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ORIGINAL PAPER

Effects of herkogamy and inbreeding on the mating system of *Mimulus luteus* in the absence of pollinators

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Abstract Self-pollination mechanisms are often invoked to explain plant reproduction in environments devoid of pollinators. However, populations may evolve a diverse variety of strategies depending on the ecological context of pollination. In this study we examined the pollination environment, inbreeding depression, and herkogamy of *Mimulus luteus* (Phrymaceae) in two consecutive years in an attempt to evaluate the extent to which these factors determine the current level of autogamy in this population. Results indicate that pollinators were almost absent in the study site in 2005 and 2006. Likewise, no evidence of significant inbreeding depression for seed production and seed germination was detected in the 2 years. Because *M. luteus* presents hermaphrodite flowers with 'movement herkogamy' (touch-sensitive stigmata that close upon contact), and anther–stigma separation, we evaluated the role of these traits in self-fertilization. First, we assessed the effects of pollen source (self- and xenogamous pollen, and control solution) on stigmata dynamics after pollen deposition, and seed production. We observed that stigmata that received pollen (self- and xenogamous hand-pollinated) remained closed for a longer time than flowers that received no pollen (control flowers). Seed production, however, was unaffected by pollen source, indicating that movement herkogamy does not prevent self-fertilization in this population. Second, a phenotypic selection analysis revealed that seed production increased with a reduction of anther–stigma separation in absence of pollinators (bagged flowers), suggesting that low herkogamy levels are promoted in this population. Our results indicate that lack of inbreeding depression, ineffective movement herkogamy, and selection against anther–stigma separation are factors that may contribute to the prevalence of autogamous reproductive mechanisms when *M. luteus* faces environments with scarce mating opportunity.

Keywords Chile · Monkey flower · Inbreeding depression · Phenotypic selection · Selfing · Stigma behavior

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Introduction

One of the important questions in pollination evolutionary ecology in the last decades relates with the mechanisms that maintain mixed reproductive strategies in plants (Schemske and Lande 1985; Lloyd and Schoen 1992; Kalisz and Vogler 2003). A mixed reproductive strategy refers to the capacity of species to reproduce through both self-fertilization and outcrossing (Schemske and Lande 1985; Brunet and Eckert 1998). Variation in the pollinator environment has been invoked as the major factor accounting for the maintenance of mixed systems, because it promotes outcrossing strategies when pollinators are available, and stimulates reproductive assurance mechanisms when pollinator-mediated mates are scarce ("the best of both worlds" hypothesis, Becerra and Lloyd 1992; Kalisz et al. 2004). The absence of pollinators or the maleficence in pollen deposition (i.e., heterospecific pollen deposition, insufficient pollen loads, incompatible conspecific pollen) often result in pollen limitation (Hegland and Totland 2008; Knight et al. 2005; see review in Ashman et al. 2004), which in the long term may promote transitions in the reproductive system from xenogamy to autogamy (Kalisz and Vogler 2003; Moeller and Geber 2005; Morgan and Wilson 2005). Environments with few pollinators may favor different strategies for flower evolution depending on the presence and magnitude of inbreeding depression (low fitness in self progeny). While selection may favor selfing avoidance traits such as heterostyly and dichogamy when inbreeding depression is high or moderate (Charlesworth and Charlesworth 1987; Willis 1993a), traits that permit self-pollination may be favored when inbreeding depression is low (Takebayashi and Delph 2000). In this way, species inhabiting environments with variable and unpredictable pollinators may present mild levels of inbreeding depression and evolve flower traits favoring self-pollination and reproductive assurance (Vickery 2008). Even though theoretical and empirical studies have tested the importance of the ecological context on the evolution of self-pollination (Herlihy and Eckert 2002; Kalisz and Vogler 2003; Moeller and Geber 2005; Morgan et al. 2005), few plant systems permit the simultaneous assessment of more than one mechanism involved in self-pollination. This lack of information is unfortunate because increasing evidence indicates that flower traits often act in integrated rather than independent form (e.g., Armbruster and Schwaegerle 1996; Murren et al. 2002; Ordano et al. 2008).

In general, segregation of female and male functions within flowers has a direct impact on plant fitness, which has been long related with changes in self-pollination levels (see review in Barrett 2002). Any reduction in anther–stigma separation is expected to promote self-pollination, especially in populations with deficiency of pollinators (Lloyd and Schoen 1992). Reductions in anther–stigma separation, however, have been also related with an increase in the level of homozygosity (Karron et al. 1997) and low inbreeding depression (Carr et al. 1997; Takebayashi and Delph 2000), especially in peripheral and low-density populations (Herlihy and Eckert 2005; Moeller and Geber 2005). While the costs and benefits of herkogamy for plant fitness have been extensively studied (see review in Barrett 2002), there is a second and less pervasive type of spatial segregation between female and male functions described as "movement herkogamy" which involves the closure of the stigma lobes upon physical stimulation (Fetscher 2001; Barrett 2002). This behavior is a case of thigmonastism, a mechanic-sensitive response to physical stimuli that is present in some plant families (reviewed in Braam 2005). Specifically, movement of stigmata after mechanical stimulus has been detected in some species of the Lamiales order (families Bignoniaceae, Lentibulariaceae, Martynaceae y Phrymaceae; see Fetscher and Kohn 1999). Movement herkogamy has been suggested to play an important role in the evolution of selfing in the *Mimulus guttatus* complex, a North American lineage of *Mimulus* (Ritland and Ritland 1989). Stigmata movement has been related to different functions, such as self-pollination avoidance (Darwin 1876; Richardson 2004), the immobility of the pollen grains on the stigma, the reduction of female interference on pollen export (Fetscher 2001; Barrett 2002; Fetscher et al. 2002), and the avoidance of heterospecific pollen deposition on stigmata (Waser and Fugate 1986). In addition, the stigma condition (open/closed) has been reported to have important consequences for pollinator behavior. For example, depending whether stigma remains open or closed, hummingbirds change the pattern of flower visitation and pollen export in the bush monkey flower *Mimulus aurantiacus* Curtis (Fetscher et al. 2002).

