



Pollen limitation and natural selection on floral characters in the yellow monkeyflower, *Mimulus guttatus*

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Summary

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• In flowering plants, pollen limitation has been proposed to intensify selection on floral characters important in pollinator attraction, but may also select for traits that increase seed set through autonomous selfing.

• Here, a factorial design (\pm pollen addition, \pm pollinator removal) was used to investigate how the pollination environment affects selection on floral morphology via female fitness in a mixed-mating population of the yellow monkeyflower, *Mimulus guttatus* (Phrymaceae).

• Female fitness was strongly pollen-limited, with supplementally pollinated plants setting 37% more seeds than open-pollinated individuals. Strong positive selection was found on flower length, weak positive selection on flower width : length ratio and no selection on stigma–anther distance in both open-pollinated and supplementally pollinated treatments. By contrast, flowers with relatively narrow corollas and low stigma–anther distances were favored in the pollinator exclusion treatment.

• These results provide mixed support for the idea that pollen limitation intensifies selection on floral characters. Despite strong phenotypic selection, natural pollen limitation did not mediate selection on characters associated with either pollinator attraction or self-fertilization. However, the novel pattern of selection on severely pollen-limited plants suggests that reproductive assurance against pollinator loss may have been directly involved in the floral evolution of closely related selfing taxa.

Key words: floral evolution, mating system, *Mimulus* (monkeyflowers), phenotypic selection, pollen limitation.

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Introduction

Interactions with animal pollinators have been a major factor in generating patterns of flowering plant diversity (Fenster *et al.*, 2004), and natural selection by pollinators continues to shape the morphology of flowers (Campbell, 1989; Alexandersson & Johnson, 2002; Maad & Alexandersson, 2004) and the architecture of inflorescences (Harder & Barrett, 1995). When seed production is limited by pollen receipt, as appears common (Burd, 1994; Knight *et al.*, 2005), theory suggests that selection for attractive floral traits via female fitness may be particularly strong (Ashman & Morgan, 2004). However, for the many animal-pollinated plants with perfect flowers, strong pollen limitation may also select for traits that promote reproductive

assurance through increased autonomous self-pollination (Morgan & Wilson, 2005; Porcher & Lande, 2005). Because floral trait values associated with high autonomous selfing rates (small corolla size, low herkogamy, low protandry) are generally the opposite of those associated with efficient outcrossing, selection for attractiveness and selection for efficient selfing are unlikely to act in parallel. Thus, despite a strong theoretical framework, the nature and outcome of natural selection in pollen-limited populations may often reflect opposing forces and be difficult to predict.

Pollen limitation in ecologically or geographically marginal habitats has almost certainly played a role in the repeated evolution of autonomous selfing (Barrett & Harder, 1996; Goodwillie *et al.*, 2005; Moeller, 2006). The reproductive assurance

value of autonomous selfing is clear in comparisons of populations or species with distinct morphologies (Fishman, 2000; Goodwillie, 2001; Elle & Carney, 2003), but natural selection on characters related to mating system variation has rarely been demonstrated (but see Kalisz *et al.*, 2004; Moeller & Geber, 2005). One powerful approach to investigating the functional relationships between floral characters and fitness is to combine phenotypic selection analysis with experimental manipulations of the pollination environment (Wade & Kalisz, 1990). Such approaches explicitly test whether hypothetical mechanisms of natural selection actually contribute to any observed relationship between fitness and the trait(s) of interest.

In this study, experimental manipulation of the pollination environment was used to address how pollen limitation affects natural selection on floral traits associated with mating system variation in *Mimulus guttatus* species complex (Phrymaceae, formerly Scrophulariaceae). *M. guttatus*, our study species and the putative progenitor of other taxa in the complex (Fenster & Ritland, 1994), is variable in mating system but predominantly outcrossing (Dudash & Ritland, 1991; Willis, 1993). The flowers of *M. guttatus* display classic adaptations for outcross pollination by bees: a tubular corolla with showy flared petals and a 'landing pad', protandry, approach herkogamy (stigma exerted beyond anthers in corolla throat), and a touch-sensitive stigma. High rates of autonomous self-fertilization have evolved multiple times within this group (Fenster & Ritland, 1994). In general, the selfing taxa (e.g. *M. nasutus*, *M. micranthus*, *M. laciniatus*) have relatively small flowers with reduced protandry and little or no stigma-anther separation. Because the selfing taxa are cross-compatible with *M. guttatus*, the species complex has become a model system for understanding the genetic basis of floral and mating system evolution. The frequent evolution of selfing in the *M. guttatus* complex and previous detection of pollen limitation in one *M. guttatus* population (Dudash & Ritland, 1991) suggest that natural selection for selfing as reproductive assurance may have been important in the evolutionary history of the group.

