

Mating System Evolution under Strong Pollen Limitation: Evidence of Disruptive Selection through Male and Female Fitness in *Clarkia xantiana*

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ABSTRACT: Selection on floral traits in hermaphroditic plants is determined by both male and female reproductive success. However, predictions regarding floral trait and mating system evolution are often based solely on female fitness. Selection via male fitness has the potential to affect the outcomes of floral evolution. In this study, we used paternity analysis to assess individual selfing rates and selection on floral traits via male and female fitness in an experimental population of *Clarkia xantiana* where pollen limitation of seed set was strong. We detected selection through both female and male fitness with reinforcing or noninterfering patterns of selection through the two sex functions. For female fitness, selection favored reduced herkogamy and protandry, traits that promote increased autonomous selfing. For male fitness, selection on petal area was disruptive, with higher trait values conferring greater pollinator attraction and outcross siring success and smaller trait values leading to higher selfed siring success. Combining both female and male fitness, selection on petal area and protandry was disruptive because intermediate phenotypes were less successful as both males and females. Finally, functional relationships among male and female fertility components indicated that selfing resulted in seed discounting and pollen discounting. Under these functional relationships, the evolutionarily stable selfing rate can be intermediate or predominantly selfing or outcrossing, depending on the segregating load of deleterious mutations.

Keywords: Bateman's principle, inbreeding depression, paternity analysis, reproductive assurance, selfing and outcrossing, sexual selection.

Introduction

The majority of flowering plants are hermaphroditic; therefore, individual fitness is gained through both female and male reproductive success. In addition, individual flowers

often contain both male and female sexual organs, which can lead to interference between sexual functions. Specifically, pollen removal and pollen receipt determine male and female fitness, respectively, and may necessitate different traits or behaviors for optimization. Theoretical models of sexual selection often predict conflicting selection on traits influencing male and female function, which may result in trait combinations that fail to maximize fitness through either gender (Morgan 1992; Ashman and Morgan 2004; Bedhomme et al. 2009; Jordan and Connallon 2014). Therefore, floral trait evolution reflects a balance between selection via male and female function (van der Pijl 1978; Lloyd and Yates 1982; Lloyd and Webb 1986; Harder and Barrett 1995).

Bateman's principle (1948) predicts greater variation in male than female fitness because the former is likely limited by mating opportunities, whereas the latter is likely limited by resources. In an outcrossing plant that produces copious pollen, male fitness may increase with continual pollinator visitation, whereas female fitness will increase only until all ovules are fertilized (Bateman 1948; Lloyd and Yates 1982; Delph and Ashman 2006). Because there may be a greater opportunity for selection through male fitness, it may disproportionately affect patterns of floral evolution (Bateman 1948; Delph and Ashman 2006). By contrast, under strong pollen limitation, variances in male and female fitness may be more similar; therefore, traits that influence pollen receipt may experience stronger selection. Moreover, those traits that enhance pollinator visitation may positively influence both male and female fitness and result in noninterfering selection (selection through one gender function only) or parallel selection (similar selection through both gender functions; Ashman and Diefenderfer 2001; Ashman and Morgan 2004; Delph and Ashman 2006).

In self-compatible species, individuals can gain male and female fitness through both pollinator visitation and auton-

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omous self-fertilization (Johnston et al. 2009). Under modest pollen limitation, selection through female and/or male function may favor secondary sexual characters (e.g., flower size) that enhance pollinator attraction and increase the import and export of nonself pollen (Bell 1985; Stanton et al. 1986; Ashman and Stanton 1991; Ashman and Morgan 2004; Fishman and Willis 2007). However, under severe pollen limitation, selection may also act on primary sexual characters (e.g., dichogamy and herkogamy) that affect the propensity for autonomous selfing because they provide reproductive assurance (Fausto et al. 2001; Kalisz et al. 2004; Moeller and Geber 2005; Eckert et al. 2006). Therefore, in highly pollen-limited populations, selection on secondary versus primary sexual characters may be complex, environmentally dependent, and influenced to different extents by male versus female fitness (Ashman and Morgan 2004; Sicard and Lenhard 2011; Karron et al. 2012).

An increase in selfing may be automatically favored by selection because individuals make greater genetic contributions to the next generation by transmitting two copies of their genome to selfed offspring while simultaneously exporting pollen to other individuals (Fisher 1941; Jain 1976). However, two forces may oppose this automatic advantage to selfing: inbreeding depression and pollen discounting. Inbreeding depression is the reduction in fitness of selfed relative to outcrossed offspring (Charlesworth and Charlesworth 1987), and pollen discounting is the trade-off between the propensity to export pollen and the propensity to reproduce via self-fertilization (Holsinger et al. 1984; Holsinger 1991; Harder and Wilson 1998). Experiments have shown that autonomous self-fertilization can reduce the probability of pollen export (Chang and Rausher 1998; Fishman 2000; but see Stone et al. 2014) and therefore can offset the automatic transmission advantage of selfing (Harder and Wilson 1998).

Despite the theoretical prediction of bimodality in selfing rate (Lande and Schemske 1985), plant species with intermediate selfing rates are common (Goodwillie et al. 2005, 2010; Eckert et al. 2006). The prevalence of intermediate selfing in plant species has led to the speculation that it may be an evolutionarily stable strategy (ESS) under some circumstances (Johnston 1998; Goodwillie et al. 2005, 2010; Johnston et al. 2009). Johnston et al. (2009) developed a model that predicts scenarios where mixed mating is an evolutionarily stable mating strategy by explicitly considering the functional relationships among the three components of reproductive fitness: outcrossed ovules, selfed ovules, and ovules sired through successful pollen export. In these models, mixed mating may be an optimal strategy when there is a positive correlation between the number of outcrossed ovules and the number of selfed ovules, a situation that may occur with frequent geitonogamous pollen movement. By contrast, when populations experience seed discounting (a negative relationship between the number of outcrossed ovules and the num-

ber of selfed ovules) and pollen discounting, intermediate outcrossing rates are less likely (Johnston et al. 2009). To our knowledge, these relationships have not been quantified from field data because few studies have simultaneously obtained accurate estimates of male fitness, female fitness, and individual selfing rates (but see van Kleunen and Burczyk 2008; Stone et al. 2014).

Clarkia xantiana (Onagraceae) is an annual plant with a geographic range spanning the southern Sierra Nevada Mountains and Transverse Ranges of California (Eckhart and Geber 1999). The taxon contains two subspecies that differ in mating system: ssp. *xantiana* is primarily outcrossing and ssp. *parviflora* is primarily selfing, although both subspecies are self-compatible and mixed mating (Runions and Geber 2000; Pettengill and Moeller 2012). In a previous experiment, Moeller and Geber (2005) examined the ecological factors that drive population and subspecies divergence in mating system. In a field experiment where population size and community context (presence vs. absence of pollinator-sharing *Clarkia* congeners) were manipulated, it was found that small populations isolated from congeners suffered high pollen limitation. In addition, natural selection in those populations favored floral traits that facilitated autonomous selfing (i.e., reduced herkogamy and protandry) because selfing provided reproductive assurance (Moeller and Geber 2005). However, because this previous study examined only female fitness, inferences about selection on floral traits may not be robust.

