



## The influence of insects and hummingbirds on the geographical variation of the flower phenotype in *Mimulus luteus*

Rodrigo Medel, Angela Valiente, Carezza Botto-Mahan, Gastón Carvalho, Fernanda Pérez, Nélida Pohl and Luis Navarro

R. Medel (rmedel@uchile.cl), A. Valiente, C. Botto-Mahan, G. Carvalho and F. Pérez, Dept de Ciencias Ecológicas, Facultad de Ciencias, Univ. de Chile, Casilla 653, Santiago, Chile. – N. Pohl, Dept of Ecology and Evolutionary Biology, 321 Steinhaus Hall, Univ. of California, Irvine, CA 92697, USA. – L. Navarro, Dept de Biología Vegetal, Facultad de Ciencias, Univ. de Vigo, ES-36200 Vigo, Spain.

We describe the geographical variation of corolla and nectar guide size in seven populations of *Mimulus luteus* (Phrymaceae) in central Chile, and examine whether flower phenotypes associate with taxonomic composition and flower visit patterns of pollinators across populations. Flowers showed higher variation in nectar guide size than corolla size. Mean corolla size increased with the proportion of bees and decreased with the proportion of lepidopterans in the pollinator assemblages. Nectar guide size increased with the proportion of hummingbirds in the pollinator assemblages. When the frequency of flower visits rather than taxonomic composition was considered, the results revealed similar patterns. Because these traits previously have been described as targets of bee- and hummingbird-mediated selection in *M. luteus*, our results have implications for understanding the processes that determine flower diversification in Chilean *Mimulus*. Although we cannot rule out ecological sorting as an explanation for the geographical association between pollinators and flower phenotypes, changes in the prevalence and importance of bees and hummingbirds across populations appear to account, at least in part, for the flower phenotypic variation across populations. The extent to which insect and hummingbird pollination in *M. luteus* produces pollinator-mediated divergence among populations needs to be examined in future studies.

Pollinator-mediated selection is traditionally considered to be a major factor shaping the evolution and diversification of flower phenotypes (Darwin 1862, Stebbins 1970, Faegri and van der Pijl 1979, Waser 1983, Fenster et al. 2004). One way in which plants are thought to speciate is through the formation of ecotypes adapted to local pollination environments (Armbruster 1985, Galen 1989, Robertson and Wyatt 1990, Johnson 1997, see review in Waser and Campbell 2004). The preference of pollinator species for some flowers and not others within populations often creates a covariance between flower phenotypes and plant fitness that influences the subsequent evolution of flower characters under pollinator-mediated selection. For example, flower traits involved in pollination attraction, such as corolla size (Campbell 1989, Galen

1989, Campbell et al. 1996, Caruso 2000), corolla shape (Herrera 1993, Endress 1999), corolla color (Nagy 1997, Campbell et al. 1997), aromatic and visual nectar guides (Bolwig 1954, Waser and Price 1985, Medel et al. 2003), and sexual attractants to insects (Schiestl et al. 2003) have been described to evolve under pollinator-mediated selection. However, flower adaptation may change across populations depending on the species composition of the pollinator assemblage, the relative efficiency and visitation rate of pollinator species, and the nature of the fitness tradeoffs associated with phenotypic adjustments to particular pollinator species (Aigner 2001). These variables often change in unpredictable ways from one population to another, thereby causing substantial variation in the selective regimes, which makes it difficult to extract

broad generalizations on the mechanisms that underlie the evolution of the flower phenotype.