Here, the focus is on the relationship between individual floral morphology and female fitness under experimental pollination conditions in a single annual population of *M. guttatus* (Iron Mountain, OR, USA). Previous studies have demonstrated substantial genetic variation in flower size characters in this population (Kelly & Willis, 2001) and in other populations of *M. guttatus* (Carr & Fenster, 1994; Robertson *et al.*, 1994; Van Kleunen & Ritland, 2004), so phenotypic selection measures are relevant to understanding the evolution and maintenance of floral variation in the *M. guttatus* complex. First, we analyze differences in mean fecundity among supplemental, open and autonomous selfing pollination treatments to ask: is female fitness naturally pollen limited; how much autonomous selfing occurs under conditions of complete pollinator loss? Second, we measure phenotypic selection on naturally varying floral characters in our pollination treatments to ask: is the strength and direction of natural selection on floral

characters (via female fecundity) different under conditions of complete pollen limitation, natural pollen limitation, and unlimited pollen receipt? More specifically, does pollen limitation intensify selection for trait values associated with increased attraction and/or increased self-fertilization?

Materials and Methods

Study species and population

The common yellow monkeyflower, *Mimulus guttatus* DC (Phrymaceae, formerly Scrophulariaceae, $2n = 28$), is a self-compatible wildflower native to western North America. *M. guttatus* is highly variable in life history characters, edaphic tolerances, and floral and vegetative morphology among populations (Vickery, 1978). *M. guttatus* is the most common species of the yellow monkeyflower species complex, a group of closely related taxa showing broad ecological and morphological diversity. In particular, routine self-fertilization and reduced flower size have evolved multiple times within the complex.

This study focuses on a population of *M. guttatus* located on Iron Mountain in the Oregon Cascades. In a typical year, this population consists of tens of thousands of plants blanketing an approx. 1 ha snowmelt seep on a steep northeast-facing slope. The growing season is restricted to the time between snowmelt in early June and the complete drying of the site in late July. Plants at this site are annual, produce a single flower, and mature a single fruit (see the Results section), which simplifies experimental manipulations and the estimation of reproductive fitness. Iron Mountain *M. guttatus* plants are primarily pollinated by bees and the population has a mixed, but predominantly outcrossing, mating system. Estimated self-fertilization rates ranged from 0.09 to 0.29 across 3 years (Willis, 1993; Sweigart *et al.*, 1999) and there is no evidence of biparental inbreeding or within-population genetic structure (Sweigart *et al.*, 1999; Kelly & Willis, 2002). The population exhibits substantial inbreeding depression for seed-to-seed fitness measured in the field (mean cumulative ID = 0.69; Willis, 1993). In addition, the population exhibits substantial genetic variation for floral characters associated with mating system variation across the *M. guttatus* complex (Kelly & Willis, 2001; Kelly & Arathi, 2003). Because that variation does not appear to result primarily from the segregation of rare deleterious recessive alleles, much of it should be available for adaptive evolution (Kelly & Willis, 2001).

Experimental design and floral measurements

In July 1999, floral characters and seed number of plants in a factorial set of treatments manipulating the pollination environment were measured. Plants in all treatments were intermingled along three horizontal transects across the steep Iron Mountain site, spanning the range of moisture availability and plant density at the site. The floral marking and measuring

were repeated in each of the two weeks of the peak flowering season that year (14–16 July and 20–22 July). Means, standard errors and coefficients of variation (CV) were calculated. Temporal and spatial variation in each flower size character was analyzed by ANOVA, with date and transect as fixed factors.

To test for pollen limitation and tease apart the relationships among floral characters, autonomous self-fertilization, and female fitness in the field, a full factorial experiment manipulating access to outcross pollination was conducted. Plants (total starting $n = 407$) were assigned to one of four treatments: pollinator exclusion (+CAGE, –ADD), pollen addition (–CAGE, +ADD), pollinator exclusion + pollen addition (+CAGE, +ADD) and open pollination (–CAGE, –ADD). Comparisons of seed number per fruit among these treatments allow estimation of autonomous selfing rates (pollinator exclusion vs open pollination) and pollen limitation (open pollination vs the two pollen addition treatments). The pollinator exclusion + pollen addition treatment controls for effects of caging other than pollinator exclusion.

