter (1988). The cubic spline procedure is a nonparametric fitting function that provides a quantitative prediction of fitness across a range of trait values. Because the form of the fitness surface can vary depending of the trait interval on which the local estimation is performed (λ), we used the trait interval that minimized the prediction error of the model. The standard error for each predicted regression surface was estimated by bootstrapping the dataset 5000 times.

RESULTS

Nectar measurements

Nectar volume differed significantly between samples taken at 900, 1300, and 1700 hours (one-way ANOVA: $F_{2, 110} = 4.34$, $P = 0.015$; mean ± 1 SE at 900 hours, 0.726 ± 0.069, $N = 41$; at 1300 hours, 1.097 ± 0.127, $N = 41$; at 1700 hours, 1.374 ± 0.131, $N = 31$). A posteriori Scheffé contrasts revealed significant differences between 900 and 1700 hours ($P = 0.020$) but not between 900 and 1300 hours ($P = 0.853$), or between 1300 and 1700 hours ($P = 0.086$). These results were consistent after including corolla and guide size as covariates (ANCOVA, $F_{2, 108} = 3.57$, $P = 0.031$), indicating that nectar tends to accumulate over the day in this species. Results from multiple-regression analyses of corolla and guide size on nectar volume revealed low and nonsignificant regression coefficients for samples taken at 900 hours (slope; corolla size = -0.012 ± 0.634, $P = 0.985$; guide size = -0.002 ± 0.620, $P = 0.998$, $N = 41$), at 1300 hours (corolla size = 0.393 ± 0.941, $P = 0.679$; guide size = -0.150 ± 0.321, $P = 0.643$, $N = 41$), and at 1700 hours (corolla size = 0.696 ± 0.483, $P = 0.160$; guide size = -0.058 ± 0.140, $P = 0.683$, $N = 31$), indicating that corolla and guide size are poor descriptors of individual variation in nectar production. Sugar concentration did not differ among flowers having extreme nectar guide phenotypes (mean % sugar; large-guided individuals, 20.64 ± 0.837; small-guided individuals, 20.22 ± 0.850; $t_8 = 0.357$, $P = 0.730$). Even though we compared nectar concentration between phenotypes in the morning only, therefore limiting our conclusions to this specific period, nectar production was independent of guide size at 900, 1300, and 1700 hours, thus turning unlikely that phenotypes differ in nectar concentration at other times of day. Overall, these two lines of evidence indicate that corolla and guide size are associated with neither nectar volume nor sugar concentration, therefore rejecting the hypothesis that nectar guides have a functional role in nectar advertisement.

Pollinator preference

Flowers had a low chance of being visited during the six-day observation period (mean visits per hour per flower were 0.0025 ± 0.00091), suggesting that the pollination environment for female fitness was limiting
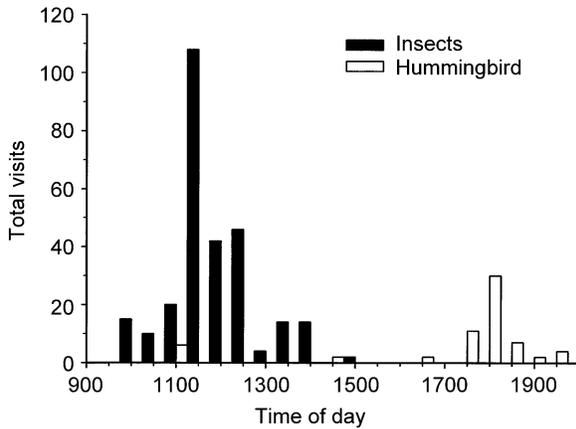
FIG. 2. Frequency of flower visits made by the hummingbird and insect species over the day. Total visits to independent flowers of *M. luteus* from several days are shown, with each bar representing one half-hour period.
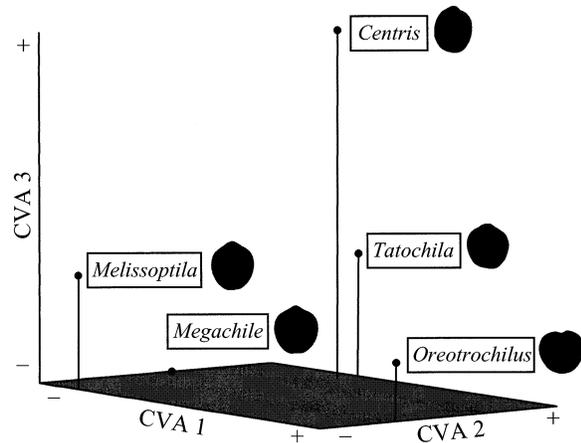

