

EFFECTS OF HERBIVORY AND INBREEDING ON THE POLLINATORS AND MATING SYSTEM OF *MIMULUS GUTTATUS* (PHRYMACEAE)¹

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Most models of mating system evolution predict mixed mating to be unstable, although it is commonly reported from nature. Ecological interactions with mutualistic pollinators can help account for this discrepancy, but antagonists such as herbivores are also likely to play a role. In addition, inbreeding can alter ecological interactions and directly affect selfing rates, which may also contribute to maintaining mating system variation. We explored herbivore and inbreeding effects on pollinator behavior and selfing rates in *Mimulus guttatus*. First, individual spittlebug (*Philaenus spumarius*) herbivores were applied to native plants in two populations. Spittlebugs reduced flower size, increased anther–stigma distance, and increased selfing rates. A second experiment factorially crossed spittlebug treatment with inbreeding history (self- vs. cross-fertilized), using potted plants in arrays. Spittlebugs did not affect pollinator behavior, but they reduced flower size and nearly doubled the selfing rate. Inbreeding reduced the frequency of pollinator visits and increased flower-handling time, and this may be the first report that inbreeding affects pollinator behavior. Selfing rates of inbred plants were reduced by one half, which may reflect early inbreeding depression or altered pollinator behavior. The contrasting effects of herbivory and inbreeding on selfing rates may help maintain mating system variation in *M. guttatus*.

Key words: California; herbivory; herkogamy; inbreeding; mating system; *Mimulus guttatus*; Phrymaceae; pollinator behavior.

Most models of mating system evolution in plants predict mixed mating (production of both self- and cross-fertilized seeds) to be unstable, although it is commonly observed in natural populations (Vogler and Kalisz, 2001). Therefore, research into the evolution of plant mating systems has often focused on identifying the origins and maintenance of variation in selfing rates (Charlesworth et al., 1990; Uyenoyama et al., 1993; Barrett, 2003).

One important source of variation in selfing stems from the behavior of pollinators (Barrett, 2003). Insects often visit more flowers on plants with larger floral displays, which can increase selfing rates via geitonogamy (Karron et al., 2004). Similarly, isolated plants often produce more selfed offspring (Karron et al., 1995). When pollinators are scarce or unreliable, self-fertile plants can increase their reproductive output by maturing autogamously self-pollinated seeds (Kalisz et al., 2004). Localized foraging patterns can exact a fitness cost associated with selfing due to reduced pollen export (Fishman, 2000), and this cost can lead to mixed mating (Holsinger, 1991).

In addition to mutualistic pollinators, antagonists such as herbivores are also likely to influence mating system dynamics, although their effects are poorly understood. Herbivores are often the most important environmental variable affecting plant fitness (Marquis, 1992), and they can shape selection on a wide variety of plant traits, including reproductive traits (Marquis, 1992). Herbivore damage is often found to alter pol-

linator behavior (Strauss, 1997; Steets and Ashman, 2004), which may indirectly influence the mating system. Herbivores can also alter the expression of inbreeding depression (Carr and Eubanks, 2002; Hayes et al., 2004; Ivey et al., 2004), which is a primary factor opposing the advantages of selfing (Charlesworth et al., 1990; Uyenoyama et al., 1993). Therefore, the scant attention paid to the influence of antagonistic interactions on plant mating systems seems conspicuous (but see Agrawal and Lively, 2001; Ashman, 2002).

Inbreeding can contribute to mating system variation as well. For example, the selfing rates of inbred progeny have been found to be lower than those of their outbred siblings in some crops (Link, 1990; Damgaard and Loeschcke, 1994). Similarly, greenhouse experiments have reported lower autogamous seed set for the inbred progeny of mixed mating species (Jain, 1978; Karoly, 1994). Thus, some of the traits underlying selfing and autogamy rates can be subject to directional dominance, and inbreeding may result in a change in mating system expression. In addition, inbreeding can alter traits associated with pollinator attraction, such as flower number and size (Wyatt, 1988; Karoly, 1994), pollen production (Carr and Dudash, 1996), or nectar volume (Ouborg et al., 2000). These changes may alter pollinator behavior in ways that can affect mating patterns, including selfing rates (Kalisz et al., 2004; Karron et al., 2004).

Here we describe two experiments to identify sources of variation in the mating system of *Mimulus guttatus* DC (Phrymaceae). The first (“Native plants”) involved native plants in two natural populations and considered the effect of herbivory on floral traits and selfing rates. The second (“Experimental arrays”) involved potted plants of known heritage placed in field arrays, and this explored the effects of both inbreeding and herbivory on floral traits, pollinator behavior, and selfing rates. Together, the results of these experiments addressed the following questions: (1) What is the effect of herbivory on floral traits and pollinator behavior? (2) What is the effect of

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inbreeding on floral traits and pollinator behavior? (3) Can selfing rates be altered by herbivory or inbreeding?

MATERIALS AND METHODS

Study organisms—*Mimulus guttatus* is an herb native to open, wet habitats throughout much of western North America. It produces one to many perfect yellow flowers that are typically paired at nodes along erect racemes. Each flower can produce several hundred seeds. *Mimulus guttatus* is an annual, except in sites that remain wet year-round, where it can persist throughout the year and spread rhizomatously (Dole, 1992). *Mimulus guttatus* population sizes can fluctuate greatly through both time and space, and they are subject to frequent extinction and recolonization events (Vickery, 1999), suggesting that founder effects, bottlenecks, and inbreeding commonly influence its ecology and evolution. The mating system of *M. guttatus* is highly variable; population-level outcrossing rates range from about 75% selfing ($t = 0.25$) to complete outcrossing ($t = 1.0$), with a species mean $t \approx 0.6$ (Ritland and Ritland, 1989; Dudash and Ritland, 1991; Willis, 1993). Inbreeding depression is commonly observed in *M. guttatus* (Carr and Dudash, 1996; Carr and Eubanks, 2002; Ivey et al. 2004). Voucher specimens of plants from each population studied herein are housed in the Illinois Natural History Survey herbarium (ILLS).

The meadow spittlebug, *Philaenus spumarius*, is a widespread generalist herbivore that feeds on xylem (Weaver and King, 1954). Spittlebugs were the most abundant herbivore species on *M. guttatus* in our coastal California study sites. Infested plants typically host 1–2 spittlebug nymphs (C. Ivey and D. Carr, personal observation). A single spittlebug nymph can exact significant costs to the survival and reproductive success of *M. guttatus* in the field (Ivey et al., 2004). The spittlebugs used in these experiments originated from one population in Marin County, California, USA (38°07' N, 122°56' W).

Native plants experimental design—Forty pairs of plants were marked prior to flowering at each of two *M. guttatus* populations located along stream margins in Napa County, California, USA (population A: 38°42' N, 122°24' W; population B: 38°34' N, 122°15' W) in April 2003. Plants were paired by size, apparent developmental stage, and proximity (members of a pair were <50 cm apart). One member of each pair was assigned by coin toss to receive a single spittlebug nymph. Plants were monitored daily during the three weeks of our field season to ensure that spittlebugs remained on plants. Once a feeding site has been established, however, spittlebug nymphs typically remain on a single plant until emergence as an adult (C. Ivey and D. Carr, personal observation).

