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EFFECTS OF INBREEDING IN *MIMULUS GUTTATUS* ON TOLERANCE TO HERBIVORY IN NATURAL ENVIRONMENTS

CHRISTOPHER T. IVEY,^{1,3} DAVID E. CARR,¹ AND MICKY D. EUBANKS²

¹*Blandy Experimental Farm, University of Virginia, 400 Blandy Farm Lane, Boyce, Virginia 22620 USA*

²*Department of Entomology and Plant Pathology, 301 Funchess Hall, Auburn University, Alabama 36849 USA*

Abstract. Inbreeding, which is common in plants, may increase the vulnerability of populations to natural enemies. Similarly, natural enemies may increase the expression of inbreeding depression in their hosts, resulting in altered selection on host mating-system evolution. To examine effects of inbreeding on tolerance to herbivory, we transplanted experimentally self- and cross-fertilized plants into four field populations of *Mimulus guttatus* and applied single *Philaenus spumarius* (spittlebug) nymphs to half. At the end of the growing season, we scored plants for five fitness components (reproductive effort, biomass, survival, probability of producing flowers or buds, and probability of bolting). Inbreeding reduced population-level tolerance to spittlebug herbivory with respect to plant aboveground biomass. Inbreeding effects on tolerance varied significantly among plant families for three fitness traits, indicating the opportunity for selection by herbivores to improve tolerance in inbreeding populations. These results also indicate that herbivores can alter inbreeding depression in plants. Our results mirror earlier greenhouse studies of inbreeding effects on plant–herbivore interactions, and demonstrate that these effects can be manifested in natural settings as well. This study indicates that inbreeding in natural populations can affect fitness not only directly, but also indirectly through altered interactions with natural enemies.

Key words: herbivory; inbreeding; mating system; *Mimulus guttatus*; *Philaenus spumarius*; self fertilization; spittlebugs; tolerance.

INTRODUCTION

Inbreeding, or mating between relatives, is widespread and common in natural plant populations (Vogler and Kalisz 2001) and can result in fundamental evolutionary consequences (Keller and Waller 2002). Inbreeding reduces heterozygosity throughout the genome and, as a consequence, is one of the primary factors influencing the distribution and extent of genetic diversity in populations (Hamrick and Godt 1989). It is accompanied by broad changes in phenotype and typically has negative fitness consequences, resulting in inbreeding depression (Husband and Schemske 1996).

In recent years, ecologists have begun to explore the significance of inbreeding in natural populations, in particular with respect to interactions involving natural enemies (Strauss and Karban 1994, Núñez-Farfán et al. 1996, Ouborg et al. 2000, Carr and Eubanks 2002, Carr et al. 2003). Much of this interest has stemmed from a concern for protected populations, in which the likelihood of inbreeding is typically higher due to small

population sizes (Cassinello et al. 2001). Small populations may face increased demographic risks if inbreeding reduces defenses against natural enemies. Natural enemies can also change the expression of inbreeding depression (Carr and Eubanks 2002), which is the primary barrier to the evolution of self-fertilization in most models of mating system evolution (Lande and Schemske 1985). Attack by a natural enemy therefore may change the spread or persistence of alleles that determine inbreeding rates in a natural population (Agrawal and Lively 2001, Ashman 2002).

Only a few previous studies have examined the impact of inbreeding on plant–herbivore interactions. Strauss and Karban (1994) found that inbreeding could maintain high resistance to thrips in *Erigeron glaucus* plants. A study of *Datura stramonium* found no inbreeding effect on the amount of damage imposed by flea beetles and grasshoppers (Núñez-Farfán et al. 1996), although this species is highly selfing and no evidence for inbreeding depression was found for any post-germination fitness trait. Furthermore, neither of these studies examined the effects of inbreeding on tolerance to herbivory (sensu Strauss and Agrawal 1999) or the influence of herbivory on inbreeding depression. In contrast, inbreeding was found to decrease

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³ E-mail: cti3c@virginia.edu

tolerance to herbivory in a greenhouse study involving *Mimulus guttatus* and spittlebugs; the negative effects of spittlebugs on flower number and plant biomass were greater in self-fertilized plants (Carr and Eubanks 2002). Spittlebugs also nearly doubled the expression of inbreeding depression in *M. guttatus*, suggesting that populations with herbivores experience a stronger barrier to the evolution of selfing. These effects, however, were observed in only one of two populations studied.