*Mimulus* is a highly diverse plant genus of about 120 species currently placed in the family Phrymaceae (Beardsley and Olmstead 2002). *Mimulus* species are model systems for the study of evolutionary processes in nature due to the diversity of traits that allow us to assess the genetics of adaptation in copper tolerance (Macnair 1983), the evolution of mating systems and inbreeding depression (Willis 1993, Dudash and Carr 1998), and the role of different pollinator species in pollinator-mediated speciation (Bradshaw et al. 1995, 1998, Bleiweiss 2001). For instance, ecological and genetic evidence suggests that adaptive divergence in *M. lewisii* and *M. cardinalis* has occurred in relation to the divergent selection imposed by bees and hummingbirds in western North America (Vickery 1990, 1992, 1995, Sutherland and Vickery 1993, Bradshaw et al. 1995, 1998, Schemske and Bradshaw 1999, Bradshaw and Schemske 2003, Ramsey et al. 2003). Despite extensive studies on the evolutionary processes in *Mimulus* in North America, our understanding of patterns of evolutionary change in South American populations remains poorly developed. The relatively low genetic distances amongst taxa suggest that the South American clade is relatively young and became established from ancestors in western North America (Beardsley et al. 2004). Presently, the genus extends from Colombia to Chile and Argentina; most species inhabit humid localities along the western margin of the Andes (von Bohlen 1995). Previous reports on *M. luteus* suggest that hummingbirds and bees exhibit divergent preferences for corolla size and nectar guide size causing disruptive selection on a local scale (Medel et al. 2003). In this

paper, we test for geographical associations between flower traits of the Andean monkeyflower *Mimulus luteus*, and the proportion and visitation rate of different pollinator taxa in *Mimulus* populations in central Chile, in an attempt to assess the potential role of the structure of pollinator assemblages as a driver of large-scale geographic variation in flower phenotype in this species.

## Materials and methods

*Mimulus luteus* var. *luteus* L. (Phrymaceae) is a hydrophilic perennial herb species that inhabits the southern South American Andes (von Bohlen 1995). Flowers are hermaphroditic, self-compatible, have inserted anthers, and a bilobed stigma that closes upon contact (von Bohlen 1995). The five-lobed yellow corolla has a conspicuous red nectar guide spot on the lower lobe, which does not reflect in the UV (Botto-Mahan et al. 2004). Most plants produce 1–4 zygomorphic flowers that differ more between than within individuals (Medel et al. 2003). Flowers remain open an average of 3.6–5.9 d when pollinated and not pollinated, respectively. Nectar (ca 20% sugar concentration) tends to accumulate during the day, and, as in most *Mimulus* species, *M. luteus* produces a small amount of nectar (range: 0.7–1.4  $\mu\text{l d}^{-1}$ , Medel et al. 2003). To characterize the flower phenotype of *M. luteus*, we sampled seven populations along a latitudinal transect of ca 800 km (Fig. 1, Table 1) in Chile. In each population, we randomly chose flowers for phenotype measurements (one flower per plant) and took photographs from a frontal perspective perpendicular to the corolla plane. Since previous studies indicated that

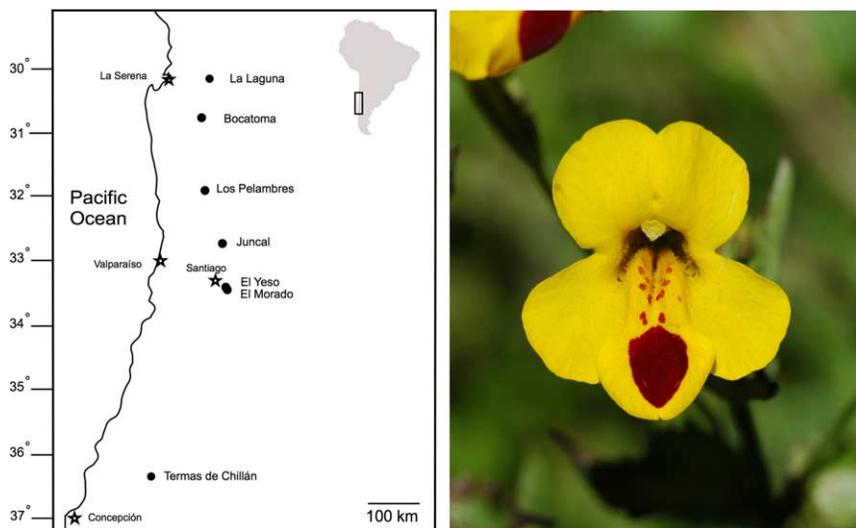


Fig. 1. Left: location of the sampled populations of *Mimulus luteus* in central Chile. Stars represent cities nearest to study sites. Right: a flower of *M. luteus*, a frontal perspective. A colour enhanced version of Fig. 1 is available in the online pdf version.