Native plants experiment data collection—At population B, we measured corolla width (at the widest point), corolla tube length (from the base of the calyx to the distal end of the tube), corolla size (the product of corolla length and width), and the minimum distance between anthers and stigma (AS) on the youngest fully expanded flower on the primary inflorescence branch of each plant. We were unable to collect these data at population A because the plants had not flowered before our field season ended in late May 2003. We returned in late June to collect seeds from both sites. Fruits from each experimental plant were collected in individual coin envelopes, and all seeds from each plant were combined (i.e., individual fruits were not distinguished) prior to sampling for selfing rate estimates (see Materials and Methods, Mating system estimation).

Experimental arrays design—In June 2001, open-pollinated seeds from 55 *M. guttatus* plants were collected from population M13W (38°33' N, 122°22' W), which was located in a roadside ditch in Napa County, California. During fall 2001, a single, randomly chosen plant from each seed family was grown in a pollinator-free greenhouse at the Blandy Experimental Farm in Boyce, Virginia, USA, and flowers that had been emasculated in bud were hand-pollinated to produce self and full-sib outcross progeny following the methods of Carr and Eubanks (2002). To produce outcross seeds, flowers on maternal plants were randomly paired with sires, each sire serving only once as pollen donor. In March 2003, several seeds from each inbreeding treatment (cross-

or self-fertilized) within each family were sown into separate randomly positioned pots in a California greenhouse. After germination, 16 families were randomly chosen and two seedlings from each inbreeding treatment within each family ($n = 64$ plants) were randomly chosen and transplanted into individual 71-mm pots. In mid-April, as plants began to bolt, one plant from each inbreeding treatment within each family was randomly assigned to receive a single spittlebug nymph. This resulted in a factorial design with two levels of inbreeding (self and outcross) and two levels of herbivory (spittlebug and control).

Plants were divided into four circular arrays of 16 plants each. Within each array, each of the 16 families was represented only once to minimize biparental inbreeding and thereby maximize the power of our selfing rate analysis (see Materials and Methods, Mating system analysis). Each inbreeding \times herbivore treatment combination was replicated four times within an array, and plants were arranged such that each plant was bordered by both inbreeding treatments as well as both herbivory treatments. This design should have maximized the opportunity for pollinators to choose among treatments. Arrays were placed in wading pools at the corners of a square (150 m per side) in an abandoned pasture in Napa County, California. Arrays were rotated among pools every 2 d to minimize the effects of local variation in pollinator activity and other environmental factors. No known native population of *M. guttatus* occurred within 3 km.

Experimental arrays data collection—We recorded the number of open flowers each day pollinators were observed (“floral display”). In addition, we measured corolla width, corolla length, corolla size, and AS on one fully expanded flower on each plant as before. We also recorded the total number of flowers produced by each plant throughout the experiment (“total flowers”) and the number of fruits with seeds (“total fruits”) produced by these flowers. Seed production was estimated by spreading each plant’s seed crop evenly over a 77 cm² rectangular grid, randomly choosing 10 individual 1-cm² cells, and counting seeds within these squares (sensu Leclerc-Potvin and Ritland, 1994). Mean seed number within the subsampled cells was multiplied by 77 to estimate the total number of seeds produced by open flowers during the experiment (“total seeds”).

Over the 14 d of peak flowering for the plants, we recorded the behavior of floral visitors for 30 min at each array, moving among arrays until each observer had watched all arrays each day (total 30 h of observation). On each of the seven observation days, 1–3 observers collected behavioral data. We recorded the number of insects arriving to forage (visit defined by head entering corolla tube), number of flowers visited per plant, and time foraging per plant. In addition, we recorded the identity of visitors to order (Lepidoptera and Coleoptera), family (most Diptera and Hymenoptera), or genus (*Bombus* and *Apis*). After the experiment, plants were moved to a greenhouse while seeds ripened. All fruits from flowers that were open during the experiment were collected, and seeds from these fruits were used to estimate seed production and selfing rates.

Mating system estimation—For both experiments, approximately 50 seeds from each plant were sown into individual 102-mm pots in a greenhouse, and 10 emerged seedlings were randomly chosen from each pot for allozyme analyses. Seedlings were crushed in a modified Tris-sucrose buffer (Williams and Waser, 1999), and extracted allozymes were resolved on starch gels (following Ritland and Ritland, 1989). Six putative loci on four enzyme systems were resolved (DIA-1, DIA-2, PGD-1, PGD-2, PGI-1, and PGM-1). Variation among progeny isozyme phenotypes was analyzed using the Newton-Raphson procedure in the computer program MLTR (Ritland, 2002) to generate a maximum-likelihood multilocus estimate of the proportion of self-fertilized progeny produced (s_m), parental fixation indices (F), and biparental inbreeding, which was calculated as the difference between multilocus (s_m) and the mean of single-locus (s_s) estimates of selfing. We also estimated two other mating system components, the correlation of paternity (r_p), or the probability that two siblings share the same outcrossed pollen parent, and the correlation of selfing (r_s), which is an estimate of the normalized variance in selfing across families (Ritland, 2002). Estimates were based on the mean of 1000 bootstrap replicates of the data with the progeny array as the unit of resampling, and

standard errors were estimated by the standard deviation of bootstrap values. Parental genotypes were inferred using a multilocus Monte-Carlo modification of the Brown and Allard (1970) procedure (Ritland, 2002).

Analysis—Plant traits (Native plants experiment)—Because of variation in phenology, we were able to collect floral trait data on 80% of the plants marked at population B, representing 21 complete pairs (25 experimental, and 32 control plants total), so we used an incomplete block ANOVA to compare spittlebug treatments on morphology at population B in which plant pair was the blocking factor.

Plant traits (Experimental arrays)—We used mixed model factorial ANOVA (PROC MIXED, SAS Institute, 2001) to compare flower morphology, flower number, fruit set (total fruits/total flowers), total seeds, and seed set (total seeds/total fruits). These models included inbreeding treatment (self- or cross-fertilized), herbivore treatment (spittlebug or control), and inbreeding \times herbivore interaction as fixed effects and plant family as a random effect. This approach does not account for variation due to position in the field (which was rotated every 2 d; see Materials and Methods, Experimental arrays design) or variation associated with the groups of 16 plants that rotated among field positions. These factors, if significant, would have limited our ability to detect treatment effects. To correct for heteroskedasticity, total seeds and seed set were square-root transformed prior to analysis. The remaining variables required no transformation.

Pollinator behavior—We examined three components of pollinator behavior: (1) the total number of visitors arriving to each plant (“arrival frequency”), (2) the mean number of flowers visited per plant (“flowers visited”), and (3) the mean foraging time per flower (“flower-handling time,” calculated as the number of flowers visited divided by the time foraging on a plant). This estimate of flower-handling time includes the time insects were foraging on flowers as well as time moving between flowers on a plant. To test for treatment effects on arrival frequency, we first summed the number of visits received by each plant from each insect visitor category across all observations. We then used a mixed-model ANOVA to compare treatment effects; this model included inbreeding treatment, herbivore treatment, insect visitor category, and all interactions as fixed effects. We also included plant family as a random effect. To compare treatment effects on flowers visited and flower-handling time we first averaged these response variables within plants for each visitor category on each day of observation. We then applied a repeated measures mixed-model ANOVA to these mean values. For both models, we assigned compound symmetric variance-covariance matrices (on the basis of best model fit) and the subject of repeated measures was the individual plant. Otherwise, the structure of these models was similar to the analysis of arrival frequency. Flowers visited and flower-handling times were log-transformed prior to analysis to correct for heteroskedasticity and improve normality. Arrival frequency required no transformation.