A single flower per plant was used as the focal flower for measurements and treatments; because the plants are annual and the vast majority produce only a single fruit (> 80%, see the Results section), single flowers are representative of whole-plant characteristics. For CAGE plants, cylindrical pollinator-exclusion cages made of fine mesh were placed over plants the day before opening of the focal flower. For ADD plants, open focal flowers were supplementally hand-pollinated with pollen from glasshouse-grown plants of the IM62 inbred line (transported to the site each day). IM62 was derived from the Iron Mountain population and has uniformly high pollen production and fertility (Fishman & Willis, 2001). To minimize variation in pollen delivery, all ADD flowers were supplementally pollinated by a single experienced human pollinator using forceps to transfer pollen. Because *M. guttatus* has a sensitive stigma (the stigma lobes close temporarily when touched and permanently after successful fertilization), the supplemental pollinations were performed in the afternoon of the day of anthesis to allow for prior open pollination.

Because flower size characters can vary with floral age, all focal flowers were measured on the first day of opening. The day before measurement, a small piece of laboratory tape was used to mark the pedicel of one fully expanded bud on each experimental plant. The following day, corolla width, corolla tube length, and stigma–anther separation (distance between the top of the upper anther pair and the base of the stigma lobes) were measured using an engineering ruler marked in 0.01 inch increments (see Fishman *et al.*, 2002 for details of the floral measurements). The values were later converted to mm. The corolla width : tube length ratio was also calculated for each flower as an indicator of flower shape.

The cages were removed from +CAGE treatment plants 1 wk after flower measurement and manipulation, at which point flowers were no longer receptive. Plants were checked regularly and the seeds collected from each experimental fruit

before dehiscence of the fruit capsule. At that time, the total number of matured or maturing fruits on each plant were also counted, since the opportunity for further reproduction was past. During the interval before fruit collection, plants that died postflowering but before opportunity for fruit maturation were also noted.

Many (~20%) measured plants died before seeds could be collected. Plants were categorized as dead if they were missing (i.e. the plant tag could be found but not the marked flower) or present but completely shriveled. Plants that were senescent (dry) but still standing were included in the seed production and selection analyses.

Effects of pollination environment on female fitness

To examine the effects of pollen availability on female fitness, the seed number data were analyzed by ANOVA with pollinator exclusion (CAGE) and pollen addition (ADD) treatments and their interaction as factors. Plants that died in the interval between flowering and fruit maturity were not included in the female fitness analyses because drought death is unlikely to be the result of pollinator activity and mortality was equal across treatments (Pearson χ^2 with 3 d.f. = 3.66, $P > 0.3$). Because there was no difference in the mean or SE of seed number between the caged and uncaged flowers with pollen addition, the data were re-categorized into three pollination treatments: pollinator exclusion, +CAGE, –ADD; open pollination, CAGE, –ADD; and supplemental pollination, +CAGE, +ADD and –CAGE, +ADD) for further analyses. Because the caged and pollen-supplemented treatment was included as a control for effects of caging other than pollinator exclusion (and no such effects occurred; see the Results section) this is an appropriate recategorization for the later analyses. The results from analyses with three categories were indistinguishable from those with all four categories with the exception of increased significance resulting from increased sample size in the supplemented category. Spatial and temporal variation in fecundity was analyzed under the different pollination environments with a full factorial ANOVA, including pollination treatment, transect and date as main effects.

Phenotypic selection analyses

Comparison of the strength and direction of phenotypic selection on floral characters in the treatments manipulating access to outcross pollination allows testing of two hypotheses about the nature of selection on floral characters. First, comparison of the open-pollinated and supplementally pollinated treatments tests whether pollinators mediate any selection observed under natural pollination conditions. For example, we might see relaxed selection on corolla width in the supplementation treatment if this character affected attractiveness to pollinators and variation in attractiveness affected fitness. Second, comparison of the pollinator exclusion treatment

with the other treatments tests whether selection changes when autonomous selfing is the only means of reproduction.