In this article, we test whether patterns of selection via male fitness reinforce, counteract, or otherwise modify patterns of selection via female fitness. We also examined how variation in the magnitude of inbreeding depression modifies patterns of selection and influences mating system evolution. We ask the following questions: (1) Is there selection on floral traits through male fitness? (2) If so, is it similar or different in direction and magnitude to selection acting via female fitness? (3) Are patterns of selection via male fitness driven primarily by pollinator visitation and pollen export? (4) Is there evidence for pollen and/or seed discounting? (5) Do functional relationships among fertility components (selfed ovules, outcrossed ovules, and ovules sired by pollen export) predict stable intermediate outcrossing rates under pollen limitation?

Material and Methods

Floral Biology

Subspecies of *Clarkia xantiana* are both annuals with self-compatible flowers that are borne in racemes with one to hundreds of flowers. *Xantiana* has comparatively large flowers, herkogamy, and marked protandry. *Parviflora* has smaller flowers and little or no herkogamy and protandry (Eckhart and Geber 1999; Runions and Geber 2000; Moeller 2006).

Xantiana also has greater pollen:ovule ratios than *parviflora*, indicative of a difference in mating system (Delesalle et al. 2007; Delesalle and Mazer 2009). Within each taxon, there is population variation in floral traits and mating system (Runions and Geber 2000; Moeller 2006; Briscoe Runquist and Moeller 2014).

Self-fertilization occurs in both subspecies. In subspecies *xantiana*, selfing rates range among populations from 0.27 to 0.38 (mean = 0.32; Moeller et al. 2012). Selfing in *xantiana* occurs almost entirely because of geitonogamy and biparental inbreeding (Moeller et al. 2012). Selfing rates are challenging to estimate in *parviflora* because individual populations contain insufficient molecular variation to detect outcrossing events (Pettengill et al. 2016); however, selfing occurs readily at anthesis (prior or competing selfing) rather than following an episode where outcrossing can occur (Runions and Geber 2000; Moeller 2006). Inbreeding coefficients (F) estimated from seeds (not mature flowering plants) are significantly greater in *parviflora* (mean = 0.60) than *xantiana* (mean = 0.38), with no overlap in values for sampled populations (Pettengill et al. 2016). Inbreeding depression has not been quantified in *C. xantiana*. In other diploid *Clarkia* species, inbreeding depression has been measured in greenhouse and field common gardens, with moderate to strong values for outcrossing populations (~0.5–0.7) and weaker values for habitual selfers (~0.2–0.5; Holtsford and Ellstrand 1990; Holtsford 1996; Barringer and Geber 2008).

Manipulation of Phenotypic Variation

Exploiting population differentiation in floral traits within and between subspecies, we conducted crosses among five populations in an effort to create an experimental population with a broad phenotypic range, quasi-normal distributions of trait variation, and reduced correlations among floral traits. The broad phenotypic variance and reduced correlation among traits improve statistical power to detect selection and help distinguish among the possible targets of selection (Mitchell-Olds and Shaw 1987; Wade and Kalisz 1990).

We reciprocally crossed five populations of the two subspecies (four *xantiana* and one *parviflora*) in all combinations in one generation (resulting in 25 cross types) to generate experimental progeny whose phenotypes spanned the extremes of the two subspecies that occur naturally in zones of admixture but that also include intermediate phenotypes (for trait distributions, see Moeller and Geber 2005). The progeny of crosses between populations and subspecies do not exhibit hybrid sterility or inviability (Briscoe Runquist et al. 2014).

Experimental plants were grown in pots (164-mL containers; Stuewe and Sons, Corvallis, OR) at the University

of California–Davis and then transported to a shade house near field sites, where plants developed to flowering. Growing plants in a common garden before the field experiment minimized variation in plant size and vigor among individuals and allowed us to isolate the effects of the biotic environment on mating success. Plants were transported to and maintained in the field throughout the experiment. Following the experiment, plants completed development in the shade house.

Experimental Design

Our previous experiment included 10 experimental populations for which population size and community context were manipulated (Moeller and Geber 2005). The results of that work showed that (1) small populations had strongly reduced mean female fitness relative to large populations, despite having the same composition of genotypes (i.e., pollen limitation; see also Moeller 2004, where flowers in small populations suffered ~27% reduction in seed set relative to flowers receiving supplemental pollen); and (2) small populations that were isolated from pollinator-sharing congeners experienced selection via female fitness on herkogamy ($\beta = -0.17$) and protandry ($\beta = -0.16$). In small, isolated populations, female mating success was low for individuals with high protandry and herkogamy (and therefore a limited propensity to autonomously self). In all other treatments, there was no evidence of selection on floral traits via female fitness. Therefore, it was of interest to understand how variation in male fitness might reinforce or counteract selection via female fitness. Because of the extensive effort required to characterize male fitness, even in experimental populations, we have focused on one small, isolated experimental population where there was pollen limitation and selection on mating system traits.

A comprehensive description of field methods can be found from Moeller and Geber (2005), but we provide a brief overview here. The focal experimental population was placed in a natural field site in the Kern River Canyon (35°33'34.7" N, 118°34'44.3" W); this area falls within the center of the distribution of *xantiana*. The experimental population was isolated from naturally occurring conspecific and congeneric individuals by >200 m. Fifty potted plants were placed in multiple container racks (0.6 × 0.3 m) that were secured to the hillside; racks were spaced 0.6 m apart. The population size and density fall within the range of natural populations. Before the initiation of the field experiment, all open flowers were removed such that all flowers included in the experiment opened and senesced in the field. We measured four floral traits on two flowers per plant. Petal length and width were measured on the uppermost petal of each flower; width was measured at the widest part of the petal. Herkogamy was measured as the distance between the stigma and the closest an-

ther; the measurement was taken when the stigma surface was completely exposed. Dichogamy was quantified using daily records of individual flower development and was expressed as the difference in time between the onset of anther dehiscence (of the long anthers) and stigma receptivity (fully reflexed stigmas; Holtsford and Ellstrand 1992). There were significant correlations among floral traits. Petal size was positively correlated with protandry ($r = 0.67, P < .0001$) and herkogamy ($r = 0.68, P < .001$). Protandry was also positively correlated with herkogamy ($r = 0.57, P < .001$). There were no significant correlations between petal shape and any other floral trait. Variance inflation factors indicated that trait correlations did not seriously affect selection gradient analyses, because all estimates were below 2.5 (Hair et al. 1995). We also counted the total number of flowers per plant (mean \pm SE = 4.6 ± 0.23). This trait was not significantly correlated with petal area ($r = 0.05, df = 45, P = .72$), petal shape ($r = -0.11, df = 45, P = .46$), protandry ($r = 0.21, df = 45, P = .14$), or herkogamy ($r = 0.13, df = 45, P = .36$).

Clarkia xantiana is nearly exclusively pollinated by bees, particularly specialist solitary bees and bumblebees (Moeller 2005, 2006). Visitors to the experiment were very similar to those of natural populations. We quantified pollinator visitation on six consecutive days from the second through the seventh day of the experiment. During 1 h per day, two researchers recorded visits by bees to each rack of plants in six 10-min periods, keeping track of visitation to each individual plant. To examine the effect of display size on visitation, we divided the number of visits per 10-min observation period by the total number of fruits matured per plant. This metric correlated strongly with visits per plant, and the results were qualitatively the same using either response variable.