FIG. 3. CV ordination of the mean guide shape of the flowers visited by each pollinator species. The first three canonical axes accounted for 75.3%, 16.0%, and 4.0% of the overall variation in the elliptic Fourier coefficients. Shape reconstructions were performed at the 16th harmonics.

in the study site. Pollinator species did not visit flowers at random over the day. On the contrary, because the observed time overlap between taxa was lower than the simulated indices in 964 out of 1000 times, there was significant time segregation between insects and hummingbirds (observed overlap = 0.073, simulated mean overlap = 0.211 ± 0.003, $P = 0.036$). While insect species foraged mainly during the morning hours, showing a peak activity during the interval 1130–1200 hours, the hummingbird *Oreotrochilus leucopleurus* was active mainly at dusk, showing maximum activity during the interval 1800–1830 hours (Fig. 2). The bee *Centris nigerrima* was the most frequent flower visitor, accounting for 52.8% of the 339 visits in 30.12 h of observation, followed by *Megachile semirufa* (19.3%) and the hummingbird *O. leucopleurus* (19.3%). Even though pollinators did not differ in visitation rate (one-way ANOVA, $F_{4,145} = 0.48$, $P = 0.751$), they differed in the number of flowers probed per approach (one-way ANOVA, $F_{4,51} = 4.96$, $P = 0.002$; Table 1).

Pollinators differed in the guide and corolla size of the flowers they visited (one-way ANOVA; guide size, $F_{4,198} = 9.14$, $P < 0.001$; corolla size, $F_{4,198} = 6.13$, $P < 0.001$). The effect of guide size on pollinator preference remained significant after removing the effects of corolla size (ANCOVA, $F_{4,197} = 6.058$, $P < 0.001$), indicating that there are independent effects of corolla and guide size on pollinator preference. Similarly, the mean nectar guide shape differed between pollinator species (MANOVA, Wilks' $\lambda_{256,625}$ on the 64 elliptic Fourier coefficients = 0.111, $P < 0.001$). Fig. 3 illustrates the separation in the reconstructed mean guide shape among the five species as found from the canonical variates analysis (CVA). While most insect species chose flowers with guides pointing to the corolla tube, the hummingbird *O. leucopleurus* showed a preference for flowers with heart-shaped guides. Subsequent comparison of the flowers visited by insects

all together and the bird, revealed that insects visited flowers with guides and corollas 1.72 and 1.25-fold larger, respectively, than the hummingbird species did (one-way ANOVA; guide size, $F_{1,198} = 23.95$, $P < 0.001$; corolla size, $F_{1,198} = 21.35$, $P < 0.001$; Fig. 4). Regarding shape, insects and hummingbirds differed in the mean guide shape of the flowers they visited (MANOVA; Wilks' $\lambda_{64,159} = 0.331$, $P < 0.001$; Fig. 4), indicating that guide shape was an important component of the floral phenotype that influenced pollinator attraction.

Selection analysis

Corolla size was the only floral trait showing a significant selection differential (Table 2), which implies that total selection shifted the mean of this character 0.202 SD (28.7 mm²) toward increasing size. Selection
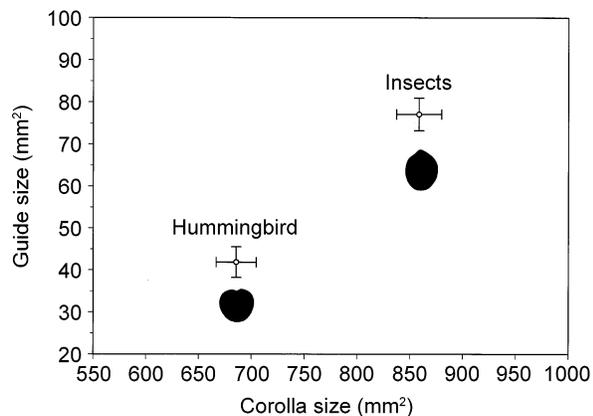

FIG. 4. Mean corolla and guide size of flowers visited by the hummingbird and insect species. Bars indicate ±1 SE. The mean guide shape of the flowers visited by each pollinator group is also depicted.