Table 1. Description of study localities including location, elevation, number of flowers sampled (N), mean corolla size (Size<sub>cor</sub>; SE in parentheses), and mean nectar guide size (Size<sub>gui</sub>; SE in parentheses). CV<sub>cor</sub> and CV<sub>gui</sub> are coefficients of variation for corolla size and nectar guide size, respectively. Pearson's product-moment correlation coefficients for corolla size and nectar guide size are given (r<sub>cor-gui</sub>). \*\*\* p < 0.001, \*\* p < 0.01, \* p < 0.05.

Locality	N	Size <sub>cor</sub> (mm <sup>2</sup> )	CV <sub>cor</sub>	Size <sub>gui</sub> (mm <sup>2</sup> )	CV <sub>gui</sub>	r <sub>cor-gui</sub>
La Laguna (30°14'S, 70°01'W, 3149 m)	52	543.4 (17.5)	23.2	10.48 (1.37)	94.1	0.374**
Bocatoma (30°44'S, 70°27'W, 2197 m)	67	455.3 (19.4)	34.8	16.70 (0.66)	32.6	0.717***
Los Pelambres (31°45'S, 70°30'W, 2730 m)	297	960.2 (8.2)	14.8	42.38 (2.25)	70.1	-0.052
Juncal (32°51'S, 70°08'W, 2284 m)	140	801.2 (4.1)	6.1	45.76 (3.21)	85.1	0.054
El Yeso (33°37'S, 70°01'W, 2580 m)	124	805.3 (21.0)	29.1	18.73 (1.76)	104.4	0.206*
El Morado (33°48'S, 69°59'W, 2599 m)	65	575.4 (19.1)	26.7	34.01 (2.34)	55.5	0.531***
Termas de Chillán (36°54'S, 71°24'W, 1834 m)	62	875.0 (15.4)	13.9	0 (0)	0	0

corolla size and nectar guide size are relevant traits for insect and bird pollinators (Medel et al. 2003), we recorded corolla size (mm<sup>2</sup>) and nectar guide size (mm<sup>2</sup>) from the area of the frontal projection of flowers (Fig. 1) using the software ImageTool 2.0 104 (UTHSCSA, San Antonio, Texas, USA). We described the pollination assemblage in each population by recording species that contacted the floral sexual organs when foraging. Observations were performed in periods of 30 min from 7:00 to 18:00 h during 2–20 sunny days per population. All observations were made in the Austral summer seasons of 2000–2007. Since pollinator assemblages often change substantially in species composition from year to year (Herrera 1988, Horvitz and Schemske 1990, Price et al. 2005), our analysis may suffer from a biased temporal sampling. However, samples from different years revealed a relative invariance in the pollinator assemblage in some populations (e.g. Los Pelambres (2000–2001) = 100% similarity; Juncal (2006–2007) = 100%; El Yeso (2003–2005) = 85%; El Morado (2005–2006) = 100%), suggesting that between-year variation in species composition is relatively minor in our study system.

We analyzed between-population differences in corolla and nectar guide size using one-way ANOVA on log-transformed data. Pairwise significance among populations was tested using Tukey's HSD. In addition, we examined whether corolla and nectar guide size exhibit a relationship with 1) the proportion of pollinator species belonging to different orders (i.e. Hymenoptera, Diptera, Lepidoptera, and hummingbirds) in each population's pollinator assemblage, and 2) the percentage of flower visits made by each pollinator taxon with respect to the total number of flower visits recorded at each population. The association between variables was estimated by Spearman's nonparametric correlation analysis. Because corolla size and nectar guide size were correlated in some populations (Table 1), the observed correlation between a pollination assemblage characteristic and one flower trait may result from a hidden relationship of the pollination assemblage characteristic and the other flower trait. In correlations involving corolla size we

therefore kept nectar guide size constant, and vice versa, using partial Spearman correlation as described by Sokal and Rohlf (1995). To assess the statistical significance of the partial correlations, partial coefficients (r<sub>p</sub>) were transformed to a Student's t distribution using  $t = r_p \sqrt{(n-3)/(1-r_p^2)}$ . The two-tailed p-value was estimated according to the location of the t-value with respect to its expected distribution.