In this paper, we present the results of a field study aimed to evaluate the role of the pollination environment, anther–stigma separation, 'movement herkogamy', and inbreeding depression, on the reproductive success of the Andean monkey flower, *Mimulus luteus* L. (Phrymaceae). More specifically, in this paper we will attempt to answer the following questions, (1) is there significant inbreeding depression in this population? (2) does pollen source determinate stigma behavior ('movement herkogamy') and seed production?, and (3) what is the importance of stigma–anther separation for seed production in the absence of pollinators? The answers to these questions will permit us to answer the more general, (4) what are the mechanisms that permit reproduction of *M. luteus* in environments without pollinators?

Materials and methods

Natural history and study site

Mimulus luteus var. *luteus* L. (Phrymaceae) is an herb that inhabits meadows in the Andes mountain range, from 30° to 45°S in Chile (von Bohlen 1995). It presents solitary hermaphrodite flowers with yellow tubular corollas and a conspicuous red spot in the lower lobe, which acts as nectar guide (Medel et al. 2003; Botto-Mahan et al. 2004). Flowers of *M. luteus* have a stigma exerted above anthers, which has been named "approach herkogamy" by Barrett (2002). Unpollinated flowers remain open 3–9 days, mean = 5.9; pollinated flowers remain open 2–4 days, mean = 3.6 (Medel et al. 2003). The species presents a wide variation in the composition of pollinator assemblages across populations, ranging from populations devoid of pollinators to populations with taxonomically diverse pollinator assemblages (Carvallo 2006; Medel et al. 2007; Cooley et al. 2008). This study was performed at El Morado National Park, Chile (33°37′28″S 70°01′53″W, 2580 m elevation) in the summer seasons of 2005 and 2006. Our sampling effort was concentrated in a 310.5 m^2 monospecific patch of *M. luteus* surrounded by a vegetation matrix that included *Adesmia echinus* (Papilonaceae), *Berberis empetrifolia* (Berberidaceae), *Caiophora coronata* (Loasaceae), *Calceolaria biflora* (Scrophulariaceae), *Calceolaria purpurea* (Scrophulariaceae), and *Ribes cucullatum* (Saxifragaceae). In 2005 and 2006, replicated 1 m^2 plots were set up and the number of flowers per plot counted (mean ± SE, 2005: 214.1 ± 25.6 flowers, $n = 34$ plots; 2006: 229.7 ± 31.3 flowers, $n = 25$ plots). Plots were located at least 5 m one from each other. We identified the floral visitors through direct observations in replicated 30 min periods between 0800 and 2100 hours in a cumulative observation time of 1770 min (1020 min in 2005, and 750 min in 2006).

Mating system and inbreeding depression

We determined the mating system and described the magnitude of inbreeding depression in 2005 and 2006. Even though inbreeding depression is expressed in several life history traits, selfers often express the cost of inbreeding in the early stages of plant development (Husband and Schemske 1996) through pollen sterility (Willis 1993a), low seed number (Hayes et al. 2005), or reduced germination (Heschel and Paige 1995). In this study we will focus on seed production and germination rate as fitness indicators. Each year, we tagged and bagged 120 flower buds from independent individuals and assigned 30 randomly chosen flowers to one of four treatments: (1) autonomous selfing, flowers bagged with 15 × 20 cm silk bags to avoid pollinator entrance to flowers. This treatment permitted to estimate the dependency of pollen vectors for seed production. (2) Self hand-pollination, flowers emasculated and hand-pollinated using their own pollen and bagged again. This treatment allowed us to infer the self-compatibility level of plants. (3) Xenogamous hand-pollination, flowers emasculated, pollinated with pollen from an individual distant 1 m from the focal plant, and re-bagged for the rest of the flower lifespan. (4) Control, flowers intact and exposed to natural pollination. Because *M. luteus* is a clonal plant, 1 m of distance may be insufficient to separate genets. To avoid using the same individual (clone) for xenogamous hand-pollination, and knowing that nectar guides show a low variability in flowers of the same plant and a high variation between individuals (Medel et al. 2003), we chose plants with a strong difference in nectar guide patterns. In this way, flowers with notoriously different nectar guide patterns were considered for experimental crossings. At the time of fruit maturation ($\sim$3 weeks after initiation of experiments), all capsules were collected, labeled, and transported to the laboratory for measurements of seed production and germination. Seed viability was assessed in Petri dishes, using one dish per capsule. Absorbent paper was used as substrate. The substrate was humidified daily with 20 ml of water. Plates were kept under natural light conditions for 6 days, after which the seed germination rate was recorded on a per fruit basis. To determine whether seed production and germination rate differed between years and between treatments, we performed two-way ANOVAs on angular transformed-data. Because variation in resource availability across years may bias estimates of reproduction system and pollinator dependency levels, we standardized the seed production per fruit to the highest seed production observed each year (2005: 2210 seeds; 2006: 987 seeds). The values of seed production and germination obtained from the different experimental treatments allowed estimation of the magnitude of inbreeding depression. We computed the inbreeding depression index (δ) using $[1 - (W_S/W_O)]$ (Charlesworth and Charlesworth 1987, 1990; Keller and Waller 2002), where W_O is the mean value from xenogamous hand-pollination (treatment 3, see above) and W_S is the mean value from self hand-pollination (treatment 2). We tested whether $\delta_{\mathrm{Observed}} \geq 0$ using 1000 Montecarlo iterations in Pop-Tools (Hood 2000).