TABLE 2. Pollinator-mediated selection coefficients on the floral traits of *Mimulus luteus* ($N = 300$).

Character i	S_i'	β_i' (SE)	γ_{ii}' (SE)	CS γ_{ij}' (SE)	GS γ_{ij}' (SE)	CV1 γ_{ij}' (SE)	CV2 γ_{ij}' (SE)
Plant height	0.098	0.078 (0.060)	0.301 (0.091)**	0.052 (0.066)	−0.038 (0.066)	0.058 (0.283)	0.226 (0.324)
Corolla size (CS)	0.202**	0.200 (0.059)**	0.353 (0.078)***		−0.067 (0.070)	−0.210 (0.299)	0.073 (0.301)
Guide size (GS)	0.005	−0.025 (0.059)	0.252 (0.098)*			0.187 (0.250)	−0.564 (0.340)
CVA 1 shape (CV1)	−0.040	0.011 (0.058)	6.338 (1.377)***				−0.332 (1.399)
CVA 2 shape (CV2)	0.002	−0.007 (0.058)	2.580 (0.752)**				

Notes: Column head abbreviations are spelled out in column 1. Plant height is included as covariate. S_i' is the standardized differential of selection. Directional (β_i'), quadratic (γ_{ii}'), and correlational (γ_{ij}') standardized gradients are depicted. Values in parentheses are 1 SE.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

for increasing corolla size was also detected in linear analysis after controlling for potential selection on correlated characters, suggesting a low importance of indirect selection acting upon this character. Gradients of directional selection for plant height, guide size, and guide shape were low and nonsignificant (Table 2). Regarding nonlinear selection, positive and significant

γ_{ii} coefficients were detected for all characters, indicating that disruptive selection was prevalent in this study (Table 2). Inspection of the selection surface on corolla size revealed a slight decrement in the fitness function up to -2 SD in the character followed by a strong pattern of directional selection for increasing size (Fig. 5a). The pattern of selection upon guide size
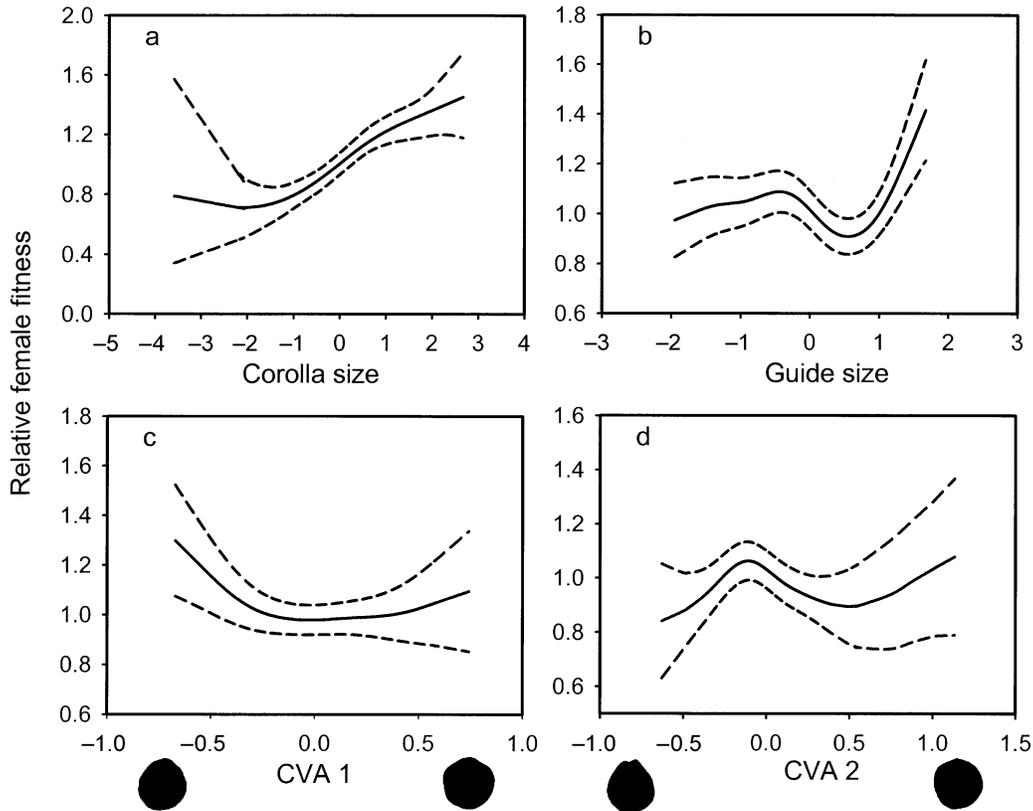