## Results

Mean corolla size and mean nectar guide size varied greatly among the seven populations (Table 1). The coefficient of variation for nectar guides was almost three times greater than that for corolla size (71 and 27%, respectively). Populations differed statistically in corolla size (one-way ANOVA:  $F_{6,800} = 204.0$ ,  $R^2$  model = 0.60,  $p < 0.001$ , Fig. 2A), and a posteriori Tukey's HSD contrasts ( $\alpha = 0.05$ ) revealed that nearby populations are not more similar than distant populations (Fig. 2A). Similarly, populations differed strongly in nectar guide size (one-way ANOVA:  $F_{6,676} = 147$ ,  $R^2$  model = 0.57,  $p < 0.001$ , Fig. 2B). Post-hoc contrasts revealed that most population differences in nectar guide size did not parallel differences in corolla size (Fig. 2A, B), suggesting that these traits did not evolve in a correlated way across populations.

Half of all visitor species were Hymenoptera, followed by Lepidoptera (35%), Diptera (10%) and hummingbirds (5%) (Appendix 1). The frequency of visits was highest for Hymenoptera (average across populations 57%, range = 20–100%), followed by hummingbirds (average 24%, range: 0–69%), lepidopterans (average 15%, range: 0–80%), and dipterans (average 5%, range: 0–25%). Corolla and nectar guide size were associated in different ways with the proportion of species in each taxonomic category across populations. Corolla size was positively associated with the proportion of bees ( $r_s = 0.78$ ,  $p = 0.038$ ;  $r_p = 0.82$ ,  $p = 0.031$ ) and negatively with the proportion of lepidopterans across populations ( $r_s = -0.85$ ,

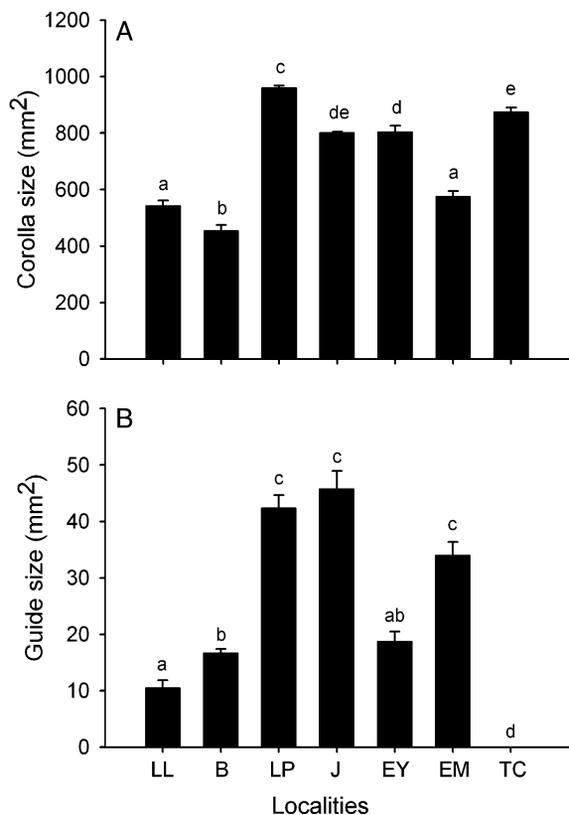


Fig. 2. Mean corolla (A) and nectar guide size (B) in populations of *Mimulus luteus*. Bars above columns are 1 SE. Populations are arranged along the x-axis in a north-south direction. LL = La Laguna, B = Bocatoma, LP = Los Pelambres, J = Juncal, EY = El Yeso, EM = El Morado, TC = Termas de Chillán. Columns showing different lowercase letters differ statistically at  $\alpha = 0.05$  in Tukey's HSD tests.