To facilitate comparison with previous studies (Willis, 1996) measuring phenotypic selection only on single characters, both univariate and multivariate selection analyses were run. To estimate total directional selection (S' , equivalent to β' ; (Lande & Arnold, 1983), we first regressed female fitness on single floral characters independently in each pollination treatment. For these and other selection analyses, seed number values (female reproductive fitness) were standardized to a mean of 1 within each treatment (individual value/treatment mean). Character values were standardized to mean 0 and unit variance ((individual value – treatment mean)/treatment SD) within each treatment. To identify heterogeneity of slopes (treatment \times character interaction effects) among the pollination treatments, ANCOVA with each character was conducted, plus the treatment and the treatment \times character interaction. Because fitnesses were standardized within each treatment, these latter analyses compare the proportional effect of each trait on fitness under different pollination conditions. The quadratic regression coefficients (γ) for each character were also estimated, which provide an indication of nonlinear (potentially disruptive or stabilizing) selection. However, because the γ -values were generally not significantly different from zero (with a few exceptions, see the Results section), S' values are presented from the simple linear regressions here.

To further tease apart relationships between each floral character and female fitness, multivariate regressions were performed including tube length, corolla width : length ratio and stigma–anther separation. To reduce the problem of multicollinearity (Lande & Arnold, 1983) as a result of the strong correlation between flower length and corolla width, corolla width was not included in the multivariate analyses. Corolla length was chosen as the primary flower size character because it is likely to parallel most closely ovary size, and corolla width : length ratio used as the metric of flower shape. As with the single character analyses, we first ran separate analyses for each treatment, then a complete ANCOVA, including treatment and all treatment \times character interactions, to test for significant difference in slope among treatments. The partial regression coefficients from these analyses estimate β' , the directional selection gradient, for each character (Lande & Arnold, 1983). Models were also run that included quadratic terms, but the nonlinear terms were never significant in the multivariate analyses and those results are not presented here. All statistical analyses were performed using JMP 5.1 (SAS Institute, 2003).

Results

Floral variation

Overall, the flowers of Iron Mountain *M. guttatus* plants varied substantially in size; the largest flowers were over four times as wide and three times as long as the smallest flowers (CV = 0.20

Table 1 Seed production of *Mimulus guttatus* in the pollination manipulation experiment ($n = 78$ – 82 individuals per treatment)

Treatment	Seed number LSM	SE	CV
–CAGE, –ADD	32.96 b	3.86	1.10
–CAGE, +ADD	45.23 a	4.11	0.89
+CAGE, +ADD	45.25 a	3.91	0.97
+CAGE, –ADD	5.93 c	3.95	1.82

Letters indicate means significantly different ($P < 0.05$) from each other by Tukey's HSD contrasts of LSMs.

and 0.16 for width and length, respectively). Both of these characters were normally distributed (Shapiro–Wilks W -test, $P > 0.05$). Stigma–anther separation was even more variable, with the uppermost anther ranging from 3.8 mm below the stigma to 0.76 mm above the stigma (CV = 0.67). The distribution of stigma–anther separation values deviated significantly from normality ($P < 0.001$), but fit a lognormal distribution (KSL goodness-of-fit test, $P = 0.15$). Some of the floral variation is explained by large-scale environmental variation; date was a significant factor in the ANOVAs for corolla width and tube length ($n = 401$, $P < 0.05$). On average, flowers on the earlier date were larger (LSM contrasts, $P < 0.05$). By contrast, both date and transect affected stigma–anther separation ($P < 0.05$), with flowers on the middle transect and the later date consistently having smaller stigma–anther distances. No interactions were significant.

The two corolla size measures were highly correlated with each other ($r = 0.80$, $P < 0.0001$) and significantly, but more weakly, correlated with stigma–anther distance ($r = 0.27$ and 0.30 for tube length and corolla width, respectively, both $P < 0.0001$).

Pollen limitation of seed number and rate of autonomous self-fertilization

Both pollen addition and pollinator exclusion strongly affected seed number (Table 1). The full model ANOVA was highly significant, as were each of the individual factors (CAGE and ADD) and their interaction (all $P < 0.001$). Because the distribution of seed numbers was highly skewed (lognormal) in the data set as a whole and within each treatment, the analysis was also repeated on log-transformed data. The results of that analysis were not qualitatively different, so the untransformed results are presented here to simplify interpretation of fitness differences.