FIG. 5. Cubic spline estimates for (a) corolla size (smoothing λ parameter $= -8$), (b) guide size ($\lambda = -10$), (c) individual scores on the first CV axis for guide shape ($\lambda = -4$), and (d) individual scores on the second CV axis for guide shape ($\lambda = -4$). Depicted outlines are mean guide reconstructions at the 5th and 95th percentiles from each CV score distribution. Dashed lines represent ± 1 SE estimates from 5000 bootstrap replications.

showed an accelerated gain toward -0.5 and 1.5 SD in the character with a minimum value about 0.5 SD (Fig. 5b). Significant disruptive selection was also detected on the two first canonical score axes describing variation in the nectar guide shape (Table 2, Fig. 5c, d). Selection favored extreme phenotypes along the axis CVA1, indicating that the shape of the medium to upper section of nectar guides was important in accounting for differences in relative fitness (Fig. 5c). Likewise, the selection surface along the axis CVA2 revealed two peaks with an accelerated fitness function toward slightly bifurcated guides, suggesting that guide bifurcation was an important signal to pollinators (Fig. 5d). Coefficients for correlational selection were low and nonsignificant, suggesting that pollinators did not select combinations of floral traits. However, because the lack of statistical significance can be attributable to weak selection or insufficient sample size, we estimated the minimum population size necessary to achieve statistical significance at the $\alpha = 0.05$ level as $(t\sigma/\gamma)^2$, where γ is the correlational selection coefficient, σ is the standard deviation of γ, and $t = 1.96$ (Johnston 1991, Medel 2000). Results indicate that detection of significant correlational selection requires a minimum sample size of 8127 individuals on the average (range for the 10 γ_{ij} coefficients $= 419–27\,442$). Because these figures exceed the observed population size in the patch (3947 individuals), we can be confident that the lack of significant γ_{ij} coefficients reflects absence of selection on the phenotypic correlations rather than insufficient sample size.

DISCUSSION

The role of guide traits on pollinator preference

Our results indicate that insect and hummingbird species visited flowers with different corolla size, guide size, and guide shape. We showed that while the insect pollinator assemblage prefers flowers with large corollas and large guides, hummingbirds prefer flowers with small corollas and small guides. Consequently, the divergent pattern of flower preference can reasonably be invoked as the proximal mechanism underlying the pattern of disruptive selection on these characters. This result raises the question as to why pollinators differ in their floral choice? Because nectar production and sugar concentration did not differ between phenotypes, nectar reward cannot be invoked as a cause for the different pollinator preference. Therefore, there is no obvious reason why insects should prefer large-guided flowers. One possibility is that guides signal pollen reward, but no pollen-gathering bees were observed at the study site, so we can be relatively confident that nectar is the main reward for pollinator species. Another possibility is that that bees and butterflies use floral pigments as cues that help them to reduce searching and handling time as a way of minimizing the high energetic cost of flight (e.g., Heinrich 1975,