Although the majority of experimental studies of inbreeding have been performed in controlled environments, the expression of inbreeding effects under more natural conditions may have a more direct bearing on conservation concerns and evolutionary theory. In general, inbreeding depression has been found to be more severe under field conditions (Keller and Waller 2002). For example, Dudash (1990) found inbreeding depression to be higher in a native site than in greenhouse or common garden settings for *Sabatia angularis*. In some cases, however, the reverse pattern or no effect has been observed (Byers and Waller 1999). Even the existence of inbreeding effects in nature is considered by some to be controversial (Frankham 1995), because organisms are expected to avoid inbreeding under natural conditions or to have purged their genetic load if they are regular inbreeders (Lande and Schemske 1985, but see Byers and Waller 1999).

Here, we report the results of a study examining the effects of inbreeding in *Mimulus guttatus* (Scrophulariaceae) on tolerance to herbivory by spittlebugs, *Philaenus spumarius* (Homoptera: Cercopidae), in four native *M. guttatus* populations in northern California.

METHODS

Study organisms.—Inbreeding is common in *Mimulus guttatus*, although its mating system varies widely; outcrossing rates vary from about 75% selfing ($t = 0.25$) to complete outcrossing ($t = 1.0$), with a species mean $t \approx 0.6$ (Carr and Eubanks 2002). The meadow spittlebug, *Philaenus spumarius*, is a widespread generalist herbivore. Spittlebugs were the most abundant herbivore of *M. guttatus* populations in coastal California, and infested plants typically hosted one or two spittlebug nymphs (Carr and Eubanks 2002). Previous studies of spittlebug impacts in natural populations of *M. guttatus* do not exist to our knowledge; a single spittlebug nymph, however, caused up to 30% loss in biomass and 20% decrease in flower number in greenhouse-grown *M. guttatus* (Carr and Eubanks 2002).

Study sites.—In 2001, we collected 28–56 seed families from each of four populations of *Mimulus guttatus* in Napa County, California, USA. Two sites (M13W and M13E) occurred in roadside ditches and, because of seasonal drought, are strictly annual, as is typical for this species. Some plants in the other two sites (RC and SB) appeared to be growing perennially, which can

occur in sites that remain wet throughout the year. Voucher specimens of native plants collected from each population have been deposited with the Illinois Natural History Survey herbarium (ILLS). Spittlebug nymphs had been previously observed feeding on *M. guttatus* at all sites. All spittlebugs used in the experiment were collected from a single population at the Point Reyes National Seashore in Marin County, California (38°07' N, 122°56' W) (Carr and Eubanks 2002).

Experimental design.—During the fall of 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 hand pollinated to produce self and full-sib outcross progeny following the methods of Carr and Eubanks (2002). To produce outcross seeds, maternal plants were randomly paired with other plants in the same population, each family serving only once as pollen donor. Progeny seeds were germinated in a greenhouse in California. On 13 and 14 April, two randomly chosen seedlings (one randomly assigned as a control, the other to herbivore treatment) per pollination treatment per family were transplanted to a randomized position within each of two blocks in their home sites, except M13E, which held three blocks. This resulted in a total of 224 (M13E), 220 (M13W), 112 (RC), or 116 (SB) seedlings per block and 1568 plants in the entire experiment. At the time of transplant, experimental plants were similar to the distribution of native seedling sizes at each of the sites. In mid-April, one first- or second-instar spittlebug nymph was placed on plants assigned to the herbivore treatment. Plants were monitored daily and spittlebugs replaced on experimental plants or removed from control plants if necessary. One week after the application of spittlebugs, 35 plants that had been assigned the spittlebug treatment, 16 of which were selfs, had to be removed from the experiment, because spittlebugs repeatedly rejected the plants and would not establish a feeding site, probably due to the unusually small size of these plants. This nonrandom removal of plants may have slightly biased our results against finding herbivore effects. Spittlebugs fed on plants for a maximum of 20 (M13W) to 31 (RC) d. In late May, we harvested plants from the two driest sites (M13E and M13W). Plant harvest at the two wetter sites was delayed until mid-June (RC) and mid-July (SB) to permit further plant growth. We recorded the number of buds with expanded pedicels and the number of open flowers or fruits on each plant, and collected all above-ground plant material. Each plant was also categorized into one of three developmental stages: (1) rosette, (2) bolting, or (3) with buds or open flowers. Finally, we recorded the survivorship of all plants between the time of spittlebug application and harvest. Plant material was dried to a constant weight in a 50°C oven and weighed to the nearest 0.001 g.