Plant traits and pollinator behavior—Pollinator behavior is often influenced by flower size (Elle and Carney, 2003) or flower number (Mitchell et al., 2004), so we examined the effects of these traits on pollinator behavior. These analyses also provided insight into the effects of experimental treatments on behavior while controlling their effects on floral traits. Rather than examine all possible pairwise relationships among the variables we measured, we chose four that we expected a priori to be informative. These included (1) mean number of arrivals per 30 min vs. daily floral display size, (2) number of flowers visited vs. daily floral display, (3) total arrivals vs. corolla size, and (4) flower-handling time vs. corolla size. The relationships in (1) and (2) involved responses that were measured through time, thus we used repeated measures mixed-model ANCOVA, to which we assigned compound-symmetric variance-covariance matrices, and the subject of repeated measures was individual plant. The relationships in (3) and (4) involved responses that did not include a temporal component, so for these we used mixed-model ANCOVA. In addition to the covariates (floral display or corolla size), all of these models included inbreeding, spittlebug treatment, and their interaction as fixed effects and plant family as a random effect. Preliminary analyses (not

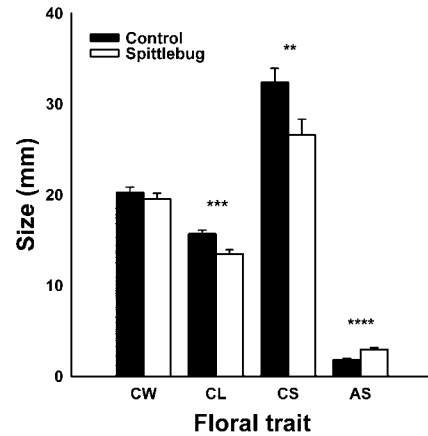


Fig. 1. Corolla width (CW), corolla length (CL), corolla size (CS = CW \times CL), and anther-stigma separation (AS) of native *Mimulus guttatus* plants at a Napa County, California, USA, field site (population B) (means + 1 SE). Unshaded bars indicate plants experimentally treated with attack by a spittlebug herbivore nymph prior to flowering. *** $P < 0.01$, **** $P < 0.001$, **** $P < 0.0001$ in an incomplete block ANOVA comparing treatments.

shown) revealed no significant differences among visitor categories in the slopes of any of these relationships, so insect visitor was not included as a term in the models presented here. Our approach to constructing ANCOVA models followed that described by Quinn and Keough (2002) and Littell et al. (1996). Specifically, because tests involving main effects are influenced by the levels of other factors, we tested for homogeneity of slopes for each fixed effect independently before combining the results for final models. Terms that were found not to be significant in the earlier phases of model construction were excluded from final models (Quinn and Keough, 2002). Flower-handling time, floral display size, and number of flowers visited were log-transformed prior to analysis to improve homoskedasticity and normality; corolla size required no transformation.

Mating system estimates—For both experiments, we used two-sided z tests to compare estimates of mating system parameters based on the distribution of the differences between treatments in the bootstrap replicates.

RESULTS

Plant traits—Native plants experiment—Spittlebugs reduced corolla length and corolla size of flowers on native plants at population B by 14% and 18%, respectively (Fig. 1). Spittlebugs also caused a 63% increase in AS in these plants (Fig. 1), which may reflect their influence in reducing corolla length, since filaments are attached to the base of the corolla in *Mimulus*.

Experimental arrays—On greenhouse-grown plants used in the experimental arrays, spittlebugs reduced corolla width and corolla size by 7% and 12%, respectively (Table 1, Fig. 2). Spittlebugs also caused a 26% increase in AS in these plants, although this was only marginally significant (Table 1). There was no significant effect of spittlebugs on floral display, total flowers, fruit set, total seeds, and seed set (Table 1, Fig. 2).

Inbreeding reduced corolla width (15%), corolla length (10%), and corolla size (23%) among plants used in the experimental arrays, but there was no effect of inbreeding on AS (Table 1, Fig. 2). Inbreeding also decreased mean floral display (24%), total flowers (24%), fruit set (28%), total seeds (46%), and seed set (51%; Table 1, Fig. 2). Spittlebugs significantly altered the effect of inbreeding on mean floral display and total

TABLE 1. Effects of inbreeding and spittlebug herbivores on morphological and fitness traits of *Mimulus guttatus* grown in experimental arrays. F values (ndf = 1, ddf = 45 for all tests) for fixed effects and χ^2 values (df = 1) from likelihood ratio tests for random effects are shown. † $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Effect type	Source	Corolla width	Corolla length	Corolla size	Herkogamy	Floral display	Total flowers	Fruit set	Total seeds	Seed set
Fixed	Inbreeding	20.69****	14.21***	19.48****	0.21	9.05**	8.55**	11.51**	37.86****	19.10****
	Spittlebug	4.53*	3.52†	4.68*	3.71†	0.29	0.36	0.05	1.56	2.47
	Inbreeding × Spittlebug	0.07	0.02	0.04	0.05	5.83*	4.78*	0.49	1.14	0.65
Random	Family	0.38	1.36	1.08	7.40**	4.47*	5.45**	0.90	5.62**	0.01

flowers (Table 1); for both traits, inbred plants had significantly fewer flowers than outbred plants in the absence of spittlebugs, but this difference disappeared when spittlebugs were present (Fig. 2, Tukey-Kramer post-hoc tests at $\alpha = 0.05$). This result indicates that spittlebugs reduced the expression of inbreeding depression for these traits.

Pollinator behavior—We observed 386 total visits during 30 h of observation time, and these were dominated (67.4%) by Syrphid flies. Bumble bees (*Bombus* spp.) represented only

3.4% of visits observed, and honey bees (*Apis mellifera*) represented 7.0% of visits. To facilitate analysis, we combined honey bee and bumble bee visits into a single category (“large bees”), and combined visits from all remaining insects except Syrphid flies (muscid flies, 9.1%; Lepidoptera, 4.9%; halictid bees, 4.1%; Coleoptera, 3.1%; other, 1.0%) into a single category (“other”).