Waser and Price 1981). Little (1983) suggested the term ''mimicry based on naiveté'' to describe situations where naive pollinators, such as newly emerged insects, are attracted toward flowers on the base of their innate behavior, which is elicited by certain floral signals such as shape, color, and odor (see also Lunau et al. 1996). As they gain knowledge of these cues, they can use them to guide their searches and improve their foraging performance on subsequent bouts. While this situation has been described in the literature (e.g., Nilsson 1980, Ackerman 1981, Fritz 1990), empirical evidence for this phenomenon is still limited. A third, not mutually exclusive but perhaps more parsimonious, explanation relates to the different flower visitation schedule used by insects and hummingbirds. It is possible that insects make the first decision by using visual cues such as large corolla and guide size in the morning, leaving flowers with smaller corollas and guides to accumulate nectar over the entire day. Under these circumstances, the hummingbird may avoid the flowers emptied in the morning by insects, concentrating its foraging effort on the flowers unvisited by insects. It is known that hummingbirds often learn to avoid nectar-emptied flowers by using spatial and floral color cues to guide their choice (e.g., Gass and Sutherland 1985, Sutherland and Gass 1995, Hurley 1996, Hurley and Healy 1996, Meléndez-Ackerman et al. 1997). The extent to which *Oreotrochilus leucopleurus* uses corolla and guide size as cues for flowers with small quantities of nectar or senses directly the nectar availability is currently under experimental study through nectar and guide phenotype modifications.

Event though several studies have documented the importance of corolla shape in determining plant reproduction and pollinator-mediated selection (e.g., Pellmyr 1988, Herrera 1993a, b, 2001, Andersson 1994, Johnson et al. 1995, Nagy 1997, Galen and Cuba 2001, Thompson 2001), studies on the role of nectar guide shape are notoriously absent in the literature. Most evidence suggesting a functional role for guide shape comes from choice studies on model flowers in the laboratory (e.g., Manning 1956, Free 1970, Lehrer et al. 1995, Dafni and Kevan 1996, Lehrer 1997, Johnson and Dafni 1998, West and Laverty 1998). In this paper we provide evidence that guide shape affected not only pollinator preference but also the plant's female fitness. More specifically, flowers with nectar guides projecting toward the corolla tube had a higher chance of being visited by insects than by the hummingbird *O. leucopleurus*, suggesting that the spatial information provided by the nectar guide may affect insect landing (see also Laverty 1980). On the contrary, flowers with heart-shaped guides had a higher chance of being visited by the hummingbird than by the insect species. The divergent guide shape preference shown by pollinators can be suggested as the mechanism underlying the pattern of disruptive selection on the prin-

cipal component axes that summarize guide shape variation.

Pollinator-mediated selection

In spite of the significant β and γ_{ii} coefficients for nectar guide phenotypes, linear and nonlinear selection models accounted for a low proportion of variance in female fitness (4.7 and 2.7%, respectively), indicating that factors other than those assessed in this study are a major source of variation for plant fitness. Unmeasured traits such as flower longevity, corolla tube length, flower color and scent, and ultraviolet reflection may account for an important fraction of the unexplained variance in seed production. In addition, environmental effects, such as microhabitat type, microclimate, and temperature conditions, can often explain more variance in seed production than floral characters involved in pollinator attraction (Zimmerman and Pyke 1988, Herrera 1993a, O'Connell and Johnston 1998, Totland 2001). In the context of this research, the heavy metal concentration in the soil environment may be an important factor influencing fitness variation. It is known that high copper substrates may select for increased tolerance capacity through changes in the seed: ovule ratio in *Mimulus guttatus* (Searcy and Macnair 1990, Macnair et al. 1993, Tilstone and Macnair 1997). Experimental studies documented that this ratio decreased by an average of 24% when pollen came from plants sensitive rather than tolerant to copper (Searcy and Macnair 1990). Since copper tolerance ecotypes have been recently described in *M. luteus*, seemingly as a consequence of mining activities (Ginocchio et al. 2002), and our population is in a copper mining area, it is likely that individual variation in tolerance capacity account for an important proportion of variance in seed production.