Female Fitness

Female mating success was estimated from only the flowers that opened in the field (mean \pm SE = 4.6 ± 0.23). We collected each fruit separately from a plant and counted the number of seeds per fruit. We also counted the number of unfertilized ovules from each fruit because the total number of ovules varied to some extent among genotypes. Because of variation in ovule number per fruit, we calculated female mating success as the fraction of ovules that fully developed into seeds. We expressed female fitness as seed set per flower by multiplying the estimate of seeds per ovule by the average number of ovules per fruit (to account for potential ovule number variation present in some populations) and then divided by the number of flowers displayed during the experiment. We were also interested in the proportion of female fitness that represented outcrossing events. To calculate female outcrossed fitness, we multiplied an individual's

total fitness (seeds/ovules) by the individual outcrossing rate ($1 - \text{selfing rate}$).

Male Fitness

We collected leaf tissue for DNA extraction from all parental individuals in the population. After the field experiment concluded, we collected all of the offspring of each individual. From each maternal pool of offspring, we randomly selected 10 seeds (or used all offspring when plants produced 10 or fewer) for genotyping. We evenly sampled 10 offspring per plant in order to obtain enough information to estimate a selfing rate for each individual. Selfing rate in this experiment (estimated on germinated seedlings) indicates a realized selfing rate (i.e., after possible early-acting inbreeding depression has been expressed). In the analyses, we also resampled offspring in proportion to each individual's total seed production. Offspring seed were germinated, and tissue was collected and frozen for DNA extraction. This resulted in 478 seedlings used for genotyping.

DNA was extracted using Qiagen kits, and DNA was quantified for polymerase chain reaction (PCR). Of 478 offspring, we successfully obtained tissue and high-quality DNA samples for 430 individuals. Of the 50 parents, we were able to successfully obtain high-quality DNA samples from 47 individuals. We amplified eight microsatellite loci (Moeller et al. 2011; also available on request) using PCR and conducted fragment analysis on an ABI 3730xl capillary instrument. The results of fragment analysis were scored using Peak Scanner Software v1.0 (Life Technologies). We assigned all alleles for each locus by eye. When necessary, we reamplified and regenotyped loci for individuals to confirm alleles. From most individuals, we were able to obtain complete or nearly complete genotype information; 93% of all individuals were genotyped at six or more loci, and 55% were genotyped for all eight loci. For a few individuals, PCR was challenging across all loci most likely because of plant chemistry, which is a common problem in *Clarkia*.

Statistical Analyses

Quantification of Male Fitness. We conducted paternity analyses using Cervus 3.0 (Kalinowski et al. 2007) to assign each of the 430 offspring to one of the 47 genotyped potential fathers. Analysis was restricted to individuals that had at least four loci genotyped to ensure inclusion of all possible parents (although most parents had complete genotype information). In the simulation used to generate critical values for paternity assignment, we included an average of 75% loci typed and a 1% mistyping rate. Inbreeding and self-fertilization were allowed in the simulation portion of the analysis. For this population, we included an average of 25% relatedness among parents and an average rate of inbreed-

ing of 40%. Selfing may have been particularly common in this population because individuals with minimal herkogamy and protandry had the highest female fitness (Moeller and Geber 2005). Because homozygosity can affect the likelihood of excluding paternity in some cases (homozygotes are more likely to be assigned), we explored the relationship between homozygosity and male fitness. We found no significant correlation between heterozygosity and fitness ($\rho = 0.009$).

The presence of null alleles in microsatellite data sets can influence paternity assignment and are detected typically by examining deviations from Hardy-Weinberg equilibrium. Given that our experimental population was composed of genotypes from multiple populations and included crosses among those populations, there is no reason to expect that our population should be in Hardy-Weinberg equilibrium (Wahlund effect). Nevertheless, we evaluated the frequency of null alleles in two ways. First, estimates of the proportion of null alleles by *Cervus* were all below 0.2 (mean = 0.13, range = 0.07–0.19); null allele frequencies below this value are not likely to strongly influence paternity assignment (Dakin and Avise 2004). We also compared all mother-offspring combinations to determine whether there were instances of offspring that failed to share an allele found in the mother, which would suggest a failure for the allele to amplify in the offspring. We found 22 instances of this among 2,749 locus \times mother combinations (0.8%). This suggests that the incidence of null alleles is infrequent and unlikely to have an important influence on paternity assignment.

Of the 430 genotyped offspring, 419 were typed at four or more loci, and of the 47 parents, 45 were typed at four or more loci (91% of the offspring by genotype matrix was filled). Of the 419 offspring included in the paternity analysis, 397 offspring had a mother that was genotyped at four or more loci (89% of the parent by genotype matrix was filled). Of these offspring, 319 were assigned paternity at the 80% confidence level. Of the 22 offspring with no maternal genotyping information in the analysis, 20 were assigned paternity using the likelihood analysis without a known mother. Offspring with identical genotypes to their mothers were assumed to be the result of selfing. This resulted in 341 offspring with paternity assignments that were used for all downstream analysis.

As described above, we evenly sampled offspring among maternal plants in the experiment (10 offspring per plant). Using all of those offspring with assigned paternity, we estimated a selfing rate for each individual maternal plant. For estimation of male fitness, it was essential to obtain a randomly sampled pool of offspring produced by the population as a whole. Therefore, we could not use the raw estimates established on the basis of the ~ 10 genotyped offspring per plant because this disproportionately equalizes fitness across plants. Rather, we used resampling to gener-

ate a pool of offspring where each plant's contribution to the pool is reflective of its total offspring production. To do this, we randomly resampled a fraction of the offspring at a frequency proportional to the number of seeds produced by the plant prior to conducting downstream analyses. We resampled 250 offspring 1,000 times to generate 1,000 estimates of offspring produced per plant in the population and then divided by the number of experimental flowers (an estimate of display size) to obtain 1,000 estimates of male fitness measured in offspring per flower. We then conducted each phenotypic selection analysis 1,000 times using the resampled data sets. All reported results were averaged from results of the 1,000 resampled data sets, and a coefficient was deemed significant if >95% of the P values fell below the $P = .05$ threshold. We conducted this process twice to verify the robustness of results from resampling, and they were qualitatively and quantitatively very similar.

Variance in Male and Female Fitness. Using a Levene's test, we tested a key prediction of Bateman's principle that variance in male fitness is greater than female fitness because females invest more heavily in fewer offspring, whereas males produce many inexpensive gametes.

Phenotypic Selection. We measured phenotypic selection via female fitness, male fitness, and combined fitness (female + male fitness). We also quantified the fraction of male and female fitness resulting from outcrossing to specifically identify trait variation that influenced pollen export and outcrossed pollen receipt. Last, we reanalyzed phenotypic selection via female fitness on our data set including only the 47 parents that had estimates of male fitness. We estimated net selection (differentials) and direct selection (gradients) from coefficients of simple and multiple linear regressions, respectively, of relative fitness on standardized floral traits, according to the approach of Lande and Arnold (1983). All analyses were conducted in R (R Development Core Team 2014) using the base statistics functions and the *car* package v. 2.0-21 (Fox and Weisberg 2011).