$p = 0.016$ ;  $r_p = -0.84$ ,  $p = 0.021$ ) (Fig. 3A, B). Nectar guide size correlated positively with the proportion of hummingbirds ( $r_s = 0.84$ ,  $p = 0.018$ ;  $r_p = 0.81$ ,  $p = 0.033$ ) (Fig. 3C). The proportion of dipterans did not correlate with corolla or nectar guide size. When the percentage of flower visits was considered, corolla size did not show any significant associations using uncorrected correlation. However, partial correlation analyses revealed a borderline-significant association between corolla size and the percentage of visits by hymenopteran species ( $r_p = 0.74$ ,  $p = 0.073$ , Fig. 4A). Nectar guide size showed a consistent and significant positive correlation with the percentage of visits by hummingbirds ( $r_s = 0.85$ ,  $p = 0.015$ ;  $r_p = 0.83$ ,  $p = 0.024$ ) (Fig. 4B).

## Discussion

We found a wide variation in corolla and nectar guide size among the studied *M. luteus* populations. This

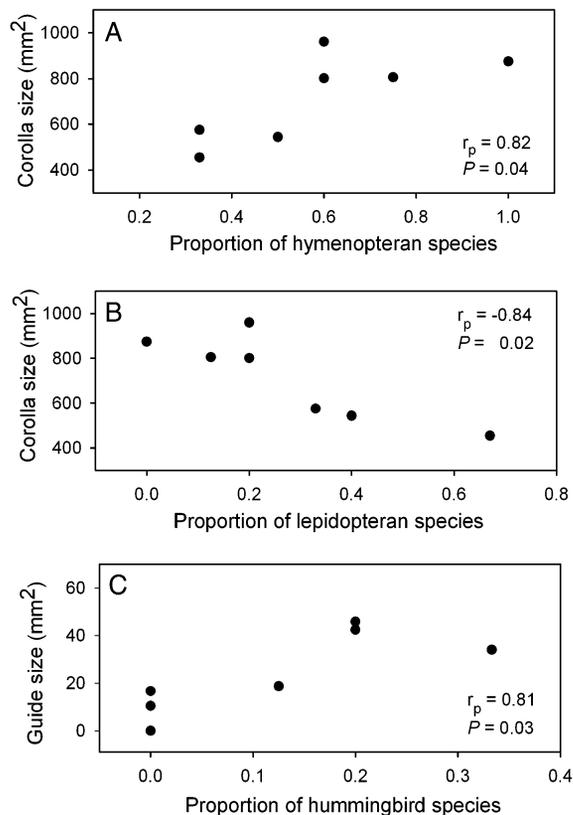


Fig. 3. Mean corolla size as a function of the proportion of (A) hymenopteran or (B) lepidopteran species in the pollinator assemblage. (C) Mean nectar guide size as a function of the proportion of hummingbird species in the pollinator assemblage. Each dot represents one population. Spearman's partial correlation coefficients ( $r_p$ ) for the relationships are given.

variation was partly related to variation in the taxonomic composition of pollinator assemblages. Both the taxonomic composition of the pollinator assemblage and the proportion of flower visits showed significant associations with corolla and nectar guide size across *M. luteus* populations. Corolla size increased with an increasing proportion of bees in pollinator assemblages, but decreased with an increasing proportion of butterflies, suggesting potential conflicting effects of bees and butterflies on flower morphology. Whether these opposite associations translate into conflicting selection upon corolla size in local populations is unknown at present. Future assessments of the pollination efficiency of the taxa involved are needed to reach more firm conclusions on this issue. Populations with a high abundance of the hummingbird *O. leucopleurus* tended to have large nectar guides, which suggests that hummingbirds may have an important role in the evolution and maintenance of the large red nectar guides in *M. luteus*. We note that only one species of