The importance of measuring reproductive success through both sex functions has been stressed by many authors (e.g., Stanton et al. 1986, Devlin and Ellstrand 1990, Campbell et al. 1991, Conner et al. 1996a, b, Schlichting and Delesalle 1997, Ashman 1998). Even though the contribution of male fitness such as pollen export and siring success to total fitness is notoriously difficult to measure in the field, recent reliable molecular methods based on paternity analysis have been developed to circumvent this problem (see review in Campbell 2000). Because we measured selection through female fitness only, shortcomings related to our selection estimates are necessary to take into account. For example, because the amount of pollen donated to other plants is expected to increase with pollinator visitation rate, the combined selection effect of the male and female function may be higher than documented in the present study. Moreover, if the floral traits here examined influence male and female sex functions in opposite ways, conclusions on the direction of selection on floral traits are necessarily contingent to the sexual function that is considered. In spite of this, recent evidence of selection on floral characters enhancing pollinator visitation rate has revealed convergent rather than divergent direction of selection through the two sexual functions (Galen 1989, Campbell et al. 1991, O'Connell and Johnston 1998, Maad 2000).

The pollinator-mediated disruptive selection documented in this study may play an important role in the maintenance of the floral variation found in *M. luteus*. It is doubtful, however, that disruptive selection alone could maintain the 76.4% variation in guide size (see *Materials and methods*: *Natural history and study site*). Both genetic and environmental factors need to be invoked to explain the extensive variation in the nectar guide phenotype. First, genetic tradeoffs may be important for the expression of floral traits under conflicting ecological pressures. If ecological or pleiotropic costs for floral attraction exist, the production and maintenance of attractive traits may imply that plants will have less resources to allocate for other functions, including antiherbivore defense (Simms and Bucher 1996, Strauss et al. 1996, Strauss 1997). For example, Galen and Cuba (2001) documented that flower shape in *Polemonium viscosum* evolves under conflicting selection, with phenotypes enhancing pollination entailing a cost in susceptibility to ant floral herbivores. Accordingly, if attractive and defensive functions conflict in *M. luteus*, natural selection may favor alternative combinations of traits (i.e., large-guided and susceptible phenotypes, and small-guided and resistant phenotypes), therefore reinforcing guide size variation. This possibility could be properly tested, because floral herbivory is frequent in *M. luteus*, reaching 13.5% four days after the beginning of anthesis (200 floral buds tagged; R. Medel, *unpublished data*) with most damage occurring on the landing petal (64%). Second, the maintenance of local variation in floral phenotypes can result from temporal variation in the pollinator assemblage (e.g., Schemske and Horvitz 1988, 1989, Campbell 1989, Johnston 1991, Herrera 1993a, Wilson 1995, Gilbert et al. 1996, O'Connell and Johnston 1998, Galen 2000, Totland 2001). If pollinator-mediated selection is intermittent between flowering seasons depending on the abiotic setting and composition of the pollinator assemblage, variation in a single floral trait can, in principle, result from weak selection in some years, directional selection when insects or hummingbirds are present, and disruptive selection in years when both hummingbirds and insects are present and the conditions are met. Even though the pollinator species were the same in 1999 and 2000, we cannot assure the constancy of the pollinator assemblage over longer timescales. Consequently, we cannot rule out fluctuating selection as a source of phenotypic variation in this species.

Although disruptive selection is commonly thought to promote reproductive isolation and divergence (Rice 1984, Kondrashov and Mina 1986, Smith 1990), evi-

dence is relatively uncommon in natural populations (Kingsolver et al. 2001). Studies performed on species of the North American *Mimulus* complex that differ markedly in flower shape, color, and nectar production have provided correlative and experimental evidence that hummingbirds and bees tend to differ in the flowers they service, leading to the suggestion that radiation in the genus has occurred by divergent pollinator-mediated selection on floral traits (e.g., Vickery 1990, 1995, 1992, Sutherland and Vickery 1993, Bradshaw et al. 1995, Schemske and Bradshaw 1999). Our results provide evidence of contemporary disruptive pollinator-mediated selection only. However, the extent to which the phenotypic selection detected in this study results in changes in the distribution of progeny phenotypes is unknown to us at present. Since the evolutionary response to selection may depend on the magnitude of additive genetic variation and genetic correlations of traits, it is necessary to perform experimental crosses between phenotypes. We are currently exploring these relationships. Hopefully, the combined information from the phenotypic selection coefficients presented here, and the measurement of the genetic basis of floral characters, will improve our understanding of how natural selection shapes floral evolution in this species.

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