Movement herkogamy

The stigma lobes of *M. luteus* close after mechanical contact (range of stigma closure time = 1–52 s; mean ± SE = 9.7 ± 0.8; $n = 157$ flowers) and remain closed for a variable time (range: 8–2245 s; mean ± SE = 788 ± 32; $n = 167$). Using a two-factor array, in the summer season of 2005 we evaluated the percentage of stigmata that remained closed and the number of seeds as a function of pollen source (self, xenogamous, and control) and number of pollination events that stigmata receives. Pollen loads were deposited on 90 first-day opened stigmata from previously bagged flowers. Pollen loads for self and xenogamous treatments were prepared by embedding the four anthers of first-day flowers in 20 μl of distilled water. The control consisted on 2 μl of distilled water only. Pollen depositions were performed on the lower stigma lobe by releasing 2 μl of a previously shaken 20 μl pollen-water solution using a micropipette (p. 10, Gilson). Information obtained in the laboratory indicates that pollen loads prepared in this manner contain a mean of 833 grains (SE = 85, $n = 7$ flowers). Because seed formation was observed in stigmata that received pollen loads in previous assays, we considered this technique as an appropriate proxy to our objective (see Kearns and Inouye 1993). To evaluate the effect of the number of pollination events on stigma behavior, each pollen treatment was applied from 1 to 3 times at intervals of 10 min between pollen depositions (3 pollen sources × 3 manual pollination events × 10 flowers). All flowers used in this experiment were emasculated and re-bagged after treatment to avoid contamination with undesirable pollen from different sources. The state of stigmata (open or closed) after 10 min of treatment deposit was considered as the response in contingency tables using pollen source and number of pollination events as entries. Frequencies were analyzed in contingency tables using a Pearson Chi-square statistics that evaluated whether the distribution of the response variable (i.e., proportion of open/closed stigmata) was similar across categories. To evaluate the treatments that show a similar pattern in stigma state, we used correspondence analysis, a graphical inductive technique that indicates the rows or columns that show a similar pattern of counts. This analysis sorts frequencies (i.e., open/closed stigmata) in orthogonal factors based on the decomposition of the Chi-square statistic associated with contingency tables (Benzécri 1992). In addition to assessments of stigma behavior, we evaluated the effects of pollen source on seed production. We collected capsules (fruits) from treatments 16 days after pollen deposition and their seeds were counted in the laboratory. The effect of pollen source and number of pollination events on seed number was analyzed in two-way ANOVA on log-transformed data. All analyses were performed using Jump 5.0.1a (SAS Institute Inc.).

Selection on anther–stigma separation

We examined the functional relationship between the stigma–anther separation with seed production in control (open) and bagged flowers. In 2006, we tagged and bagged floral buds in 200 individuals. The first day of anthesis we measured, (1) the separation between the junction of the two stigma lobes with the nearest anther, and (2) corolla tube length, using a digital caliper (precision 0.01 mm, Mitutoyo Corp.). In addition, we measured the height of the focal flower from the ground level (plant height hereafter). One hundred first day-opened flowers were bagged to evaluate the role of anther–stigma separation and corolla tube length on seed production in the absence of pollinators, leaving the remaining 100 flowers intact (i.e., unbagged). Twenty-six days after anthesis capsules were collected for seed counting in the laboratory. We considered corolla tube length, plant height, and anther–stigma separation as traits for phenotypic selection analysis. Because flowers of *M. luteus* produce low nectar volumes (0.72–1.37 μl depending of the time of day, Medel et al. 2003), most nectar is concealed at the bottom of corolla tubes and the accessibility of pollinators to nectar may be severely constrained by corolla tube length (see Fenster 1991). Similarly, because plant height is a factor that may affect pollinator behavior and reproductive success through to female function (Wolf and Hainsworth 1990; Maad 2000), we included this trait as covariate in phenotypic selection analysis.

We performed a phenotypic selection analysis to determine the strength, sign and curvature of natural selection acting upon the anther–stigma separation and corolla tube length in the presence and absence of pollinators. We estimated the differential of selection (s_i), which represents total expected change attributable to direct and indirect selection acting on the focal trait *i* in a generation. To describe the direct force of selection acting on anther–stigma separation, excluding indirect selection through correlated characters, we used the multivariate model of Lande and Arnold (1983) to estimate, (1) the linear gradient of selection, (β_i), which provides information of the direction and magnitude of the expected change on trait *i*, keeping all the remaining characters constant, and (2) the quadratic selection gradient (γ_{ii}), which describes the magnitude and curvature of selection acting on the character *i*. When $\gamma_{ii} < 0$, it implies downward concavity and stabilizing selection acting upon the focal character. On the contrary, when $\gamma_{ii} > 0$, it reflects upward concavity and disruptive selection on the character *i*. All traits were standardized to zero mean and unit variance. 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 or experimental treatments. Standardized differentials are written as s_i' and standardized linear and quadratic gradients are written as β_i' and γ_{ii}', respectively. Seed number was transformed to relative fitness by dividing individual values by the mean seed production of each group. The statistical significance of the s_i' values was tested by Pearson's product-moment correlation of relative fitness with anther–stigma separation. The significance of gradients was determined directly from the regression coefficients after the effects of all other parameters had been removed. All phenotypic traits in control and bagged conditions were transformed to fulfill the requirements of normality (Shapiro–Wilk's test), and homogeneity of variance (Levene's test). Because the Lande and Arnold (1983) methodology makes only very weak assumptions about the distribution of fitness, but makes the strong assumption that phenotypic traits are multivariate normal, we did not attempt to transform fitness data to a more normal distribution, since such transformation will lead to invalid estimates of the forces of selection. All statistical analyses were performed in Jump 5.0.1a (SAS Institute Inc.). To visualize the form of the fitness function and hence the location of the fitness maximum and minimum within the range of phenotypes, we used the univariate cubic spline routine (Schluter 1988). The cubic spline procedure is a nonparametric regression fitting function that provides a quantitative prediction of fitness across a range of a quantitative trait values. This function is useful in predicting fitness differences among individuals (y), through estimates of the fitness surface, f, as function of the independent variable z: $y = f(z) +$ random error, where y represents survival, reproductive success, or any other measure having a binomial, Poisson, or normal distribution for each z. The goal of this procedure is to estimate the surface f without making any prior assumptions about its shape. 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