Flowers that were supplementally hand-pollinated, both caged (mean seed number = 45.23) and uncaged (mean seed number = 45.25), produced 37% more seeds than unmanipulated, open-pollinated flowers (mean seed number = 32.96). This significant increase in seed number with hand-pollination (LSMs contrast, $P < 0.005$) demonstrates strong natural pollen limitation of flower-level fecundity. Over 87% of the plants in

Treatment	<i>n</i>	Corolla tube length	Corolla width	Corolla width : length ratio	Stigma–anther separation
Pollinator exclusion	82	0.556 a <i>0.218</i>	–0.021 a <i>0.215</i>	–0.546 a <i>0.195</i>	–0.190 a <i>0.185</i>
Open pollination	82	0.612 a <i>0.116</i>	0.651 b <i>0.121</i>	0.12 b <i>0.147</i>	0.190 b <i>0.136</i>
Supplemental pollination	158	0.388 a <i>0.067</i>	0.544 b <i>0.069</i>	0.167 b <i>0.071</i>	0.205 b <i>0.082</i>
Different <i>S'</i> ?		<i>P</i> = 0.34	<i>P</i> = 0.001	<i>P</i> < 0.001	<i>P</i> = 0.045

To obtain the standard errors for the slopes (in italics), each treatment was analyzed separately. Slopes in bold were significantly different from zero. To test for significant differences among the treatments (bottom row of table), a single ANCOVA was used for each character with treatment and treatment × character interaction effects included. The letters indicate slopes significantly different at the *P* = 0.05 level in that analysis.

this experiment produced only a single fruit; for these plants, this difference translates into pollen limitation of total female fitness. To account for the possibility that reallocation to other fruits could reduce the individual-level fitness effects of flower-level pollen limitation, we also compared open-pollinated with supplemented treatments with fruit number category (1 vs > 1) and its interaction with pollen addition as additional factors. The pattern of seed number variation across pollination treatments was parallel in the two fruit number categories (interaction NS, *P* = 0.8). However, this analysis also revealed that the relatively few plants that matured multiple fruits had significantly larger flowers (*P* < 0.005 for both corolla width and tube length) and produced almost twice as many seeds/fruit as plants that matured only one fruit (*P* < 0.001). As expected, hand-pollination also reduced variation in fecundity among individuals; the coefficient of variation of seed number was lower in the two supplementally pollinated treatments (0.96 and 0.89) relative to the pollinator exclusion (1.82) and open pollination treatments (1.10).

Rates of autonomous self-fertilization were low. Flowers in the pollinator exclusion treatment produced few seeds relative to both open-pollinated and supplementally pollinated flowers (mean seed number = 5.93). More than 50% (45/82) of caged, unvisited flowers matured no seeds, compared with 11 and 7%, respectively, in open- and supplementally pollinated treatments. These differences cannot be the result of effects of caging other than pollinator exclusion, as seed number was equally high in the pollinator exclusion + pollen addition treatment.

Phenotypic selection of floral characters in the different pollination environments

All of the floral characters measured showed strong associations with female fitness (seed number) under at least one of the experimental conditions. However, different patterns of

selection on floral morphology were observed under the different pollination regimes (Tables 2, 3). In the single-character analyses (Table 2), only corolla tube length showed no variation in the strength or direction of selection across treatments (treatment–character interaction NS, *P* = 0.34). Across all treatments, longer flowers produced greater numbers of seeds, generating significantly positive values of total selection for corolla tube length (*S'* = 0.402–0.612, *P* < 0.001 for each). For the other three characters, total selection differed significantly between the pollinator exclusion treatment (autonomous selfing only) and both the open pollination and supplemental pollination treatments (*P* < 0.05 for interactions). For corolla width, total selection differentials were strongly positive in the two outcrossing treatments and near zero in the pollinator exclusion treatment. This translated into a strongly negative selection (*S'* = –0.546) on width : length ratio under conditions of obligate autogamy (i.e. for a given size, narrower flowers produced relatively more seeds) vs positive directional selection in the outcrossing treatments. Similarly, total directional selection on stigma–anther separation appeared positive in the outcrossing treatments and negative in the pollinator exclusion treatment (although only the supplemental pollination treatment, which had doubled sample size, had a significantly nonzero value of total *S'*). Polynomial regressions on single characters revealed significant quadratic terms only for corolla width in the hand-pollinated treatments (γ = 0.14, *P* = 0.01) and for width : length ratio in the autonomous selfing treatment (γ = 0.37, *P* = 0.02).