Female and male fitness were examined both for the total set of offspring (total fitness) as well as for the subset that were the result of outcrossing only (outcrossed fitness). Examining outcrossed fitness separately illuminates the trait values that facilitate pollinator-mediated pollen dispersal and receipt. It also represents a special case where inbreeding depression is one and selection on traits primarily reflects outcrossing success. Paternity assignment was used to subset offspring that were the product of outcrossing events from those that were the product of selfing events. Total female fitness was calculated (as described above) as the seeds per flower relativized to the population mean of seed number per flower. Total male fitness was expressed as the total number of seeds sired per flower by each indi-

vidual plant relativized by the population mean of total seeds sired per flower. Outcrossed female and male fitness was calculated for only the outcrossing component of mating success and relativized by the population mean for outcross success. Combined fitness was obtained by assigning each individual two fitness values, one for female fitness and one for male fitness (both measured in seeds/flower). We then summed those values and relativized them to the population mean. Combined fitness (male + female) was calculated for both total (selfed + outcrossed) fitness and the outcrossed component of fitness.

Floral traits were standardized to a mean of 0 and a standard deviation of 1. Because of strong correlations between petal length and petal width, we used principal component (PC) analysis to create two new uncorrelated petal variables. The first PC serves as a proxy for petal area and explains 50% of the variation. The second PC describes aspects of petal shape and also explains 50% of the variation; large values indicate long, thin petals, and smaller values indicate shorter, wider petals. We also tested for correlations among the four floral traits.

We ran all models using only linear or linear plus quadratic terms. When quadratic differentials or gradients were significant, we used nonparametric cubic splines to determine whether there was a fitness maximum or minimum within the range of phenotypic values in the population that might indicate stabilizing or disruptive selection. We used the `smooth.spline` function in R to fit the spline and estimate the smoothing parameter using log likelihood estimation (Schluter and Nychka 1994). For all quadratic gradients, we multiplied the regression coefficient by two to obtain the correct estimate of stabilizing/disruptive selection (Stinchcombe et al. 2008). In analyses of male fitness and combined fitness that included all offspring, we eliminated one high leverage data point that had a strong influence on tests of significance. This individual had floral trait values approximately 1 SD larger than the next largest individuals. Inclusion versus exclusion of the high leverage point did not change the qualitative relationships between variables but influenced only levels of significance.

Inbreeding Depression. The response to selection based on our observed patterns of mating will depend upon the fitness of selfed versus outcrossed offspring. Because we do not have an estimate of inbreeding depression for this population, we explored the sensitivity of our inferred selection coefficients (direction and significance of selection) to variation in the magnitude of inbreeding depression. We characterized how net and direct selection changed for combined (male + female) fitness when the fitness of selfed offspring was discounted from 0 to 1 by 0.1 increments for each of our traits. Appendix B (apps. A–C are available online) shows the results for petal area and protandry because these are the only

two traits for which patterns of selection varied in response to discounting the fitness of selfed offspring.

Pollinator Visitation. To assess the patterns of selection by pollinators, we regressed pollinator visitation rates to each individual plant against relativized trait values, in an analysis similar to the phenotypic selection analyses used for female and male fitness. We also tested for correlations between pollinator visitation and male and female fitness. Last, we parsed male and female fitness into fitness gained through outcrossing alone and tested for correlations with pollinator visitation. Correlations were tested using Spearman's rank correlation tests.

Fitness Correlations. We ascertained the relationship between male and female fitness, both total and outcrossed, using a Spearman's rank correlation test.

Functional Relationships between Selfed and Outcrossed Ovules. Following the model of Johnston et al. (2009), we visualized the functional relationships among fertility components to assess the potential ESS for this population. To characterize the relationships of Johnston et al. (2009), we calculated a standardized number of outcrossed ovules (n_x), selfed ovules (n_s), and ovules sired through pollen export (male outcrossing success; n_p) for each individual plant. Selfed and outcrossed ovules within a fruit were standardized to the number of total ovules in that fruit. To calculate a standardized value for ovules sired by outcrossing, we divided the number of ovules sired through outcrossing by the total number of offspring produced by an individual (via both male and female function). Values range from 0 to 1.

Three factors determine the evolutionarily stable mating strategy: (1) the relationship between the number of outcrossed (n_x) and selfed (n_s) ovules within a fruit, (2) the relationship between the number of selfed ovules (n_s) and ovules fertilized through pollen export (n_p), and (3) the magnitude of inbreeding depression of selfed ovules. For both relationships, we conducted multiple regressions including linear and quadratic terms. When the quadratic term was significant, we further explored the shape of the relationship using a nonparametric spline. The relationship between outcrossed and selfed ovules can reveal seed discounting, where self-fertilization of seeds usurps potentially outcrossed seeds. The presence of seed discounting is highly influential in determining the ESS (Johnston et al. 2009). When the relationship between outcrossed and selfed ovules is positive, individuals gain more outcross success with an increase in selfing (e.g., geitonogamous selfing is an unavoidable by-product of pollinator attraction, and there is no seed discounting). In this case, populations are likely to evolve stable intermediate selfing rates. If the relationship between outcrossed and selfed ovules is negative, the population exhibits seed discounting

and the parameter space for the evolution of stable intermediate selfing is greatly reduced.

The relationship between ovules sired by pollen export and selfed ovules can reveal the presence of pollen discounting if the two are negatively correlated. Pollen discounting has also been expressed in the literature as the relationship between the number of seeds sired by exported pollen versus the selfing rate (see app. C; e.g., Harder and Barrett 1995). In the presence of seed discounting, this relationship influences whether the stable mating system is fully outcrossing, fully selfing, includes both extremes, or mixed mating. When there is seed discounting without pollen discounting, a high rate of outcrossing is the most likely evolutionarily stable mating strategy. In the presence of seed and pollen discounting, the evolutionarily stable selfing rate can be intermediate (mixed mating) or predominantly selfing or outcrossing, depending on the level of inbreeding depression, which, in turn, depends on the segregating load of deleterious mutations (Johnston et al. 2009).

Results

Patterns of Variation in Male and Female Fitness

Individual selfing rates varied from completely outcrossing ($s = 0$) to completely selfing ($s = 1$). The distribution of selfing rates was right-skewed with the primary mode of 0.10. The mean selfing rate was 0.18, and the median was 0.10. Selfing rate was negatively correlated with petal area ($r = -0.62, P < .001$), protandry ($r = -0.52, P < .001$), and herkogamy ($r = -0.33, P = .03$); however, it was not significantly associated with petal shape ($r = 0.10, P = .51$).

Variance in male per-flower fitness was approximately 78% greater than variance in female per-flower fitness, al-

though this difference was not quite significant (Levene's test: $F_{1,90} = 3.33, P = .07$). For outcrossed fitness alone, the variance in male per-flower fitness was approximately 26% greater than the variance in female per-flower fitness, but the difference was not significant (Levene's test: $F_{1,90} = 1.47, P = .23$). Male and female per-flower fitness were significantly positively correlated for both total fitness (outcrossed + selfed) and outcrossed fitness alone (total: $\rho = 0.50, n = 46, P < .001$; outcrossed: $\rho = 0.34, n = 45, P = .02$).

Selection Via Female Fitness

Total Fitness. Net selection differentials on protandry and herkogamy via female fitness were negative, favoring reduced trait values (tables 1, 2; fig. 1). There was a significant positive quadratic differential and gradient on protandry, indicating net and direct disruptive selection. Net selection on petal area was also significantly disruptive, but direct selection was positive and linear (table 1; fig. 1).