Pollinator visits, reproductive system and inbreeding depression

We detected a low diversity and activity of pollinators in the 2 years of study. The only flower visitors detected in 2005 were the exotic bumblebee *Bombus terrestris* (Apidae) (4 visits), and the hoverfly *Melanostoma sp.* (Syrphidae) (5 visits) in 1020 min observation

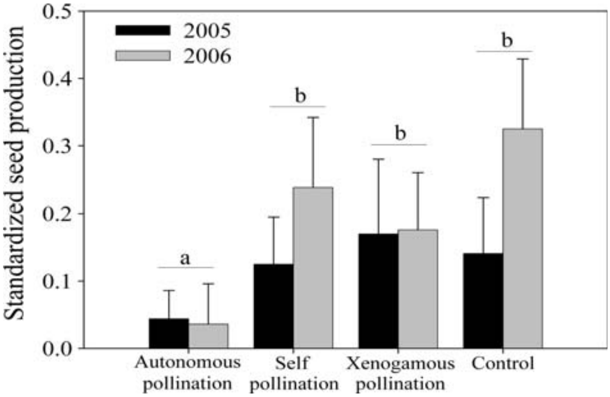

Fig. 1 Effects of mating mode on seed production for years 2005 (*black bars*) and 2006 (*gray bars*). Seed number was standardized to the highest seed production observed each year. *Bars* and *error bars* indicate mean and two standard errors, respectively. *Letter on bars* indicate the results of Tukey's post-hoc comparisons among treatments; bars with different letters differ statistically at $\alpha = 0.05$

on 7276 flowers. In 2006, the only pollinator recorded was the Andean hummingbird *Oreotrochilus leucopleurus* (Trochilidae), which performed 11 visits in 750 min observation on 5750 flowers. Based in this result, we discard out the analysis comparing the frequency of visits between pollinators and between years.

Seed production differed between years ($F_{1,196} = 5.403$, $P = 0.021$) being almost four-fold higher in 2006 than 2005 (mean seed production ± SE, 2005: 120.3 ± 20.2; 2006: 459.0 ± 57.9). Likewise, there was a significant effect of treatment on seed production ($F_{3,196} = 6.69$, $P = 0.003$) but no significant interaction term between factors was detected ($F_{3,196} = 2.13$, $P = 0.097$). *A posteriori* Tukey's contrasts among pollen treatments revealed that flowers with autonomous self-pollination produced consistently less seeds (seed number ± 1 SE; 59.7 ± 31.5; $n = 46$) than self-crossed (292.1 ± 59.0; $n = 55$), xenogamous-crossed (256.8 ± 51.6; $n = 45$), and control flowers (419.1 ± 70.5; $n = 58$) (Fig. 1), indicating that this species requires pollen vectors for reproductive success (see also Medel et al. 2003, Pohl et al. 2006). Comparison between self-crossed and xenogamous-crossed pollination treatments did not differ in seed production, indicating that *M. luteus* is a self-compatible species (Fig. 1). Regarding seed germination, no significant effect of year, treatment, and interaction between factors was detected (year: $F_{1,116} = 0.003$, $P = 0.954$; treatment: $F_{3,116} = 0.167$, $P = 0.918$; interaction: $F_{3,116} = 1.773$, $P = 0.156$), indicating that despite of year and pollen source, seed viability was unaffected. Analyses of inbreeding depression revealed low and non-significant δ-indexes for seed production and germination in 2005 or 2006 (Table 1), indicating that selfing did not reduce significantly seed production and germination in this population.

Movement herkogamy and pollen source

Observations made on 199 unbagged flowers revealed that all stigmata closed their lobes immediately after physical contact. How long they remained closed varied substantially among plants (closure time, mean ± SE: 9.7 ± 0.8 s, $n = 157$, range 0.9–51.6 s; stigma reopening time, mean ± SE: 788.3 ± 32.1 s, $n = 167$, range 8.1–2245.9 s [Carvallo and Medel 2005]). The chance of stigmata to be open or closed did not depend of the time of

Table 1 Estimated inbreeding depression index (δ) for seed production and germination in 2005 and 2006

Year	Seed production			Seed germination		
	$\delta \pm$ SE	95% CI	P	$\delta \pm$ SE	95% CI	P
2005	0.267 ± 0.102	−1.554–0.591	0.493	0.023 ± 0.084	−0.399–0.484	0.512
2006	−0.353 ± 0.082	−0.982–0.483	0.480	−0.399 ± 0.164	−1.054–0.478	0.101