The differences in selection between treatments were even clearer in the multivariate analysis, which included the correlated effect of flower size (represented by tube length) in the analyses of the other characters (Table 3). Again, tube length was strongly and positively associated with female fitness (β' = 0.457–0.650), with no significant variation across treatments. Selection on shape (width : length ratio) differed dramatically among treatments, with relatively narrow corollas having higher

Table 2 Standardized total phenotypic selection differentials (*S'*) from ANCOVAs on single floral characters of *Mimulus guttatus*

Table 3 Standardized direct selection gradients (β') from partial regression coefficients of ANCOVA of all characters of *Mimulus guttatus*

Pollination	<i>n</i>	Corolla tube length	Corolla width : length ratio	Stigma–anther separation
Treatment				
Pollinator exclusion	82	0.650 a <i>0.218</i>	−0.463 a <i>0.195</i>	−0.364 a <i>0.182</i>
Open pollination	82	0.634 a <i>0.116</i>	0.214 b <i>0.132</i>	0.038 b <i>0.124</i>
Supplemental pollination	158	0.457 a <i>0.069</i>	0.279 b <i>0.067</i>	0.025 b <i>0.077</i>
Different β' ?		<i>P</i> = 0.42	<i>P</i> < 0.001	<i>P</i> = 0.032

To obtain the standard errors for the slopes (in italics), each treatment was analyzed separately. Slopes in bold are significantly different from zero. To test for significant differences among the treatments (bottom row of table), a single ANCOVA was used with all characters, treatment, and treatment \times character interaction effects included. The letters indicate slopes significantly different at the *P* = 0.05 level.

fitness in the pollinator exclusion treatment and relatively wide ones in both the open-pollinated and supplementally pollinated treatments. In the multivariate analysis, directional selection on stigma–anther separation was significantly negative ($\beta' = -0.36$) in the pollinator exclusion treatment, suggesting that (all else being equal) flowers with smaller distances between male and female parts produced more seeds under conditions of obligate self-pollination. Unlike the single-variate analyses, which included indirect effects of floral size, there was no evidence for selection on stigma–anther distance in the other treatments ($\beta' = 0.025$ and 0.038 in the supplemental pollination and open pollination treatments, respectively).

Overall, no difference was seen in selection between the open-pollination and supplemental pollination treatments, indicating that the strong selection observed on corolla size characters in the naturally pollen-limited open-pollination treatment is not mediated by attractiveness to pollinators. However, a change was seen in the direction of selection on stigma–anther separation and corolla width : length ratio between the pollinator exclusion treatment (all seeds set by autonomous selfing) and the other two treatments (primarily outcrossing), suggesting that fundamentally different trait values are favored under conditions of pollinator loss.

Discussion

Does pollen availability limit female fitness?

Substantial pollen limitation of female fitness was found in the Iron Mountain population of *M. guttatus*: on average, supplementally hand-pollinated flowers produced nearly 40% more seeds than open-pollinated controls. Strong pollen limitation is common across plant taxa (Ashman *et al.*, 2004; Knight *et al.*, 2005), but is expected to be less severe in potentially self-fertile species. Selfing in mixed-mating populations is often thought to provide reproductive assurance by buffering against spatial or temporal variation in the pollination environment (Kalisz

et al., 2004). In addition, delayed selfing is theoretically advantageous to annual plants even when inbreeding depression is high (Lloyd, 1979). Thus, assuming appropriate genetic variation, populations with a history of strong pollen limitation might be expected to evolve mechanisms of delayed selfing that would provide reproductive assurance and reduce pollen limitation (Morgan & Wilson, 2005). Of course, the Iron Mountain *M. guttatus* population may simply not be at evolutionary equilibrium with respect to pollen limitation and self-fertilization, but it is also possible that the population is adapted as well as possible to current conditions. In particular, high rates of inbreeding depression in this population (Willis, 1993) may prevent the evolution of reproductive assurance by competing selfing, and the specifics of its floral and pollination biology may constrain opportunities for delayed selfing.