Outcrossed Fitness. When considering only offspring sired through outcrossing, there was net and direct positive selection on petal area (tables 1, 2; fig. 2). Direct selection was negative on herkogamy. For details on patterns of selection via selfed fitness, see appendix A.

Selection Via Male Fitness

Total Fitness. Selection via male fitness was evident on petal area and protandry. Net and direct selection on petal area

Table 1: Selection differentials from simple linear regression models

	Petal area			Petal shape			Protandry			Herkogamy		
	S alone	S + C _{ii}		S alone	S + C _{ii}		S alone	S + C _{ii}		S alone	S + C _{ii}	
		S	C _{ii}		S	C _{ii}		S	C _{ii}		S	C _{ii}
Total fitness:												
Female	-.13	-.06	.34	.10	.11	-.06	-.21	-.16	.44	-.21	-.24	.16
Male	.13	.48	1.00	.17	.23	.22	-.09	.01	.64 ^b	.09	.09	-.02
Combined	-.02	.22 ^a	.68	.13	.14	.04	-.16	-.07	.52	-.06	-.07	.06
Outcrossed fitness:												
Female	.21	.21 ^a	-.02	.01	.07	.02	.11	.12	.1	.01	.01	.00
Male	.55	.79	.64 ^b	.06	.15	.34	.24	.28	.20	.42	.42	.00
Combined	.37	.46	.28	.05	.08	.12	.16	.19	.14	.20	.20	.04
Pollinator visitation	.42	.47	.26	.22 ^a	.19	.17	.07	.03	-.19	.31	.31	-.37

Note: Differentials were determined using female fitness, male fitness, and combined fitness (male + female). Models included either only linear (S) regression coefficients or linear (S) and quadratic (C) regression coefficients. Selection differentials are calculated on the basis of total fitness (outcrossed + selfed; inbreeding depression = 0), outcrossed fitness (inbreeding depression = 1), and pollinator visitation. Values in bold are significant at $P < .05$.

^a Values with mean $P < .05$ and 95% of $P < .1$.

^b Values that are not quite significant ($.1 < P < .05$).

Table 2: Selection gradients from multiple linear regression models

	Petal area			Petal shape			Protandry			Herkogamy		
	β alone	$\beta + \gamma_{ii}$		β alone	$\beta + \gamma_{ii}$		β alone	$\beta + \gamma_{ii}$		β alone	$\beta + \gamma_{ii}$	
		β	γ_{ii}		β	γ_{ii}		β	γ_{ii}		β	γ_{ii}
Total fitness:												
Female	.11	.39	-.10	.10	-.01	-.12	-.21 ^a	-.26 ^a	.66	-.14	-.24 ^a	.00
Male	.34	.84	.28	.26	.15	.08	-.38	-.30	1.02 ^b	.12	-.10	1.84
Combined	.20	.61	.12	.16	.09	.02	-.28 ^a	-.25	.80 ^b	-.01	-.19	-.10
Outcrossed fitness:												
Female	.40	.65	-.44	.03	.05	-.12	-.02	-.13	.56 ^a	-.27 ^a	-.35	.36
Male	.69	1.08	.14	.20	.14	-.02	-.23	-.18	.68	.06	-.11	.06
Combined	.56	.84	-.16	.13	.10	-.06	-.11	-.15	.62	-.14	-.24	.22
Pollinator visitation	.62	.58	.09	.31	.32	.00	-.51	-.50	-.17	.17	.18	-.10

Note: Gradients were determined using female fitness, male fitness, and combined fitness (male + female). Models included either only linear (β) regression coefficients or linear (β) and quadratic (γ) regression coefficients. Selection gradients are calculated on the basis of total fitness (outcrossed + selfed; inbreeding depression = 0), outcrossed fitness (inbreeding depression = 1), and pollinator visitation. Values in bold are significant at $P < .05$.

^a Values with mean $P < .05$ and 95% of $P < .1$.

^b Values that are not quite significant ($.1 < P < .05$).

was positive (tables 1, 2; fig. 1), and there was a significant positive quadratic selection differential for petal area, indicating disruptive selection (table 1; fig. 1). Cubic spline analyses verified that male fitness had a minimum approximately 0.29 SDs below the population mean (size: smoothing parameter = 1, $\lambda = 0.004$, $df = 4.8$; fig. 1). Direct selection on petal area was significantly positive and linear (table 2). Net and direct selection on protandry via male fitness was disruptive, with a fitness minimum at approximately 1.4 SDs above the mean (tables 1, 2).

Outcrossed Fitness. For outcrossed male fitness, direct and net selection was positive on petal area (tables 1, 2; fig. 2). There was also significant positive net selection (but not direct selection) on herkogamy (table 1; fig. 2).

Selection Via Combined (Male + Female) Fitness

Total Fitness. A significant positive quadratic differential on petal area via combined male and female fitness indicated selection for extreme petal values; this pattern reflects significant disruptive selection via both female and male fitness (tables 1, 2; fig. 1). This pattern was confirmed by cubic spline analysis (local minimum 0.34 SDs below the population mean; smoothing parameter = 0.90, $\lambda = 0.002$, $df = 5.7$; fig. 1). There was also positive direct selection for larger petal area, again reflective of patterns of both female and male fitness (table 2).

For mating system traits, there was a significant positive quadratic selection differential for protandry, primarily reflecting selection via female fitness (table 1). There was no

significant direct or net selection on herkogamy, despite our observation of selection via female fitness alone.

Outcrossed Fitness. Considering only outcrossed progeny, there was significant and positive net and direct selection on petal area via combined fitness; this was due to the fact that selection via outcrossed fitness through both sex functions favored larger petals (tables 1, 2; fig. 2). Unlike patterns inferred from analysis of all offspring (total fitness), there was no evidence of disruptive selection on floral traits through outcrossed fitness (tables 1, 2). There was no selection on mating system traits via combined outcross success.

Inbreeding depression. For petal area and protandry, net and direct selection coefficients changed significantly between analyses that included all offspring and those that included only outcrossed offspring, indicating that these traits were especially sensitive to potential inbreeding depression. For petal area, there was significant net and direct positive selection on petals at inbreeding depression values of 0.6–1 and 0.5–1, respectively (fig. B1; figs. A1, B1, C1 are available online). Net quadratic differentials were positive (disruptive selection) on petal area and protandry when inbreeding depression ranged from 0 to 0.8 and 0 to 0.6, respectively. There was no significant selection on herkogamy via combined fitness, with or without inbreeding depression.