Estimated value ± SE, confidence intervals, and associated P-levels with respect to the null hypothesis $\delta_{\mathrm{Observed}} = 0$ are indicated

the day. Flowers ($n = 199$) had a similar proportion of open stigmata in the morning (mean % ± SE, 99.5 ± 0.2%, $n = 3$ days), mid-day (98.8 ± 0.4%) and dusk (98.4 ± 0.5%). For pollination assays, all stigmata closed after drop application. Results from our experiment can be sorted in two ways. First, the pollen source explained stigmata state only after three pollination depositions ($\chi^2_2 = 23.33$; $P < 0.001$) with control flowers showing a higher proportion of open stigmata relative to self- and xenogamous pollinated flowers (contingency analysis at α-level = 0.05). This result indicates that the presence of pollen delays stigma reopening (Fig. 2). Experimental flowers that received one or two pollen loads from different pollen sources did not differ in the proportion of open/closed stigmata ($\chi^2_2 = 0.00$; $P = 1.00$ and $\chi^2_2 = 1.90$; $P = 0.386$, for 1 and 2 pollen loads, respectively). Second, we evaluated the effect of the number of pollen depositions on the chance of stigmata to be open or closed within pollen source treatments (Fig. 2). Flowers that received a control solution (distilled water) showed an increase in the proportion of opened stigmata after one to three pollen loads with control solution ($\chi^2_2 = 8.400$; $P = 0.015$, Fig. 2). However, the proportion of closed/opened stigmata in self- and xenogamous hand-pollinated stigmata did not change significantly across pollen depositions ($\chi^2_2 = 1.200$; $P = 0.548$ and $\chi^2_2 = 4.845$; $P = 0.088$ for self- and xenogamous pollination, respectively, Fig. 2). Regarding seed production, flowers pollinated from different sources differed in seed production (one-way ANOVA, $F_{2,27} = 86.269$, $P < 0.001$). Control stigmata produced less seeds (mean ± SE, 2.03 ± 1.36, $n = 28$) than self-crossed (139.4 ± 22.9, $n = 28$),

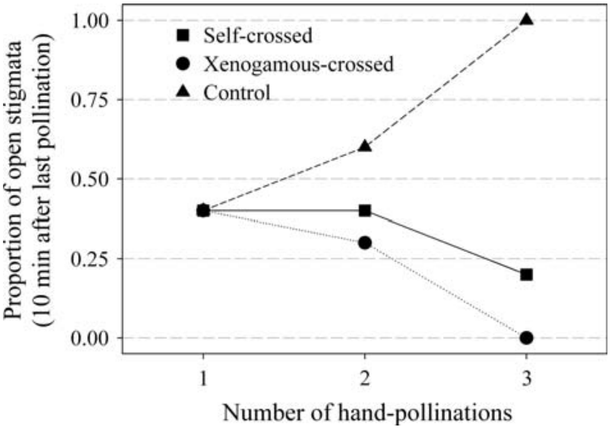

Fig. 2 Proportion of open stigmata after receiving self- (*squares*), xeno- (*circles*), or control (*triangles*) hand pollination treatments during one, two or three events. Each *symbol* represent $n = 10$ stigmata

and xenogamous-crossed dropping treatments (132.2 ± 20.7, $n = 28$). Self- and xenogamous-crossing flowers did not differ in seed production (Tukey's test, $P > 0.05$). Similarly, seed production was not affected by the number of pollination events ($F_{2,26} = 0.700$, $P = 0.504$) and the interaction between pollen source x pollination events ($F_{4,75} = 1.18$, $P = 0.326$).

Selection on anther–stigma separation

The anther–stigma separation was the only trait under significant selection (Table 2; Fig. 3). More specifically, flowers with short anther–stigma separation were favored in the bagged condition, as revealed by the significant and negative differential and gradient of directional selection (Table 2). The anther–stigma separation did not receive significant selection in the control condition (Table 2). Corolla tube length was not selected under any condition (Table 2). Plant height was similar in control and bagged flowers (mean ± SE,

Table 2 Descriptive statistics and standardized selection coefficients for flower traits in control (C) and bagged (B) treatments

Trait	Treatment	Mean (SE)	N_1	s_i'	β_i' (SE)	γ_{ii}' (SE)	N_2
Anther–stigma separation	C	5.21 (0.14)	98	0.133	0.066 (0.077)	−0.046 (0.051)	93
	B	5.52 (0.14)	99	**−0.374*****	**−0.325 (0.094)*****	0.018 (0.078)	89
Corolla tube length	C	30.29 (0.23)	100	0.127	0.128 (0.075)	0.016 (0.049)	93
	B	30.32 (0.22)	100	−0.036	−0.037 (0.101)	−0.101 (0.053)	89

N_1 and N_2 are the sample size for descriptive statistics and selection coefficients, respectively

s_i' is the differential of selection, β_i' is the multivariate gradient for directional selection, and γ_{ii}' is the multivariate gradient for stabilizing or disruptive selection; SE is standard error

Bold face values indicate statistical significance after Bonferroni adjustment

The normality of anther–stigma separation was achieved in both control ($W = 0.966$, $P = 0.074$) and bagged ($W = 0.971$, $P = 0.189$) conditions

By Homocedasticity was achieved for all variables (anther–stigma separation: $F = 0.018$, $P = 0.083$; corolla tube: $F = 0.446$, $P = 0.504$)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

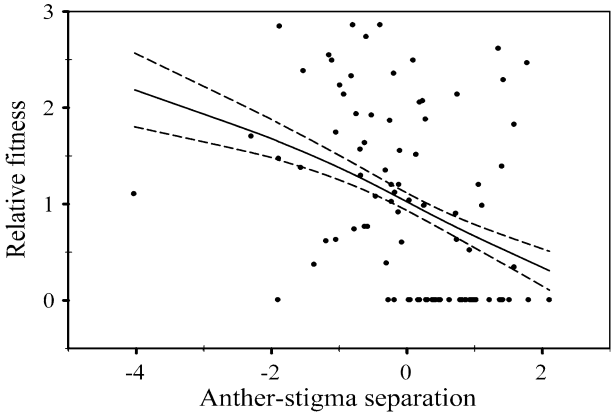

Fig. 3 Cubic spline estimate for anther–stigma separation in bagged flowers ($\lambda = 2$). *Dashed lines* represent ± 1 SE estimates from 5,000 bootstrap replications

n; Control: 0.43 ± 0.01, 100; Bagged: 0.44 ± 0.02, 100). Notwithstanding, this trait was used as covariate in selection analysis. Comparison of selection between treatments revealed that differentials of selection differed between control and bagged groups for anther–stigma separation (Fisher-z transformation, $P < 0.001$, Papoulis 1990). Similarly, directional gradients for anther–stigma separation differed between treatments as revealed by ANCOVA ($F_{1,176} = 13.95$, $P < 0.001$).