In *M. guttatus*, which has highly variable selfing rates both within populations (L. Fishman & J. H. Willis, unpublished data) and among populations and years (Willis, 1993), delayed selfing has been identified as an important mechanism of reproductive assurance in some populations (Dole, 1992) but not found in others (Dudash & Ritland, 1991). Two mechanisms of delayed selfing have been proposed for *M. guttatus*: corolla dragging (in which the epipetalous stamens come into contact with the stigma as the corolla is shed; Dole, 1992); and late stigma contact with self pollen shed into the corolla tube (Arathi & Kelly, 2004). However, these mechanisms are clearly not particularly effective under current pollination conditions at the Iron Mountain site. Unvisited flowers (as in the pollinator exclusion treatment) have very low female fitness, suggesting little reproductive assurance by delayed selfing. For visited flowers, the floral biology of *Mimulus* may preclude effective delayed self-pollination as well; *M. guttatus* has a touch-sensitive stigma and the stigma lobes generally remain closed after receiving a pollen load (L. Fishman, pers. obs.). If the natural pollen load of a single insect visitor is insufficient to fill all ovules but sufficient to stimulate permanent stigma closure, delayed selfing cannot occur. Under current conditions,

the male fitness advantages of stigma closing at visitation (Fetscher, 2001) may outweigh the benefits of producing additional low-cost, but low-fitness, inbred seeds via delayed selfing. Thus, pollen limitation of female fitness and high outcrossing rates may persist despite the apparent opportunity for additional delayed self-fertilization. Further studies of the pollination ecology of the Iron Mountain *M. guttatus* population will be necessary to test this hypothesis.

Does selection on floral characters vary with pollination environment?

Pollen limitation, by generating variation in female fecundity, may create greater opportunity for phenotypic selection on characters related to either reproductive assurance (autonomous selfing) or pollinator attraction (Ashman & Morgan, 2004). In this study, pollen limitation had the expected effect on variation in fitness: the CV of fecundity was highest in the plants forced to autonomously self-fertilize and lowest in the pollen addition treatments. Strong selection was observed on at least one character under each pollination environment. In addition, the pattern of selection changed substantially with the degree of pollen limitation, providing insight into the functional relationship between particular floral characters and female fitness.

In the open-pollination treatment, which was substantially pollen-limited, strong positive total selection differentials ($S' \sim 0.5$) were observed for both measures of flower size (tube length and corolla width) and no significant selection on stigma–anther separation. The standardized regression coefficients are very similar to values calculated 10 yr previously in the same population (Willis, 1996), suggesting that the processes underlying the relationship between traits and fitness are temporally consistent. The pattern and intensity of maternal selection were also similar to that reported in a recent study of a British Columbia population of *M. guttatus* (Van Kleunen & Ritland, 2004) and to total selection intensities reported for two California *M. guttatus* populations that were inferred to be not pollen-limited (Fenster & Ritland, 1994).

From the open-pollinated data alone, it might be tempting to conclude that pollinator preferences for large flowers generate both the variance in seed number (pollen limitation) and the association between seed number and flower size (phenotypic selection). However, our experimental treatments demonstrate that the observed relationship between floral size and female fitness has little or nothing to do with pollinator attraction. If the effects of flower size on fitness were mediated by differences in pollinator visitation and resultant pollen receipt, we would expect a relaxation of selection when pollen loads were supplemented and when pollinators were removed. Instead, consistently high selection on floral tube length (flower size) was seen under all three conditions (Tables 2, 3). Furthermore, no decrease was seen in the strength of selection on corolla width (univariate analysis) or corolla width : length

ratio (multivariate analysis) under pollen-supplemented conditions, indicating that positive natural selection on this trait in the open-pollination treatment is not mediated by attractiveness to pollinators. More likely, correlations between flower size and seed number result from individual environment (Mitchell-Olds & Shaw, 1987; Rausher, 1992), inbreeding history (Willis, 1996), or other sources of positive correlation between flower size and female reproductive potential (number of ovules per flower). This inference is further supported by the observation that plants that died between flowering and fruiting had flowers 9–10% smaller than those that survived to set seed (data not shown; Wald χ^2 for flower length = 13.6, $P < 0.001$), suggesting that the individual abiotic environment may mediate relationships between flower size and both measures of fitness under current pollination conditions.

Importantly, the inclusion of additional correlated characters (e.g. a measure of overall plant size, vigor, or ovules per flower) in the selection analyses would not necessarily have revealed this, as high correlations between traits limit the utility of multivariate selection analyses because of problems of multicollinearity (Lande & Arnold, 1983; Mitchell-Olds & Shaw, 1987). For example, a similar study of selection via male and female fitness on a British Columbia population of *M. guttatus* (Van Kleunen & Ritland, 2004) found strong total selection on both corolla width and ovule number (phenotypic correlation = 0.94) in single trait selection analyses of seed production, but only detected selection on corolla width in the multivariate selection analysis. In such a situation, it is impossible to determine which character is actually the target of selection without manipulative experiments.