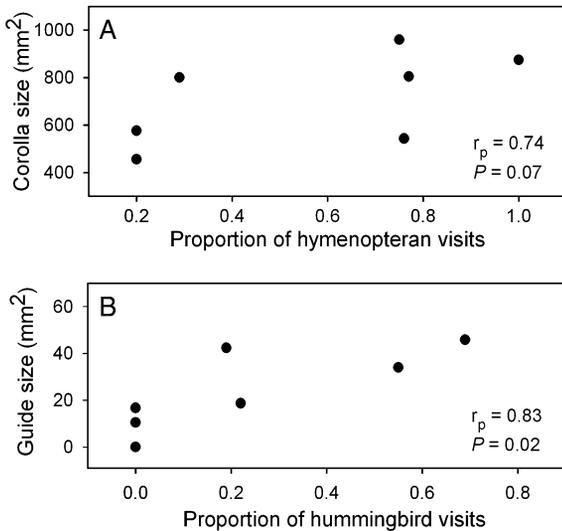


Fig. 4. (A) Mean corolla size as a function of the proportion of hymenopteran visits with respect to the total number of visits. (B) Proportion of hummingbird visits and mean nectar guide size. Each dot represents one population. Spearman's partial correlation coefficients ( $r_p$ ) for the relationships are given.

hummingbird, the white-sided hill-star *O. leucopleurus*, is involved; no other hummingbird species visited populations of *M. luteus* in our 800 km latitudinal transect. While the ultimate reason for the association between *O. leucopleurus* and red guides in *M. luteus* is not well understood, it is known that hummingbirds do not present innate preference for red flowers (Bené 1941, Miller and Miller 1971, see review in Chittka and Waser 1997). On the contrary, hummingbirds tend to visit flowers of all colors and use colors as signals of nectar reward, but not as the primary agent of attraction (Waser 1983, Sutherland and Vickery 1993, Waser et al. 1996). Previous work, however, revealed an absence of correlation between nectar volume and sugar concentration with guide size (Medel et al. 2003). It is likely that red nectar guides in *M. luteus* enhance flower detection by increasing the contrast of guides on the yellow background corollas in clumped plants with high flower numbers. More work is needed to evaluate whether *O. leucopleurus* uses nectar guides to gain quick access to flower reward, and on this basis selects plants with larger guides.

Recent theoretical and empirical advances to the geographical theory of coevolution have stimulated a renewed interest in the phenotypic expectations of the coevolutionary process across populations. For example, it has been suggested that variation in the biotic and abiotic setting across sites will influence the outcome of local selection, leading to variable patterns of selection across populations (Thompson 1988,

1994). Furthermore, the interplay of local selection and gene flow among populations, as well as other complex evolutionary forces acting outside the domain of the focal interacting species, may often lead to variable patterns of adaptation and maladaptation across populations (Gomulkiewicz et al. 2000, Nuismer et al. 2003, Rudgers and Strauss 2004). Even if corolla and guide size result from pollinator-mediated selection in some populations, processes unrelated to pollinator-mediated selection may determine the character states in other localities. For example, interactions between pollinators and herbivores or among different pollinator taxa may influence the net result of the selection process in ways not identified in studies that focus on single pollinator taxon (Herrera 2000, Herrera et al. 2002). The hummingbird *O. leucopleurus* tends to avoid flowers of *M. luteus* damaged in their lower lobe, indicating that interactive effects between hummingbird pollination and flower herbivory influence the fecundity of this species (Pohl et al. 2006). Consequently, the magnitude of the pollination-herbivory interaction may drive the flower phenotype far away from expectations based solely on single population and single interaction analyses. In addition, if evolution of floral traits is driven by non-pollinator factors, such as random drift and founder effects, pollinators may be just sorting among plant populations based on their respective flower preferences. Therefore, pollinator representation and dominance in local assemblages might potentially represent the outcome of ecological sorting rather than the force driving the adaptive evolution of the flower phenotype. This scenario provides a non-adaptive hypothesis to explain geographical associations between flower traits and pollinator assemblage composition.

Our results are consistent with previous findings stressing the ecological association of hummingbirds and bees with particular components of the flower phenotype in *M. luteus* (Medel et al. 2003, Botto-Mahan et al. 2004, Pohl et al. 2006). This scenario probably mimics, at least in part, the pattern described by Vickery (1978) for color polymorphisms and their effect on pollinator visitation (Sutherland and Vickery 1993), partial reproductive isolation, and diversification of some *Mimulus* species in western North America (Vickery 1995, Schemske and Bradshaw 1999, Beardsley et al. 2003).

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