Discussion

The ecological setting for autogamous reproduction

Changes in the pollinator environment may help to understand the factors that determine the change in reproductive strategies (Herrera 1995; Kalisz and Vogler 2003; Moeller and Geber 2005), especially in self-compatible species that show a higher potential for evolutionary change from xenogamous to autogamous strategies (Charlesworth and Charlesworth 1987; Lloyd and Schoen 1992). In this study we have described flower mechanisms that may promote autogamous reproduction in the absence of pollinators. The scarcity of floral visitants is unusual in other populations of *M. luteus* (Medel et al. 2007). Unlike previous reports indicating that pollinators are important vectors for successful reproduction (Medel et al. 2003; Pohl et al. 2006), we detected an extremely low diversity and activity of pollinators. It is likely that the frequent gusts of wind, low temperature, and even light snowing found in the site when this study was performed explain, at least in part, the almost nil pollinator activity observed (see also Arroyo et al. 1982, 2006). The lack of pollination was consistent in 2005 and 2006, which suggests that this population has experienced a history of pollination absence.

Lack of inbreeding depression

Theoretical studies suggest that the magnitude of inbreeding depression evolves in correlation with mating system (e.g., Maynard Smith 1978; Lloyd 1979; Charlesworth 1980; Lande and Schemske 1985; Charlesworth et al. 1990). Models predict that selection will favor self-fertilization only if inbreeding depression is less than 0.5. In this study, we detected no significant inbreeding depression for either seed production or germination success in 2005 and 2006 (Table 1). Studies on inbreeding depression in *M. guttatus* and *M. micranthus* have detected significant coefficients on later stages of the plant life history such as growth, survival, and aboveground plant biomass (e.g., Willis 1993a, Carr and Dudash 1996). Even less obvious effects of inbreeding depression include indirect and more complex effects upon plant fitness. For example, recent studies have reported that natural enemies may increase the expression of inbreeding depression through reduced plant quality in *M. guttatus*, which in turn, translates into a higher susceptibility to herbivore attack, ultimately reducing plant aboveground biomass and flower production (e.g., Carr and Eubanks 2002; Ivey et al. 2004; Ivey and Carr 2005). An additional cautionary note relates to the possibility that inbreeding depression expresses through the male rather than female fitness component. Although estimation of inbreeding depression through the male function is beyond the scope of this study, experiments in *M. guttatus* have demonstrated strong inbreeding depression for the proportion of viable pollen grains produced (e.g., Ritland and Ganders 1987; Willis 1993b), which suggests that both male and female fitness components need to be considered for a more complete estimation of inbreeding depression in *M. luteus*.

Movement herkogamy and anther–stigma separation

Although stigma behavior has long been interpreted as a self-pollination avoiding mechanism (Darwin 1876; Faegri and van der Pijl 1971), their functional role is comparatively less studied than anther-stigma separation. In addition to avoid self-fertilization, the stigma state may have important implications for the male function of pollen transfer through a reduction in the male–female reproductive interference. For instance, Fetscher (2001) showed that flowers of *M. aurantiacus* with closed stigmata exported more pollen than flowers with open stigmata. The ability to reduce the stigma surface may imply that less pollen is potentially trapped in the stigma lobes, hence excluding ovules from self-pollination and increasing the amount of pollen that is available for potential export (Barrett 2002). Fetscher and Kohn (1999) evaluated the potential costs and benefits of stigma closure for female fitness of *M. aurantiacus*, concluding that after receiving pollen, most stigmata remain closed for the remaining lifetime of the flower, even if stigmata received less pollen than needed for full seed set. Our results indicate that the frequency of open/closed stigmata was only dependent of the presence of pollen, regardless of pollen source (Fig. 2). Moreover, movement herkogamy did not influence seed production as revealed by the similar seed production in autogamous and xenogamous treatments, indicating that movement herkogamy was largely irrelevant as a self-pollination avoidance mechanism.

Our results show significant selection for reduced anther–stigma separation in bagged but not unbagged flowers (Table 2). The absence of selection upon flower traits in the unbagged condition (control) is intriguing and suggests that reduced herkogamy may favor autogamous reproduction of *M. luteus* only in the absence of pollinators. But why selection for anther–stigma separation was undetected in the unbagged condition? Three hypotheses can be advanced to account for this phenomenon. First, it is likely that the absence of significant association results from the presence of an undetected pollen vector. We did not evaluate the presence of nocturnal pollinators. Even though other studies have rule out the presence of nocturnal pollinators in *M. luteus* (e.g., Medel et al. 2003, 2007; Pohl et al. 2006), the lack of nocturnal observations in this study is undoubtedly a limitation that may affect the interpretation of our results and that need to be considered in future studies. Second, even though we never detected hummingbird activity on flowers out of our census periods of observation, we cannot rule out that the Andean hummingbird (*O. leucopleurus*) has performed short and quick visits to flowers. A third, less feasible possibility relates with the pollen movement by wind. Pollen movement by gusts of wind may be an important vector in the Andean range as suggested by studies on the Composite family (Arroyo et al. 1982). However, the zoophilous flower morphology of *M. luteus* turns improbable that wind has an important effect on the pollination of this species (e.g., Vickery 2008).