In contrast to natural pollen limitation, the extreme pollen limitation caused by pollinator exclusion fundamentally changed selection on floral morphology. In particular, significant negative direct selection on both corolla width : length ratio and stigma–anther separation was observed only in the pollinator exclusion treatment (Tables 2, 3). Whereas longer flowers were always favored (potentially because of an association with greater numbers of ovules), relatively narrow flowers with low stigma–anther separation were more successful as autonomous selfers. These differences make sense from a functional perspective, as physical contact between the sexual organs is a key to autonomous self-pollination. A relatively narrow corolla may be important in promoting stigma–anther contact both at anthesis and at corolla shedding, as *Mimulus* anther filaments are attached to the tubular corolla. In addition, narrower corollas may promote increased contact between the stigma and (self) pollen shed and held on the corolla, as has been observed in plants from the Iron Mountain population (Arathi & Kelly, 2004).

Overall, our results provide little support for the idea that selection on attractive floral traits increases with increasing pollen limitation (Ashman & Morgan, 2004), but they do suggest that severe pollen limitation favors traits associated with autonomous self-fertilization (Morgan & Wilson, 2005).

Of course, pollen limitation is a continuum, and subtler manipulations of the pollination environment might reveal increased positive selection on corolla width or length as competition for limited pollinator service increased. Some studies have found stronger selection on floral traits associated with attractiveness or efficiency in relatively pollen-limited populations (Caruso, 2000), but others have not (Totland, 2004; Caruso *et al.*, 2005; Vanhoenacker *et al.*, 2006). It would be logistically very difficult, but interesting, to examine how selection on *M. guttatus* flowers changed across a broader range of pollinator availabilities (Moeller & Geber, 2005), as any selection for increased attractiveness is likely to oppose the observed selection for decreased stigma–anther distance and corolla width seen under conditions of complete pollinator failure. In general, the success of our experimental approach in distinguishing pollinator-mediated selection from other sources of trait–fitness correlation reiterates the need for careful interpretation of phenotypic selection analyses (Wade & Kalisz, 1990) and detailed experimental study of the ecological context of selection on floral and mating system characters (Busch, 2005; Moeller & Geber, 2005; Moeller, 2006).

Implications for mating system evolution

The reproductive assurance value of selfing is often invoked to explain the repeated evolution of autonomous self-pollination (and associated floral morphologies) in flowering plants. However, only a few studies have demonstrated that floral traits associated with the evolution of increased selfing rates across populations are under selection via female fitness in relatively pollen-limited environments (Elle & Carney, 2003; Kalisz *et al.*, 2004). In this study of *M. guttatus*, we found that the severe pollen limitation imposed by experimental pollinator exclusion selected for trait values similar to those of highly selfing congeners (e.g. *M. nasutus*, *M. laciniatus*). Thus, natural selection to increase the efficiency of autonomous selfing in the absence of pollinators is an empirically supported explanation for the evolution of the reduced floral morphology of the selfing *Mimulus* taxa.

The evidence for unique selection on flower shape (for relatively narrow flowers) under extreme pollen limitation is particularly interesting. We did not explicitly analyze corolla width : length ratio in a quantitative trait locus mapping study of the genetics of floral differences between *M. guttatus* and selfer *M. nasutus* (Fishman *et al.*, 2002), but this character is highly divergent between the two species. In that study, *M. nasutus* flowers were about one-sixth (15%) as wide as *M. guttatus* flowers, but more than half as long. The shift to autonomous selfing in *M. nasutus* thus entails a shape shift from a flower about twice as wide as it is long to one about twice as long as it is wide. This shift is consistent with the pattern of natural selection we observed under conditions of complete pollinator loss. Furthermore, the change in shape is almost certainly not the genetic 'line of least resistance' (Schluter, 1996),

as the genetic correlation between corolla length and width in *M. guttatus* is high (Willis, 1999) and the slope of the linear relationship between width and length phenotypes is only 1.35 (values from this study). Together with our experimental results, these constraints point to quantitative trait loci with disproportionate effects on corolla width characters, as well as those affecting stigma–anther distance, as potential targets of context-dependent natural selection early in the evolution of selfing in the *Mimulus guttatus* species complex.

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