Spittlebugs had no significant effect on arrival frequency (Table 2, Fig. 3A). In contrast, inbreeding reduced arrival frequency by 34% (Table 2, Fig. 3A). The effects of spittlebugs

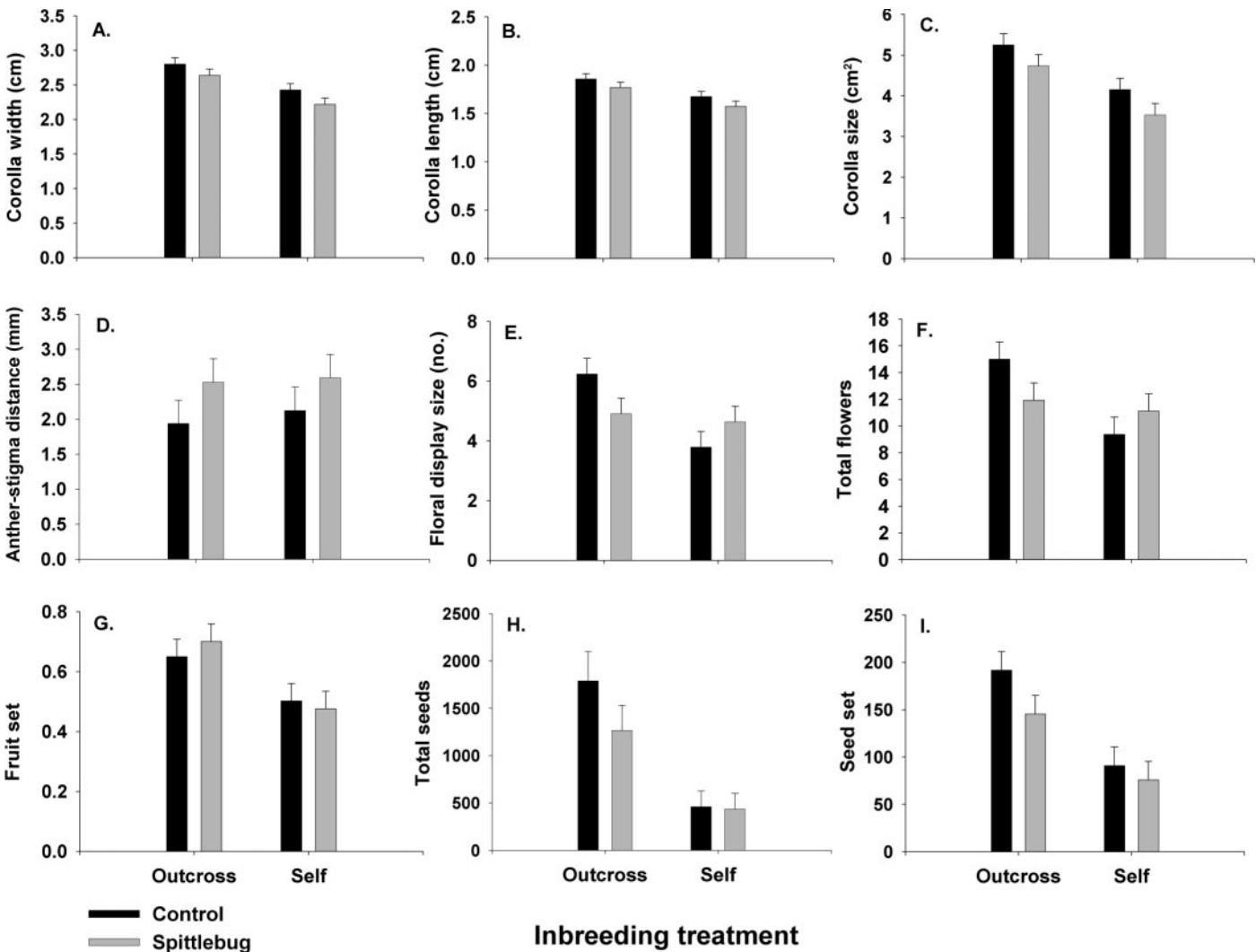


Fig. 2. Corolla width (A), corolla length (B), corolla size (C), anther–stigma separation (D), floral display size (E), total flowers (F), fruit set (G), total seeds (H), and seed set (I) of plants from experimental arrays of *Mimulus guttatus* testing responses to inbreeding and spittlebug herbivory (means + 1 SE).

TABLE 2. Effects of inbreeding and spittlebug herbivory on behavior of pollinators visiting experimental arrays of *Mimulus guttatus*. *F* values_{ndf,ddf} for fixed effects and χ^2 values (df = 1) from likelihood ratio tests for random effects are shown. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001.

Effect type	Source	Arrival frequency	Flowers visited	Time per flower
Fixed	Inbreeding (I)	12.40 _{1,165} ***	0.09 _{1,80}	5.40 _{1,62} *
	Spittlebug (S)	0.01 _{1,165}	0.00 _{1,83}	0.12 _{1,66}
	I × S	0.01 _{1,165}	1.95 _{1,81}	0.07 _{1,62}
	Visitor (V)	78.29 _{2,165} ****	5.06 _{2,242} **	8.31 _{2,158} ****
	I × V	0.90 _{2,165}	0.60 _{2,243}	0.10 _{2,158}
	S × V	0.80 _{2,165}	1.03 _{2,241}	1.19 _{2,156}
	I × S × V	0.58 _{2,165}	0.77 _{2,243}	0.13 _{2,158}
	Random	Family	0.80	0.53

and inbreeding on arrival frequency did not vary among visitors (Table 2, Fig. 3A). Large bees visited significantly more flowers on plants than syrphid flies or other visitors (Table 2, Fig. 3B, Tukey-Kramer post-hoc tests: *P* < 0.007), but there was no significant effect of spittlebugs or inbreeding on number of flowers visited, either within visitor categories or overall. Pollinators spent about 7 s (49%) longer while visiting each inbred flower, and this did not vary among visitor categories (Table 2, Fig. 3C). We found no effect of spittlebug herbivory on flower-handling time.

Plant traits and pollinator behavior—More pollinators arrived to forage on plants with larger floral displays ($F_{1,332} = 21.35$, *P* < 0.0001), and while the slope of this relationship did not differ between inbreeding treatments, the intercept did; outcross plants received more arrivals than self plants ($F_{1,49} = 11.74$, *P* = 0.001; Fig. 4A). Arrival frequency also increased with corolla size ($F_{1,61} = 4.40$, *P* = 0.04), and again, the intercept of this relationship was greater for outbred plants ($F_{1,61} = 6.92$, *P* = 0.01; Fig. 4C), but there was no interaction with inbreeding treatment. These models thus indicated that the negative effect of inbreeding on arrival frequency was not solely due to its effects on floral display or corolla size. The number of flowers visited by insects also increased with floral display ($F_{1,180} = 12.16$, *P* = 0.0006; Fig. 4B), suggesting increased potential for geitonogamy in plants with larger floral displays. Neither experimental treatment, however, had any significant effect on this relationship (*P* > 0.1). Finally, flower-handling time was longer for plants with larger corollas ($F_{1,59} = 7.40$, *P* = 0.008), yet flowers on inbred plants received significantly longer visits ($F_{1,59} = 4.57$, *P* = 0.04; Fig. 4D), even though inbred flowers were smaller (Fig. 2). Inbreeding did not significantly alter the slope of the relationship between flower-handling time and corolla size, however. The spittlebug treatment had no significant effect in any of these models (*P* > 0.1 for main effects and interaction terms).

Mating systems—*Native plants experiment*—Allozyme polymorphism was high in the two native sites. In population A the frequency of the most common multilocus allozyme genotype was 0.372, with an average of three alleles per polymorphic locus; in population B these values were 0.285 and 3.5, respectively. The fixation index for parental plants was negative for population A plants, indicating higher heterozygosity than expected from Hardy-Weinberg proportions, whereas in population B, *F* was low but significantly positive (Table 3).