Lloyd and Schoen (1992) identified three modes of self-pollination without the participation of a pollen vector: prior, competing and delayed selfing. The most important trait for any of these self-reproduction modes is anther–stigma separation. A reduced anther–stigma separation has been directly related with a high self-fecundity rate and a decreased outcrossing rate in *Mimulus* (e.g., Dole 1992; Karron et al. 1997). In the same vein, a reduced anther–stigma separation combined with corolla abscission, after flowers had the opportunity to be visited (delayed selfing, from Lloyd and Schoen 1992), permit to explain reproduction in *M. guttatus* (Dole 1992). Whether delayed selfing occurs in *M. luteus* is unknown at present. Notwithstanding, the evidence of an increase in seed production with a decreased anther-stigma separation in bagged flowers suggests a mechanism for reproductive assurance.

Conclusions

We have presented different lines of evidence that *M. luteus* has an important potential to produce seeds through self-pollination in conditions with scarce mating opportunity. This observation complements previous knowledge related with the role of pollinators as selective agents of the floral phenotype in other populations of *M. luteus* in Chile (Medel et al. 2003). While pollinator-mediated selection may promote different combinations of floral traits associated with pollinator attraction along the geographical range, such as corolla size and presence of nectar guides (e.g., Medel et al. 2003, 2007), selection for traits related with autogamous strategies seem to be important in environments without pollinators. Given that no significant inbreeding depression was detected, simple mating system models predict that complete selfing may evolve and replace mixed mating in *M. luteus*.

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References

Armbruster WS, Schwaegerle KE (1996) Causes of covariation of phenotypic traits among populations. J Evol Biol 9:261–276

Arroyo MTK, Primack R, Armesto JJ (1982) Community studies in pollination ecology in the high temperate Andes of Central Chile. I. Pollination mechanisms and altitudinal variation. Am J Bot 69:82–97

Arroyo MTK, Muñoz MS, Henríquez C et al (2006) Erratic pollination, high selfing levels and their correlates and consequences in an altitudinally widespread above-tree-line species in the high Andes of Chile. Acta Oecol 30:248–257

Ashman T-L, Knight TM, Steets J et al (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. Ecology 85:2408–2421

Barrett SCH (2002) Sexual interference of the floral kind. Heredity 88:154–159

Becerra JX, Lloyd DG (1992) Competition dependent abscission of self-pollinated flowers of *Phormium tenax*: a second action of self-incompatibility at the whole flower level. Evolution 46:90–110

Benzécri JP (1992) Correspondence analysis handbook. Marcel Dekker, New York

Botto-Mahan C, Pohl N, Medel R (2004) Nectar guide fluctuating asymmetry does not relate to female fitness in *Mimulus luteus*. Plant Ecol 174:347–352

Braam J (2005) In touch: plant responses to mechanical stimuli. New Phytol 165:373–389

Brunet J, Eckert CG (1998) Effects of floral morphology and display on outcrossing in blue columbine, *Aquilegia caerulea* (Ranunculaceae). Funct Ecol 12:596–606

Carr DE, Dudash MR (1996) Inbreeding depression in two species of *Mimulus* (Scrophulariaceae) with contrasting mating systems. Am J Bot 83:586–593

Carr DE, Eubanks M (2002) Inbreeding alters resistance to insect herbivory and host plant quality in *Mimulus guttatus* (Schrophulariaceae). Evolution 56:22–30

Carr DE, Fenster CB, Dudash MR (1997) The relationship between mating-system characters and inbreeding depression in *Mimulus guttatus*. Evolution 51:363–372

Carvallo G (2006) Biología reproductiva y mecanismos que evitan la autopolinización en el berro cordillerano, *Mimulus luteus* L. (Phrymaceae). Master Dissertation, Universidad de Chile

Carvallo G, Medel R (2005) The modular structure of the floral phenotype in *Mimulus luteus* var. luteus (Phrymaceae). Rev Chil Hist Nat 78:665–672

Charlesworth B (1980) The cost of sex in relation to mating system. J Theor Biol 84:655–671

Charlesworth D, Charlesworth B (1987) The effects of investment in attractive structures on allocation to male and female function in plants. Evolution 41:948–968

Charlesworth D, Charlesworth B (1990) Inbreeding depression with heterozygote advantage and its effects on selection for modifiers changing in the outcrossing rate. Evolution 44:870–888

Charlesworth D, Morgan MT, Charlesworth B (1990) Inbreeding depression, genetic load, and the evolution of outcrossing rates in a multilocus system with no linkage. Evolution 44:1469–1489

Cooley AM, Carvallo G, Willis JH (2008) Is floral diversification associated with pollinator divergence? Flower shape, flower color and pollinator preference in Chilean *Mimulus*. Ann Bot 101:641–650

Darwin C (1876) The effects of cross and self-fertilization in the vegetable kingdom. John Murray, London

Dole JA (1992) Reproductive assurance mechanisms in three taxa of the *Mimulus guttatus* complex (Schrophulariaceae). Am J Bot 79:650–659

Faegri K, van der Pijl L (1971) The principles of pollination ecology. Pergamon Press, Oxford

Fenster CB (1991) Selection on floral morphology by hummingbirds. Biotropica 23:98–101

Fetscher AE (2001) Resolution of male–female conflict in a hermaphroditic flower. Proc R Soc Lond B 268:525–529

Fetscher E, Kohn JR (1999) Stigma behavior in *Mimulus aurantiacus* (Schrophulariaceae). Am J Bot 86:1130–1135

Fetscher AE, Rupert SM, Kohn JR (2002) Hummingbird foraging position is altered by the touch-sensitive stigma of bush monkeyflower. Oecologia 133:551–558

Hayes CN, Winsor JA, Stephenson G (2005) Multigenerational effects of inbreeding in *Cucurbita pepo* ssp. texana (Cucurbitaceae). Evolution 59:276–286

Hegland SJ, Totland O (2008) Is the magnitude of pollen limitation in a plant community affected by pollinator visitation and plant species specialization levels? Oikos 117:883–891