Pollinator Visitation

Visitation rates to plants increased with petal area when considering petal area alone and when accounting for all other traits (tables 1, 2). When accounting for all other traits, visi-

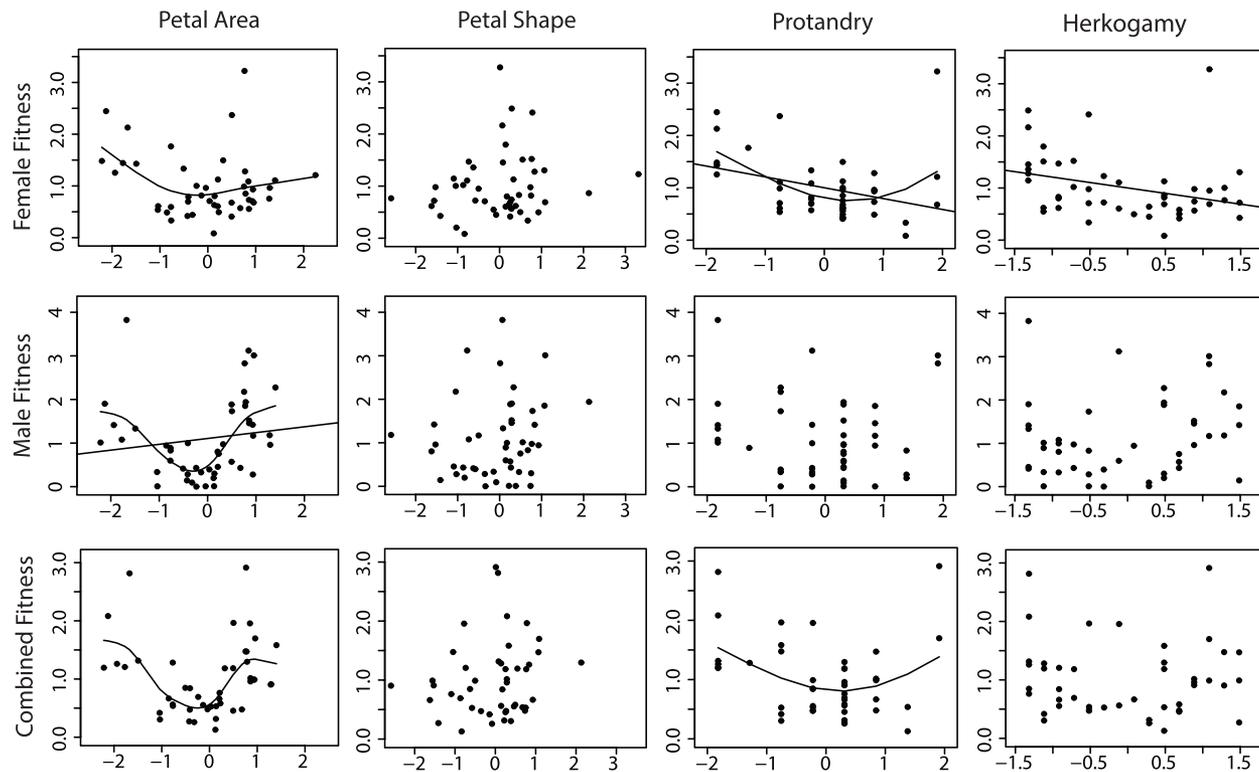


Figure 1: Phenotypic selection differentials for four floral traits using total fitness (outcrossed + selfed offspring). This is the case in which there is no inbreeding depression. Each panel shows the relationship between relative fitness and standardized trait values for female fitness (first row), male fitness (second row), and combined fitness (male + female; third row). Only significant linear or curvilinear relationships are visualized in panels. Curvilinear relationships shown are the results of spline analyses. All panels that involve fitness through male function are means of 1,000 resampled offspring data sets generated to produce a random sample of offspring (for details, see “Methods” and “Results”).

tation was also higher on plants with narrower petals and reduced protandry (table 2). Pollinator visitation was a significant predictor of outcrossed male fitness (outcrossed fitness: $\rho = 0.41$, $P < .01$) and not quite significant predictor for total (outcrossed + selfed) male fitness (total fitness: $\rho = 0.28$, $P = .06$). For female fitness, pollinator visitation was not a significant predictor of either total female fitness or outcrossed female fitness (total: $\rho = -0.004$, $P = .98$; outcrossed: $\rho = 0.21$, $P = .17$).

Relationships among Fertility Components

Within a fruit, the number of outcrossed ovules was negatively correlated with the number of selfed ovules, indicative of seed discounting ($\rho = -0.51$, $P < .001$; fig. 3A). There was no significant quadratic relationship ($\gamma = 0.11$, $P = .88$). For all fruits, only a fraction of ovules were fertilized; therefore, the proportion of ovules selfed and outcrossed within a fruit never summed to 1.

The relationship between outcrossed ovules sired through pollen export and selfed ovules was significantly quadratic, but the linear term was not significant (linear: $\beta = 1.6$, $P = .10$; quadratic: $\gamma = -3.66$, $P = .03$). A nonparametric

spline revealed a positive relationship when there were few selfed ovules and a negative relationship when the proportion of selfed ovules exceeded 0.14 (smoothing parameter = 1, $\lambda = 0.003$, $df = 4.28$; fig. 3B). The latter relationship indicates pollen discounting at selfing rates higher than 14% (see also app. C).

We assessed functional relationships among fertility components for a potential mating system ESS, as per the Johnston et al. (2009) model. In our population, we observed seed discounting and accelerating pollen discounting (greater discounting at higher selfing rates), which bears closest resemblance to the scenario shown in figure 5E of Johnston et al. (2009). In this scenario, under low segregating genetic load, the evolutionarily stable state is predominant but not complete selfing. Under higher segregating genetic load, the stable state is either complete outcrossing or predominant but not complete outcrossing.

Discussion

Variation in male fitness is central to many theoretical models of floral and mating system evolution, in part be-