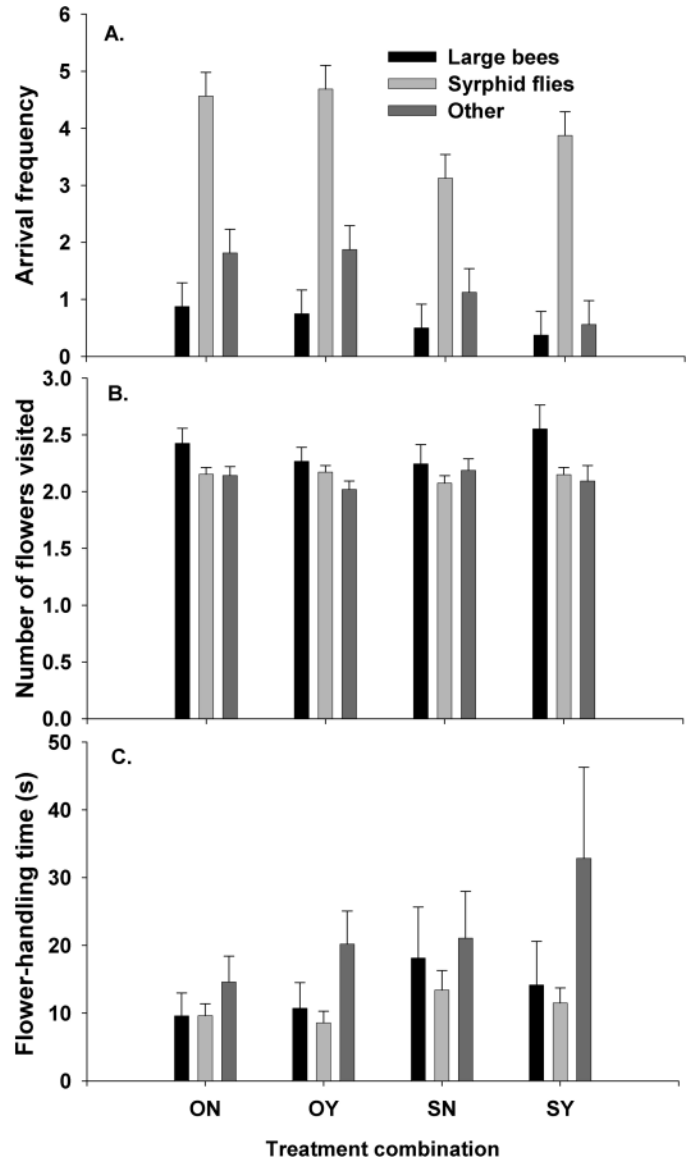


Fig. 3. Arrival frequency (number of visits per plant) (A), number of flowers visited (B), and flower-handling time (C) by floral visitors to experimental arrays of *Mimulus guttatus* testing responses to inbreeding and spittlebug herbivory treatments (means + 1 SE). Treatments: O = outbred, S = inbred, Y = herbivory, N = no herbivory.

Spittlebugs increased selfing rates by 10.4% in population A, but had no significant effect on selfing in population B (Table 3). In neither population did spittlebugs alter biparental inbreeding, the fraction of siblings that shared the same male parent (r_p), or the variation among families in selfing rates (r_s ; Table 3).

Experimental arrays—The frequency of the most common multilocus allozyme genotype was 0.269 among plants in the pollinator behavior experiment, and the mean number of alleles per locus was 2.7. The fixation index among parental plants was low (F [SE] = 0.039 [0.022]), and this value was constrained to zero to improve power for estimating other parameters.

Spittlebug attack significantly increased selfing rates nearly

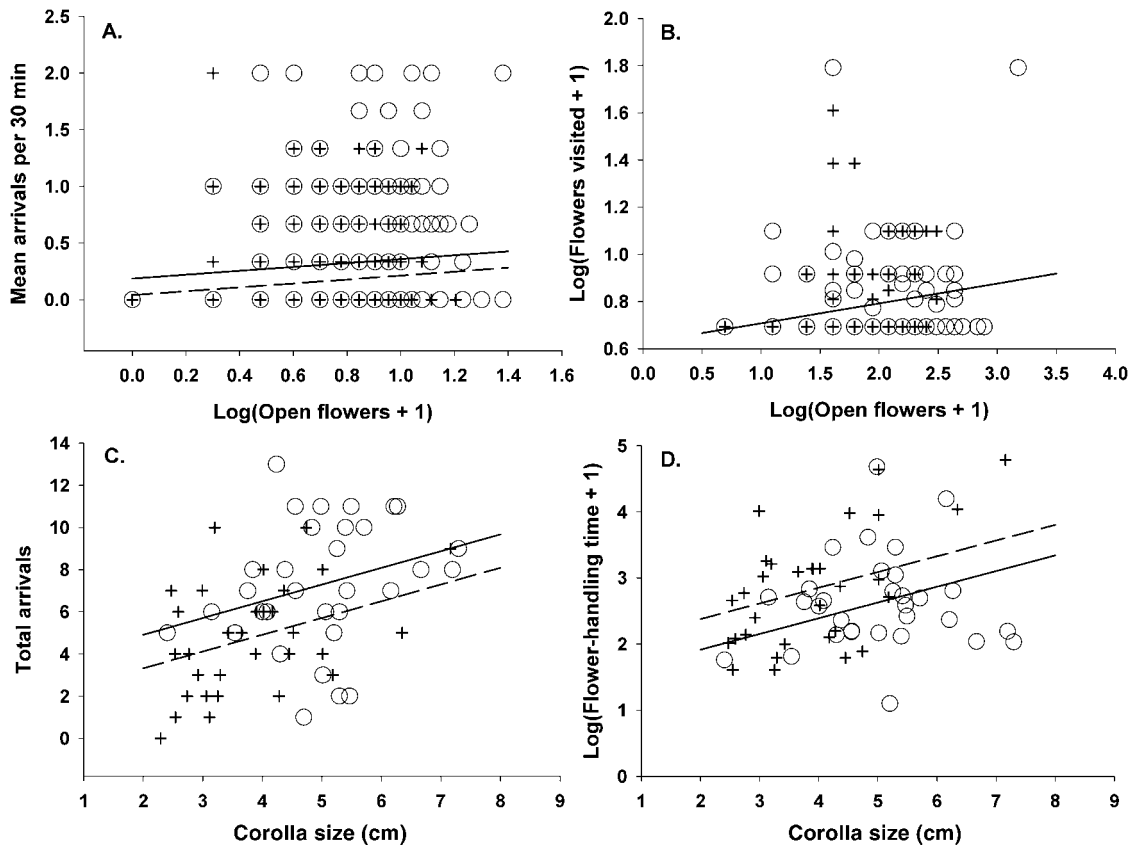


Fig. 4. Relationships between pollinator behavior and plant traits for experimental arrays of *Mimulus guttatus* testing the effects of inbreeding and spittlebug herbivory. Solid lines and open circles indicate the outbred treatment, and dashed lines and crosses indicate the inbred treatment. See Materials and Methods, Analysis for details of analyses.

twofold from 17.8% to 32.5% ($z = 2.23, P = 0.03$). In contrast, the inbreeding treatment significantly reduced selfing by about one-half, from 29.3% to 15.2% ($z = 2.24, P = 0.03$). Estimates of biparental inbreeding, correlation of paternity, and correlation of selfing were not significantly altered by experimental treatments (Table 4).