Herlihy CR, Eckert CG (2002) Genetic cost of reproductive assurance in a self-fertilizing plant. Nature 416:320–323

Herlihy CR, Eckert CG (2005) Evolution of self-fertilization at geographical range margins? A comparison of demographic, floral, and mating system variables in central vs. peripheral populations of *Aquilegia canadensis* (Ranunculaceae). Am J Bot 92:744–751

Herrera CM (1995) Microclimate and individual variation in pollinators: flowering plants are more than their flowers. Ecology 76:1516–1524

Heschel MS, Paige KN (1995) Inbreeding depression, environmental stress, and population size variation in scarlet gilia (Ipomopsis aggregata). Conserv Biol 9:126–133

Hood GM (2000) PopTools. Software for the analysis of ecological models. Version 2.5.5. Available from http://www.cse.csiro.au/poptools. Accessed July 2008

Husband B, Schemske DW (1996) Evolution of the magnitude and timing of inbreeding depression in plants. Evolution 50:54–70

Ivey CT, Carr DE (2005) Effects of herbivory and inbreeding on the pollinators and mating system of *Mimulus guttatus* (Phrymaceae). Am J Bot 92:1641–1649

Ivey CT, Carr DE, Eubanks MD (2004) Effects of inbreeding in *Mimulus guttatus* on tolerance to herbivory in natural environments. Ecology 85:567–574

Kalisz S, Vogler DW (2003) Benefits of autonomous selfing under unpredictable pollinator environments. Ecology 84:2928–2942

Kalisz S, Vogler DW, Hanley KM (2004) Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. Nature 430:884–887

Karron JD, Jackson RT, Thumser NM et al (1997) Outcrossing rates of *Mimulus ringens* genets are correlated with anther–stigma separation. Heredity 79:365–370

Kearns CA, Inouye DW (1993) Techniques for pollination biologists. University Press of Colorado, Niwot, Colorado

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. Trends Ecol Evol 17:230–241

Knight TM, Steets JA, Vamosi JC (2005) Pollen limitation of plant reproduction: pattern and process. Annu Rev Ecol Evol Syst 36:467–497

Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. Evolution 37:1210–1226

Lande R, Schemske DW (1985) The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. Evolution 39:24–40

Lloyd DG (1979) Some reproductive factors affecting the selection of self-fertilization in plants. Am Nat 113:67–79

Lloyd DG, Schoen DJ (1992) Self- and cross-fertilization in plants. I. Functional dimensions. Int J Plant Sci 153:358–369

Maad J (2000) Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. Evolution 54:112–123

Maynard Smith J (1978) The evolution of sex. Cambridge Univ Press, NY

Medel R, Botto-Mahan C, Kalin-Arroyo M (2003) Pollinator-mediated selection on the nectar guide phenotype in the Andean monkeyflower, *Mimulus luteus*. Ecology 84:1721–1732

Medel R, Valiente A, Botto-Mahan C et al (2007) The role of insects and hummingbirds on the geographical variation of the flower phenotype in *Mimulus luteus* (Phrymaceae). Ecography 30:812–818

Moeller DA, Geber MA (2005) Ecological context of evolution of self-pollination in *Clarkia xantiana*: population size, plant communities and reproductive assurance. Evolution 59:786–799

Morgan MT, Wilson WG (2005) Self-fertilization and the escape from pollen limitation in variable pollination environments. Evolution 5:1143–1150

Morgan MT, Wilson WG, Knight TM (2005) Plant population dynamics, pollinator foraging, and the selection of self-fertilization. Am Nat 166:169–183

Murren CJ, Pendleton N, Pigliucci M (2002) Evolution of phenotypic integration in *Brassica* (Brassicaceae). Am J Bot 89:655–663

Ordano M, Fornoni J, Boege K et al (2008) The adaptive value of phenotypic floral integration. New Phytol 179:1183–1192

Papoulis A (1990) Probability and statistics. Prentice Hall, Englewood Cliffs, NJ

Pohl N, Carvallo G, Botto-Mahan C et al (2006) Non-additive effects of flower damage and hummingbird pollination on the fecundity of *Mimulus luteus*. Oecologia 149:648–655

Richardson SC (2004) Benefits and costs of floral visitors to *Chilopsis linearis*: pollen deposition and stigma closure. Oikos 107:363–375

Ritland K, Ganders F (1987) Crossability of *Mimulus guttatus* in relation to components of gene fixation. Evolution 41:772–786

Ritland C, Ritland K (1989) Variation of sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). Am J Bot 76:1731–1739

Schemske DW, Lande R (1985) The evolution of self-fertilization and inbreeding depression in plants. II. Empirical observations. Evolution 39:41–52

Schluter D (1988) Estimating the form of natural selection on a quantitative trait. Evolution 42:849–861

Takebayashi N, Delph LF (2000) An association between floral trait and inbreeding depression. Evolution 54:840–846

Vickery RK Jr (2008) How does *Mimulus verbenaceus* (Phrymaceae) set seed in the absence of pollinators? Evol Biol 35:199–207

von Bohlen C (1995) El génerο *Mimulus* (Schrophulariaceae) en Chile. Gayana Bot 52:7–28

Waser NM, Fugate ML (1986) Pollen precedence and stigma closure: a mechanism of competition between *Delphinium nelsonii* and *Ipomopsis aggregata*. Oecologia 70:573–577

Willis JH (1993a) Effects of different levels of inbreeding on fitness components in *Mimulus guttatu*s. Evolution 47:864–876

Willis JH (1993b) Partial self-fertilization and inbreeding depression in two populations of *Mimulus guttatus*. Heredity 71:145–154

Wolf LL, Hainsworth FR (1990) Non-random foraging by hummingbirds: patterns of movement between Ipomopsis aggregata (Pursch) V. Grant inflorescences. Funct Ecol 4:149–157