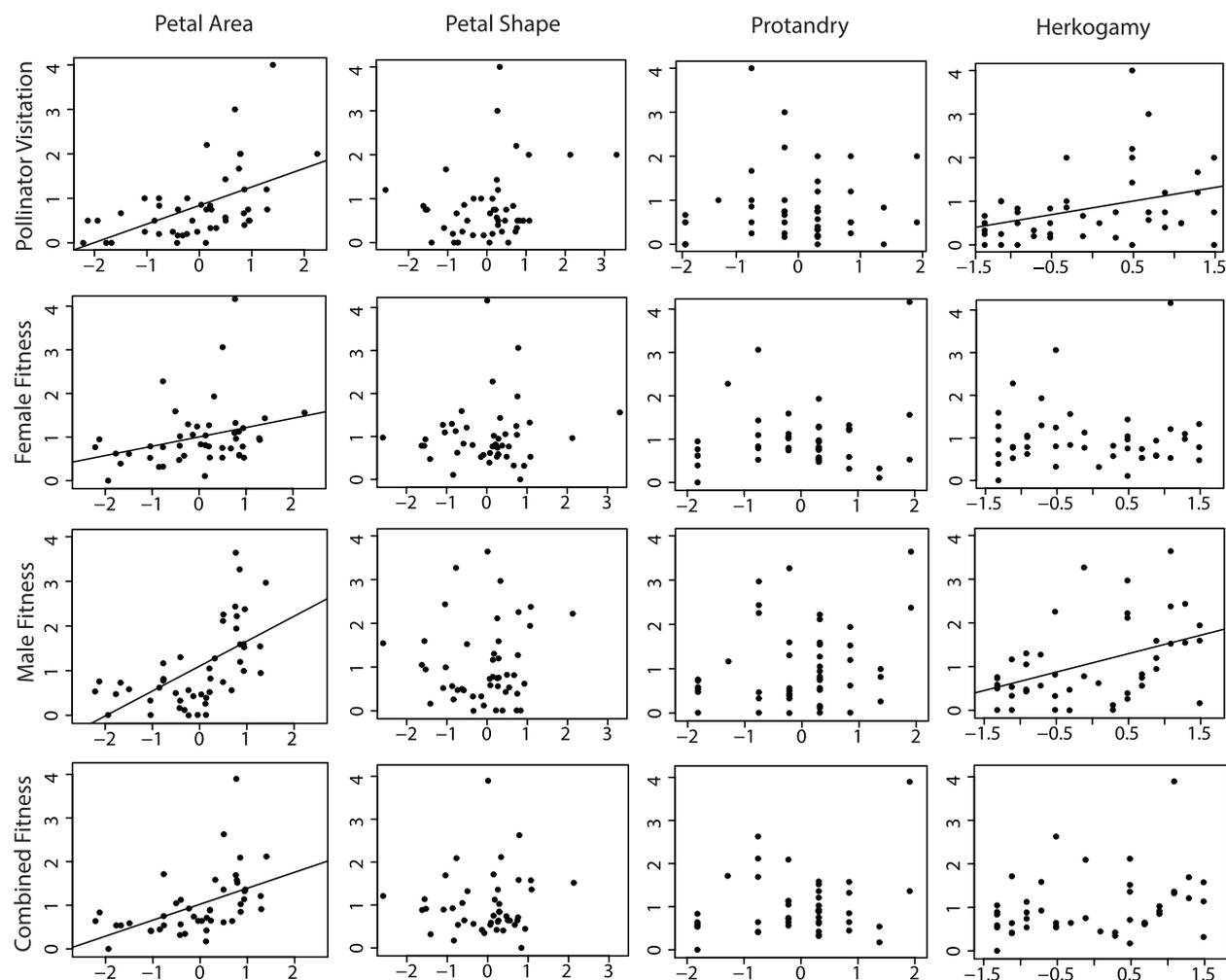


Figure 2: Phenotypic selection differentials for four floral traits using outcrossed fitness alone. This is the case in which inbreeding depression is complete and equal to 1. Each panel shows the relationship between relativized fitness and standardized trait values for pollinator visitation (first row), female fitness (second row), male fitness (third row), and combined fitness (male + female; fourth row). Only significant linear or curvilinear relationships are visualized in panels. Curvilinear relationships shown are the results of spline analysis. All panels that involve fitness through male function are means of 1,000 resampled offspring data sets generated to produce a random sample of offspring (for details, see “Methods” and “Results”).

cause the opportunity for selection via male fitness can be relatively high (Bateman 1948; Bell 1985; Stanton et al. 1986). Although there are now many studies of natural selection on floral traits, most predict the direction and extent of phenotypic evolution via only female fitness. Those that do consider male fitness typically consider proxies for it rather than direct measures (but see Devlin and Ellstrand 1990; Conner et al. 1996; Elle and Meagher 2000; Morgan and Conner 2001; van Kleunen and Ritland 2004; Wright and Meagher 2004; Lau et al. 2008; van Kleunen and Burczyk 2008; Sahli and Conner 2011; Kulbaba and Worley 2012; Austen and Weis 2016). Thus, the extent to which the pattern of selection via male and female function are parallel versus conflicting and the effect of male fitness

on predicted patterns of mating system evolution remain poorly characterized (Delph and Ashman 2006; Johnston et al. 2009). In this study, we examined selection via male fitness in an experimental population where pollen limitation to seed set was strong (with ~27% fewer seed in open pollinated flowers relative to flowers that received supplemental pollination), and selection via female fitness favored traits that increased selfing rates. We found that patterns of selection via female and male fitness were often reinforcing or noninterfering. Notably, we detected disruptive selection on both a secondary sexual attractive trait (petal area) that influences pollinator attraction and a mating system trait (protandry) that influences the likelihood of autonomous selfing. On the basis of patterns of correlations among ferti-

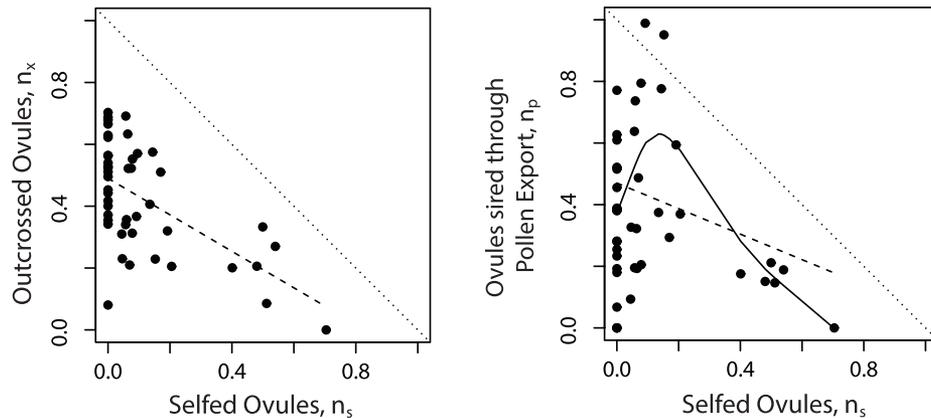


Figure 3: Functional relationships between selfed and outcrossed ovules. *Left*, relationship between the standardized number of outcrossed ovules within a fruit, n_x , and the standardized number of selfed ovules, n_s . Dotted line indicates the 1:1 and cannot be exceeded in this panel. *Right*, relationship between the standardized number of ovules sired through pollen export, n_p , and the number of selfed ovules, n_s . In both panels, solid line represents the spline relationship, and dashed line represents the linear relationship between the variables.

ity components, our results suggest that the ESS for this population depends on the level of inbreeding depression generated by the segregating load of deleterious mutations (Johnston et al. 2009). Under intermediate load, mixed mating is favored, but high and low load favor complete outcrossing and predominant selfing, respectively.

Reinforcing Patterns of Selection

One of the more striking findings of our study was strong disruptive selection on petal area, where small and large flowers gained significantly greater fitness than intermediate phenotypes. This pattern of selection was evident from both male and female fitness. Parsing selection between outcrossed and selfed offspring revealed the underlying cause of this pattern: (1) larger flowers significantly promoted outcrossing through both pollen import and export, whereas (2) small flowers significantly promoted selfing, presumably autonomous selfing (see Moeller 2006). This pattern of disruptive selection would have not have been evident without manipulating the phenotypic distribution of floral traits to produce phenotypes intermediate between the two optima found in nature and that suffered reduced fitness.

Although petal area was under selection through both male and female function, selection was strongest via male fitness. This finding is consistent with sexual selection theory and previous empirical studies in plants indicating that secondary attractive features of flowers enhance siring success through greater pollinator attraction and pollen export to a greater extent than seed set (Bell 1985; Stanton et al. 1986; Campbell 1989; Wilson 1995; Morgan and Schoen 1997; O'Connell and Johnston 1998; Ashman and Diefenderfer 2001). Indeed, in our study, individuals with larger petals had higher pollina-

tor visitation and outcross siring success. These results suggest that secondary sexual characteristics may not be directly selected during the evolution of selfing (reviewed in Sicard and Lenhard 2011). Instead, the small flowers characteristic of selfers (Goodwillie et al. 2010) likely evolve because they provide little function and are less costly to maintain.