DISCUSSION

Mixed mating systems are considered to be unstable in most models of mating system evolution (Charlesworth et al., 1990; Uyenoyama et al., 1993), although paradoxically, mixed mating is commonly observed in natural populations of *Mimulus guttatus* (Ritland and Ritland, 1989; Dudash and Ritland,

1991; Willis, 1993) and many other plant species (Vogler and Kalisz, 2001). Some previous studies seeking to explain the persistence of mixed mating in nature have considered the influence of mutualist pollinators, such as their imposition of spatial structure to mating patterns (Holsinger, 1991; Karron et al., 1995) or temporal variation in pollinator availability (Kalisz et al., 2004). Our results suggest that recognizing the influence of other ecological interactions on mating systems, in particular those involving natural enemies such as herbivores, may also contribute to resolving this paradox.

Effects of herbivory—One of our more interesting results was that spittlebugs increased selfing rates by as much as two-

TABLE 3. Mating system estimates from two native populations of *Mimulus guttatus* growing in Napa County, California, USA. Estimates (1 SE) and results from two-sided z tests comparing spittlebug treatments are shown for the multilocus selfing rate (s_m), biparental inbreeding ($s_m - s_s$), correlation among progeny pairs within families for shared paternity (r_p), and the correlation among families in selfing rates (r_s). Spittlebug indicates plants that were experimentally treated with attack by a single spittlebug herbivore nymph prior to flowering.

Parameter	Population A			Population B		
	Control	Spittlebug	z	Control	Spittlebug	z
s_m	0.263 (0.049)	0.367 (0.045)	1.96*	0.181 (0.036)	0.211 (0.032)	0.63
$s_m - s_s$	0.037 (0.023)	-0.002 (0.021)	1.26	0.039 (0.017)	0.042 (0.018)	0.13
r_p	0.232 (0.060)	0.223 (0.077)	0.12	0.119 (0.048)	0.151 (0.055)	0.41
r_s	0.529 (0.109)	0.275 (0.131)	1.48	0.479 (0.151)	0.291 (0.119)	1.11

Note: Fixation index (F [1 SE]): Population A parental $F = -0.121$ (0.027), population B parental $F = 0.070$ (0.012). Number of families analyzed (10 progeny per family): population A-control = 26, population A-spittlebug = 29, population B-control = 31, population B-spittlebug = 33.

TABLE 4. Mating system estimates from experimental arrays of *Mimulus guttatus* testing the effects of inbreeding (outcross vs. self-pollination) and spittlebug herbivory (bug vs. control). Estimates (1 SE) are shown for the multilocus selfing rate (s_m), biparental inbreeding ($s_m - s_s$), correlation among progeny pairs within families for shared paternity (r_p), and the correlation among families in selfing rates (r_s).

Parameter	Outcross		Self	
	Control	Bug	Control	Bug
s_m	0.258 (0.040)	0.360 (0.067)	0.095 (0.033)	0.257 (0.087)
$s_m - s_s$	0.035 (0.026)	0.007 (0.029)	-0.009 (0.033)	-0.039 (0.035)
r_p	0.126 (0.067)	0.207 (0.095)	0.121 (0.084)	0.311 (0.108)
r_s	0.670 (0.114)	0.591 (0.119)	0.163 (0.158)	0.448 (0.147)

Note: Number of families (progeny) analyzed: outcross-control = 16 (159), outcross-bug = 16 (160), self-control = 13 (130), self-bug = 14 (138).

fold. The mechanism for this effect, however, was not clear. In both experiments, plants attacked by spittlebugs increased herkogamy, although this effect is more likely to decrease selfing (e.g., Ritland and Ritland, 1989; Dole, 1992; Karron et al., 1997; van Kleunen and Ritland, 2004). If spittlebugs exact a resource cost to maternal plants, preferential abortion of selfed ovules could change realized selfing rates (Levri and Real, 1998), although this also would presumably reduce the effective selfing rate. A previous study reported that spittlebug attack reduced pollinator visits to *Rudbeckia* (Hamback, 2001). *Mimulus* plants that receive fewer pollinator visits might be expected to have higher selfing rates (Dole, 1992; Fenster and Ritland, 1994), but we found no evidence for an effect of spittlebugs on pollinator behavior. While the mechanism for this result remains for future experiments to unravel, we suggest that the consistent pattern observed across three sites, including naturally occurring plants in native populations, likely reflects a meaningful biological phenomenon.

Spittlebugs altered floral traits of *M. guttatus*, but they had little effect on pollinator behavior or plant fitness. In both experiments, plants attacked by spittlebugs had smaller flowers and increased anther–stigma separation, and in the experimental arrays, spittlebugs attenuated the negative effect of inbreeding on flower number and floral display size. Other workers have found that herbivore-imposed changes in flower size or number also cause a change in pollinator behavior (Strauss, 1997; Steets and Ashman, 2004). Typically, plants with larger flowers and more flowers are more attractive to pollinators (Elle and Carney, 2003; Mitchell et al., 2004). Likewise, we also found that plants with more flowers received more visits and longer visit sequences, and we found that plants with larger flowers received more visits and had a longer flower-handling time. We found, however, no significant effect of herbivory on these relationships.

Effects of inbreeding—The selfing rate of inbred plants was about one-half that of outbred plants. Similar negative effects of inbreeding on selfing rates have been reported from some crops (Link, 1990; Damgaard and Loeschke, 1994). These patterns may reflect early-acting inbreeding depression in the maturing progeny of selfed plants or other post-pollination processes. Our results suggest that the effect of inbreeding on pollinator behavior may also be involved. For example, inbred plants had fewer flowers, which should have limited opportunities for geitonogamous pollination. We found a positive relationship between floral display size and the number of flowers visited (Fig. 4B), which provides some support for this idea. Furthermore, pollinators spent longer foraging on flowers of inbred plants, which may have improved opportunities for outcross pollen transfer. The mechanism by which inbreeding

affected flower-handling time is not clear. One possibility is that fewer pollinator rewards (nectar and pollen) had been removed from inbred flowers because inbred plants received fewer visits; if so, pollinators that visited inbred flowers may have remained longer to collect the rewards. Regardless of the mechanism, this effect was strong enough to overcome an overall positive relationship between corolla size and flower-handling time (Fig. 4D).

To our knowledge, our study is the first to report that inbreeding can alter plant interactions with pollinators. In addition to its effects on flower-handling time, inbreeding reduced the number of pollinator arrivals to plants. This in part reflects the negative influence of inbreeding on floral display size (Fig. 2E) and corolla size (Fig. 2C), both of which can influence pollinator visitation (e.g., Elle and Carney, 2003; Mitchell et al., 2004). Even after accounting for these effects, however, pollinators behaved differently on inbred plants (Fig. 4). Previous studies have reported that pollen production in *M. guttatus* declines with inbreeding (Carr and Dudash, 1996) and that some pollinators can discriminate among *M. guttatus* based on pollen quality (Robertson et al., 1999), which points to another possible mechanistic basis for the effects of inbreeding on pollinator behavior. In any case, this result adds mutualisms to the list of ecological interactions that are influenced by inbreeding (Ouborg et al., 2000; Carr and Eubanks, 2002; Ivey et al., 2004), which reinforces the importance of acknowledging the influence of inbreeding in community ecological studies. The observation that inbreeding can alter important ecological interactions may in part contribute to the wide variation in the numerical dynamics of *M. guttatus* populations (Vickery, 1999).

As reported previously for *M. guttatus* (Willis, 1993; Carr and Dudash, 1996; Carr and Eubanks, 2002), we found inbreeding depression for fitness and floral traits. Interestingly, inbreeding affected all traits that we measured except anther–stigma separation, although inbreeding was found to increase herkogamy in another *M. guttatus* population (Kelly and Arathi, 2003). Another surprise was that spittlebugs reduced the expression of inbreeding depression for flower number by over 30% in our study; inbred control plants had significantly fewer flowers, but when attacked by spittlebugs this difference disappeared (Fig. 2). In contrast, a previous greenhouse study using plants from this population found spittlebugs to increase inbreeding depression for flower number nearly twofold (Carr and Eubanks, 2002). A field study of this population also found spittlebugs to increase inbreeding depression (for biomass), although this effect varied among fitness traits (Ivey et al., 2004). One of the more salient results from the few studies of inbreeding effects on plant–herbivore interactions has been

the wide variation among populations and plant families in the expression of inbreeding effects (Carr and Eubanks, 2002; Ivey et al., 2004). Inbreeding depression in five families of *Cucurbita pepo* subsp. *texana*, for example, increased in response to beetle damage in one study (Hayes et al., 2004), but decreased in another study conducted in a different year (Stephenson et al., 2004), possibly reflecting changes in weather or other environmental conditions. Such variation can have important consequences for variation in mating systems; heterogeneity in the expression of inbreeding depression, due to herbivory or other environmental factors, can select for stable mixed mating (Cheptou and Mathias, 2001).

Implications for mating systems—The patterns we observed suggest that herbivores could play a role in maintaining mixed mating in *M. guttatus*. Herbivores and inbreeding had opposing effects on selfing rates, which indicates that herbivores may contribute to variation in mixed mating in this species simply by balancing the numbers of self-fertilized progeny contributed to subsequent generations. Spittlebugs also reduced flower size, which has been linked to a reduction in male fertility in previous studies (Fishman, 2000; van Kleunen and Ritland, 2004). Shifting the relative performance of male vs. female function may contribute to variation in mating systems (Delph et al., 1997; Strauss et al., 2001; Stephenson et al., 2004). If herbivore-imposed reductions in male function are associated with a shift in selfing rates, the parameter space under which mixed mating is stable can be increased (Agrawal and Lively, 2001). We also found that spittlebugs decreased inbreeding depression, suggesting that the barriers to the spread and persistence of traits associated with selfing would be reduced in infested populations. Based on previous studies of this system, however, we expect that spittlebug effects on inbreeding depression vary among populations and families (Carr and Eubanks, 2002; Ivey et al., 2004); such variation itself can help to maintain mixed mating (Cheptou and Mathias, 2001). More generally, plasticity in selfing rates in response to herbivory or other environmental conditions (i.e., variation in genotype \times environment interactions) can be sufficient to maintain genetic variation in mating systems. Although our study was not designed to examine genotype-level effects of herbivory on selfing rates, such interactions may occur in this system. For example, a previous study found variation among family lines for tolerance to herbivory in response to inbreeding (Ivey et al., 2004).

Contemporary understanding of plant mating system dynamics has been shaped by recognition of the importance of ecological variation (Uyenoyama et al., 1993; Cheptou and Mathias, 2001; Barrett, 2003). Despite these developments, surprisingly few reports of the influence of antagonists on plant mating systems appear in the literature (but see Levri and Real, 1998; Elle and Hare, 2002; Steets and Ashman, 2004). In comparison, studies of the effects of mutualistic pollinators on mating systems are remarkably widespread (e.g., Holsinger, 1991; Kalisz et al., 2004; Karron et al., 2004). Our results underscore that interpreting mating system variation may require a broader appreciation of the ecological context in which plants occur, including their antagonistic interactions with other community members.

LITERATURE CITED

AGRAWAL, A. F., AND C. M. LIVELY. 2001. Parasites and the evolution of self-fertilization. *Evolution* 55: 869–879.

- ASHMAN, T. L. 2002. The role of herbivores in the evolution of separate sexes from hermaphroditism. *Ecology* 83: 1175–1184.
- BARRETT, S. C. H. 2003. Mating strategies in plants: the outcrossing-selfing paradigm and beyond. *Philosophical Transactions of the Royal Society of London, B* 358: 991–1004.
- BROWN, A. H. D., AND R. W. ALLARD. 1970. Estimation of the mating system in open-pollinated maize populations using isozyme polymorphisms. *Genetics* 66: 133–145.
- CARR, D. E., AND M. R. DUDASH. 1996. Inbreeding depression in two species of *Mimulus* (Scrophulariaceae) with contrasting mating systems. *American Journal of Botany* 83: 586–593.
- CARR, D. E., AND M. D. EUBANKS. 2002. Inbreeding alters resistance to insect herbivory and host plant quality in *Mimulus guttatus* (Scrophulariaceae). *Evolution* 56: 22–30.
- CHARLESWORTH, D., M. T. MORGAN, AND B. CHARLESWORTH. 1990. Inbreeding depression, genetic load, and the evolution of outcrossing rates in a multilocus system with no linkage. *Evolution* 44: 1469–1489.
- CHEPTOU, P. O., AND A. MATHIAS. 2001. Can varying inbreeding depression select for intermediary selfing rates? *American Naturalist* 157: 361–373.
- DAMGAARD, C., AND V. LOESCHCKE. 1994. Genetic variation for selfing rate and the dependence of selfing rate on mating history in *Brassica napus* (rape seed). *Heredity* 72: 570–573.
- DELPH, L. F., M. H. JOHANSSON, AND A. G. STEPHENSON. 1997. How environmental factors affect pollen performance: ecological and evolutionary perspectives. *Ecology* 78: 1632–1639.
- DOLE, J. A. 1992. Reproductive assurance mechanisms in three taxa of the *Mimulus guttatus* complex (Scrophulariaceae). *American Journal of Botany* 79: 650–659.
- DUDASH, M. R., AND K. RITLAND. 1991. Multiple paternity and self-fertilization in relation to floral age in *Mimulus guttatus* (Scrophulariaceae). *American Journal of Botany* 78: 1746–1753.
- ELLE, E., AND R. C. CARNEY. 2003. Reproductive assurance varies with flower size in *Collinsia parviflora* (Scrophulariaceae). *American Journal of Botany* 90: 888–896.
- ELLE, E., AND J. D. HARE. 2002. Environmentally induced variation in floral traits affects the mating system in *Datura wrightii*. *Functional Ecology* 16: 79–88.
- FENSTER, C. B., AND K. RITLAND. 1994. Evidence for natural selection on mating system in *Mimulus* (Scrophulariaceae). *International Journal of Plant Sciences* 155: 588–596.
- FISHMAN, L. 2000. Pollen discounting and the evolution of selfing in *Arenaria uniflora* (Caryophyllaceae). *Evolution* 54: 1558–1565.
- HAMBACK, P. A. 2001. Direct and indirect effects of herbivory: feeding by spittlebugs affects pollinator visitation rates and seedset of *Rudbeckia hirta*. *Ecoscience* 8: 45–50.
- HAYES, C. N., J. A. WINSOR, AND A. G. STEPHENSON. 2004. Inbreeding influences herbivory in *Cucurbita pepo* ssp. *texana* (Cucurbitaceae). *Oecologia* 140: 601–608.
- HOLSINGER, K. E. 1991. Mass-action models of plant mating systems: the evolutionary stability of mixed mating systems. *American Naturalist* 138: 606–622.
- IVEY, C. T., D. E. CARR, AND M. D. EUBANKS. 2004. Effects of inbreeding in *Mimulus guttatus* on tolerance to herbivory in natural environments. *Ecology* 85: 567–574.
- JAIN, S. K. 1978. Breeding system in *Limnanthes*: several alternative measures. *American Journal of Botany* 65: 272–275.
- KALISZ, S., D. W. VOGLER, AND K. M. HANLEY. 2004. Context dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* 430: 884–887.
- KAROLY, K. 1994. Inbreeding effects on mating system traits for two species of *Lupinus* (Leguminosae). *American Journal of Botany* 81: 1538–1544.
- KARRON, J. D., R. T. JACKSON, N. N. THUMSER, AND S. L. SCHLICHT. 1997. Outcrossing rates of individual *Mimulus ringens* genets are correlated with anther–stigma separation. *Heredity* 79: 365–370.
- KARRON, J. D., R. J. MITCHELL, K. G. HOLMQUIST, J. M. BELL, AND B. FUNK. 2004. The influence of floral display size on selfing rates in *Mimulus ringens*. *Heredity* 92: 242–248.
- KARRON, J. D., N. N. THUMSER, R. RUCKER, AND A. J. HESSENAUER. 1995. The influence of population density on outcrossing rates in *Mimulus ringens*. *Heredity* 75: 175–180.
- KELLY, J. K., AND H. S. ARATHI. 2003. Inbreeding and the genetic variance in floral traits of *Mimulus guttatus*. *Heredity* 90: 77–83.
- LECLERC-POTVIN, C., AND K. RITLAND. 1994. Modes of self-fertilization in