Noninterfering Patterns of Selection

Mating system traits (dichogamy and herkogamy) were largely subject to noninterfering selection, where selection operated through one gender function but not the other. Selection on protandry was stronger via female than male fitness, but inclusion of both did not strongly change predictions about the overall pattern of selection. Overall, disruptive selection on protandry was caused primarily by disruptive selection via female function; quadratic gradients for male fitness were positive (disruptive) but not quite significant. Disruptive selection appears to arise from two different phenomena: (1) individuals with low protandry were successful at autonomous selfing (see Moeller 2006), and (2) individuals with high protandry had higher rates of geitonogamous selfing (but not higher outcrossed male fitness). The latter is apparent in the analyses of selection based on selfed offspring (app. A), where patterns of selection were disruptive. Individuals with greater protandry tended to have longer-lived flowers and therefore more flowers open in an inflorescence at a given time (D. A. Moeller, personal observation). Larger displays may result in more pollinator movement among flowers within inflorescences and therefore greater geitonogamous selfing (e.g., Eckert 2000; Karron et al. 2004), especially since flowers develop sequentially in inflorescences (asynchronous dichogamy) rather than simultaneously (Harder

et al. 2004; for an example of synchronous dichogamy, see Bhardwaj and Eckert 2001). More field experiments are needed to examine the relationship between the protandry of individual flowers, display size, and the probability of geitonogamous selfing. Given that both extremes of protandry were favored through increased production of selfed offspring, it follows that if inbreeding depression is high (>0.6), disruptive selection is not observed. This is particularly interesting because the threshold of inbreeding depression where disruptive selection disappears is lower for protandry (0.6) than petal area (0.8), where high values of petal area more strongly promote outcrossing than selfing.

Herkogamy experienced negative selection through female fitness, but selection was not detected for combined fitness (male + female). These results have implications for mating system evolution, given that reduced herkogamy is considered a key factor facilitating higher rates of autonomous selfing by placing anthers and stigmas in close physical proximity (Sicard and Lenhard 2011). When we parsed mating success into outcrossed versus selfed success, there was an apparent trade-off between how herkogamy affected female versus male fitness. Reduced herkogamy positively affected female fitness by increasing autonomous selfing (app. A), whereas increased herkogamy increased outcrossed siring success. The latter may occur because greater herkogamy minimizes interference between sexual functions: when herkogamy is reduced, selfing may readily occur, but this impedes a flower's potential to export pollen. This trade-off between self-pollen deposition and pollen export is indicative of pollen discounting (Nagyaki 1976; Lloyd 1979), and our analysis suggests that variation in herkogamy may be the primary trait involved in the negative relationship we observed between outcross siring success and selfing rate (for values of selfing between 0.2 and 1; fig. C1).

Evaluating Our Results in Light of the Johnston et al. Model

Few empirical studies have tested Johnston et al.'s (2009) model of mating system evolution by assessing functional relationships between fitness gained through selfed ovules, outcrossed ovules, and ovules sired by pollen export. The model predicts that stable intermediate selfing most often evolves when seed discounting is absent. When seed discounting is present, the evolutionarily stable state is dependent on the shape of the seed discounting and pollen discounting relationships and on the segregating load of deleterious mutations that contribute to inbreeding depression.

Insofar as the functional relationships apparent in our experimental population are applicable to natural populations, they provided two key insights. First, seed discounting is evident in the negative relationship between outcrossed and selfed ovules (fig. 3A). Second, the relationship between outcross siring success and selfed ovules varied across the range

of observed selfing (fig. 3B). For highly outcrossing individuals, a bit of selfing was associated with increased pollen export. This kind of phenomenon has been observed when larger inflorescences promote greater pollinator visitation and pollen export but come at the cost of some geitonogamous selfing (Barrett 2003; Lau et al. 2008; Karron and Mitchell 2012). For individuals where autonomous selfing is more common, pollen export was comparatively reduced (fig. 3B).

The complex relationship between ovules sired via pollen export versus selfed ovules that we found in our experimental population was not explored by Johnston et al. (2009). However, it represents a type of acceleration in pollen discounting, which would allow for the evolution of intermediate selfing rates, depending on the level of inbreeding depression that is driven by the segregating load of deleterious mutations. At intermediate inbreeding depression and segregating loads (high genomic mutation rate to—and intermediate dominance of—deleterious mutations), a mixed mating system is stable. Given F values that have been measured in *Clarkia xantiana*, inbreeding depression may be sufficiently weak to allow for stable intermediate outcrossing rates. However, caution is needed because a high inbreeding depression and segregating load (high mutation rate and low dominance of deleterious mutations) favor complete outcrossing, while a low inbreeding depression and load lead to predominant selfing.

Inbreeding Depression and Patterns of Selection

Although our study examines patterns of mating in detail, we did not simultaneously quantify the magnitude of inbreeding depression in the field for this experimental population. The strength of inbreeding depression is a key factor opposing the evolution of selfing (Lande and Schemske 1985) and responses to selection on quantitative traits (Willis 1996). In lieu of a specific estimate of inbreeding depression, we explored how variation in inbreeding depression (discounting selfed offspring success from 0 to 1 in 0.1 intervals) modifies responses to the patterns of selection. Since the strength of inbreeding depression is often context dependent (Cheptou and Donohue 2011), this approach provides information that is applicable to a range of potential conditions. We reanalyzed petal area and protandry because these two traits experienced selection via combined (male + female) and total (outcrossed + selfed) fitness.

For petal area, if inbreeding depression exceeded 0.8, the pattern of selection was directional rather than disruptive and more similar to the results of selection via outcrossed offspring alone (fig. B1). This level of inbreeding depression is greater than the range that has been estimated for other predominantly outcrossing diploid *Clarkia* (0.5–0.7: Holtsford and Ellstrand 1990; Holtsford 1996;

Barringer and Geber 2008). For protandry, if inbreeding depression exceeded 0.6, the signal of net disruptive selection was no longer significant; however, direct selection remained significantly disruptive for all values of inbreeding depression (fig. B1). An inbreeding depression value of 0.6 is within the range estimated for other *Clarkia*. For herkogamy, there was no selection via combined fitness in the presence or absence of inbreeding depression. Overall, these results suggest that if inbreeding depression is strong, it could hinder the evolution of selfing under this degree of pollen limitation.

Conclusions

By manipulating trait variation in an experimental population, we generated a distribution of floral phenotypes on which we measured relationships between and patterns of trait selection through male and female fitness under pollen limitation. We used our findings and the Johnston et al. (2009) model to evaluate the ESS mating system on the basis of the strength of pollen discounting, seed discounting, and inbreeding depression. Our results suggest that evolutionarily stable mating system is mixed, if inbreeding depression and the segregating load of deleterious mutations are intermediate. Predominant outcrossing or predominant selfing are stable at high and low inbreeding depression, respectively. Our results are consistent with patterns of trait and mating system variation in nature, where populations are mixed mating but primarily outcrossing or selfing (Eckhart and Geber 1999; Pettengill and Moeller 2012; Pettengill et al. 2016).

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Left, highly selfing subspecies of *Clarkia xantiana*, *C. xantiana parviflora*. Right, highly outcrossing subspecies of *C. xantiana*, *C. xantiana xantiana*. Photo credit: David A. Moeller.