- Mimulus guttatus* (Scrophulariaceae): a field experiment. *American Journal of Botany* 81: 199–205.
- LEVRI, M. A., AND L. A. REAL. 1998. The role of resources and pathogens in mediating the mating system of *Kalmia latifolia*. *Ecology* 79: 1602–1609.
- LINK, W. 1990. Autofertility and rate of cross-fertilization: crucial characters for breeding synthetic varieties in faba beans (*Vicia faba* L.). *Theoretical and Applied Genetics* 79: 713–717.
- LITTELL, R. C., G. A. MILLIKEN, W. W. STROUP, AND R. D. WOLFINGER. 1996. SAS system for mixed models. SAS Institute, Cary, North Carolina, USA.
- MARQUIS, R. J. 1992. Selective impact of herbivores. In R. S. Fritz and E. L. Simms [eds.], *Plant resistance to herbivores and pathogens: ecology, evolution, and genetics*, 301–325. University of Chicago Press, Chicago, Illinois, USA.
- MITCHELL, R. J., J. D. KARRON, K. G. HOLMQUIST, AND J. M. BELL. 2004. The influence of *Mimulus ringens* floral display size on pollinator visitation patterns. *Functional Ecology* 18: 116–124.
- OUBORG, N. J., A. BIERE, AND C. L. MUDDE. 2000. Inbreeding effects on resistance and transmission-related traits in the *Silene-Microbotryum* pathosystem. *Ecology* 81: 520–531.
- QUINN, G. P., AND M. J. KEOUGH. 2002. *Experimental design and data analysis for biologists*. Cambridge University Press, Cambridge, UK.
- RITLAND, C., AND K. RITLAND. 1989. Variation of sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). *American Journal of Botany* 76: 1731–1739.
- RITLAND, K. 2002. Extensions of models for the estimation of mating systems using n independent loci. *Heredity* 88: 221–228.
- ROBERTSON, A. W., C. MOUNTJOY, B. E. FAULKNER, M. V. ROBERTS, AND M. R. MACNAIR. 1999. Bumble bee selection of *Mimulus guttatus* flowers: the effects of pollen quality and reward depletion. *Ecology* 80: 2594–2606.
- SAS INSTITUTE. 2001. SAS version 8.02. SAS Institute, Cary, North Carolina, USA.
- STEETS, J. A., AND T. L. ASHMAN. 2004. Herbivory alters the expression of a mixed-mating system. *American Journal of Botany* 91: 1046–1051.
- STEPHENSON, A. G., B. LEYSHON, S. E. TRAVERS, C. N. HAYES, AND J. A. WINSOR. 2004. Interrelationships among inbreeding, herbivory, and disease on reproduction in a wild gourd. *Ecology* 85: 3023–3034.
- STRAUSS, S. Y. 1997. Floral characters link herbivores, pollinators, and plant fitness. *Ecology* 78: 1640–1645.
- STRAUSS, S. Y., J. K. CONNER, AND K. P. LEHTILÄ. 2001. Effects of foliar herbivory by insects on the fitness of *Raphanus raphanistrum*: damage can increase male fitness. *American Naturalist* 158: 496–504.
- UYENOYAMA, M. K., K. E. HOLSINGER, AND D. M. WALLER. 1993. Ecological and genetic factors directing the evolution of self-fertilization. *Oxford Survey of Evolutionary Biology* 9: 327–381.
- VAN KLEUNEN, M., AND K. RITLAND. 2004. Predicting evolution of floral traits associated with mating system in a natural plant population. *Journal of Evolutionary Biology* 17: 1389–1399.
- VICKERY, R. K. 1999. Remarkable waxing, waning, and wandering of populations of *Mimulus guttatus*: an unexpected example of global warming. *Great Basin Naturalist* 59: 112–126.
- VOGLER, D. W., AND S. KALISZ. 2001. Sex among the flowers: the distribution of plant mating systems. *Evolution* 55: 202–204.
- WEAVER, C. R., AND D. R. KING. 1954. Meadow spittlebug. Research Bulletin no. 741. Ohio Agricultural Experiment Station, Wooster, Ohio, USA.
- WILLIAMS, C. F., AND N. M. WASER. 1999. Spatial genetic structure of *Delphinium nuttallianum* populations: inferences about gene flow. *Heredity* 83: 541–550.
- WILLIS, J. H. 1993. Partial self-fertilization and inbreeding depression in two populations of *Mimulus guttatus*. *Heredity* 71: 145–154.
- WYATT, R. 1988. Phylogenetic aspects of the evolution of self-pollination. In L. D. Gottlieb and S. K. Jain [eds.], *Plant evolutionary biology*, 109–131. Chapman and Hall, New York, New York, USA.