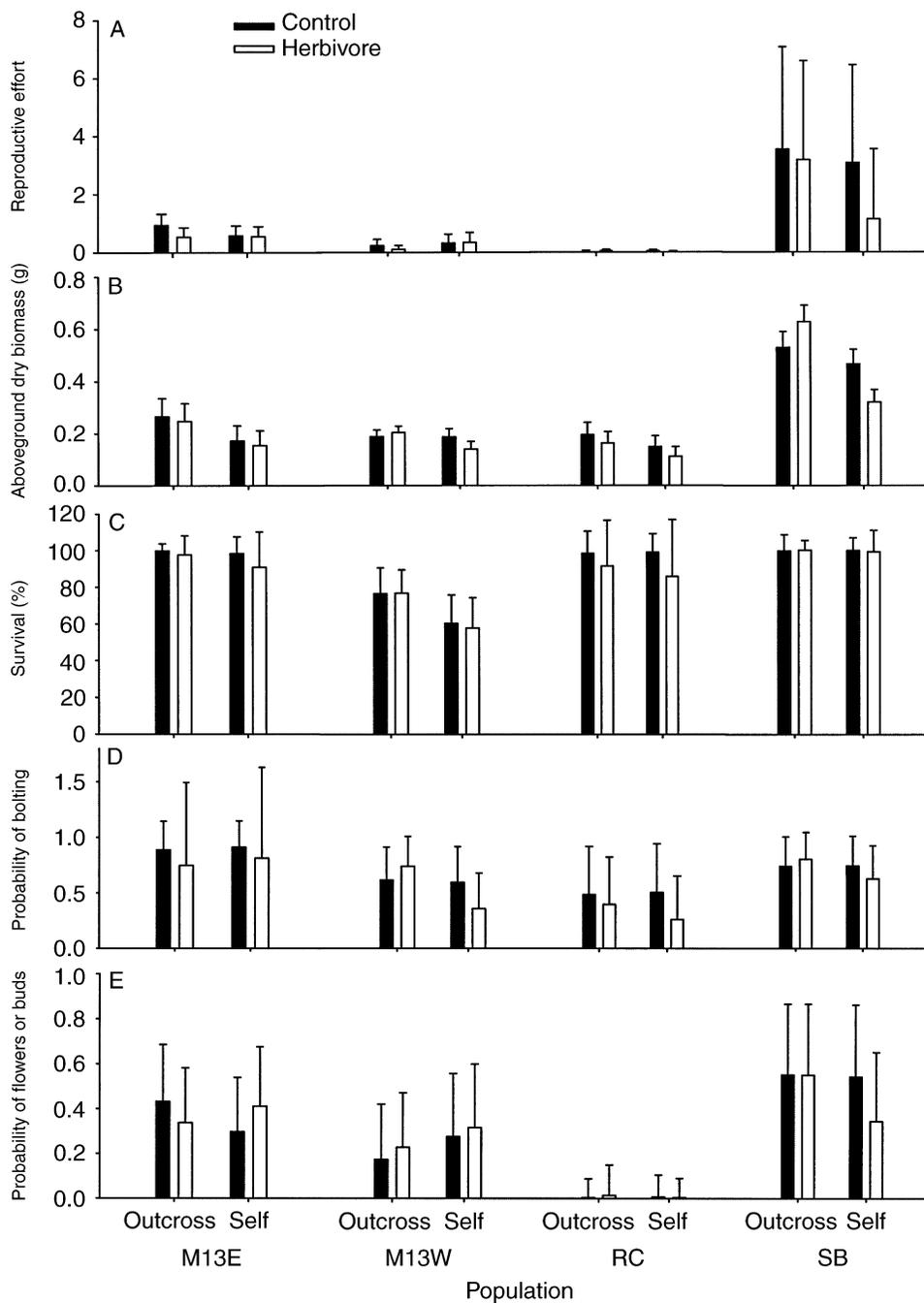


FIG. 1. Least-squares means of (A) reproductive effort (the sum of the number of flowers, fruits, and buds), (B) aboveground biomass, (C) percentage survival, (D) probability of plants reaching the bolting stage, and (E) probability of plants producing flowers or buds for inbred and outbred *Mimulus guttatus* plants with and without spittlebug herbivores in four Napa County, California, populations. Bars represent +1 SE.

Analysis.—We used MANOVA to examine inbreeding effects on tolerance to herbivory in two fitness components of plants: (1) reproductive effort, measured as the sum of the number of flowers, fruits, and

buds; and (2) aboveground dry biomass, which is strongly correlated with total seed mass in natural populations of *M. guttatus* from northern California ($0.68 < r < 0.80$; Fenster and Ritland 1994). The model had

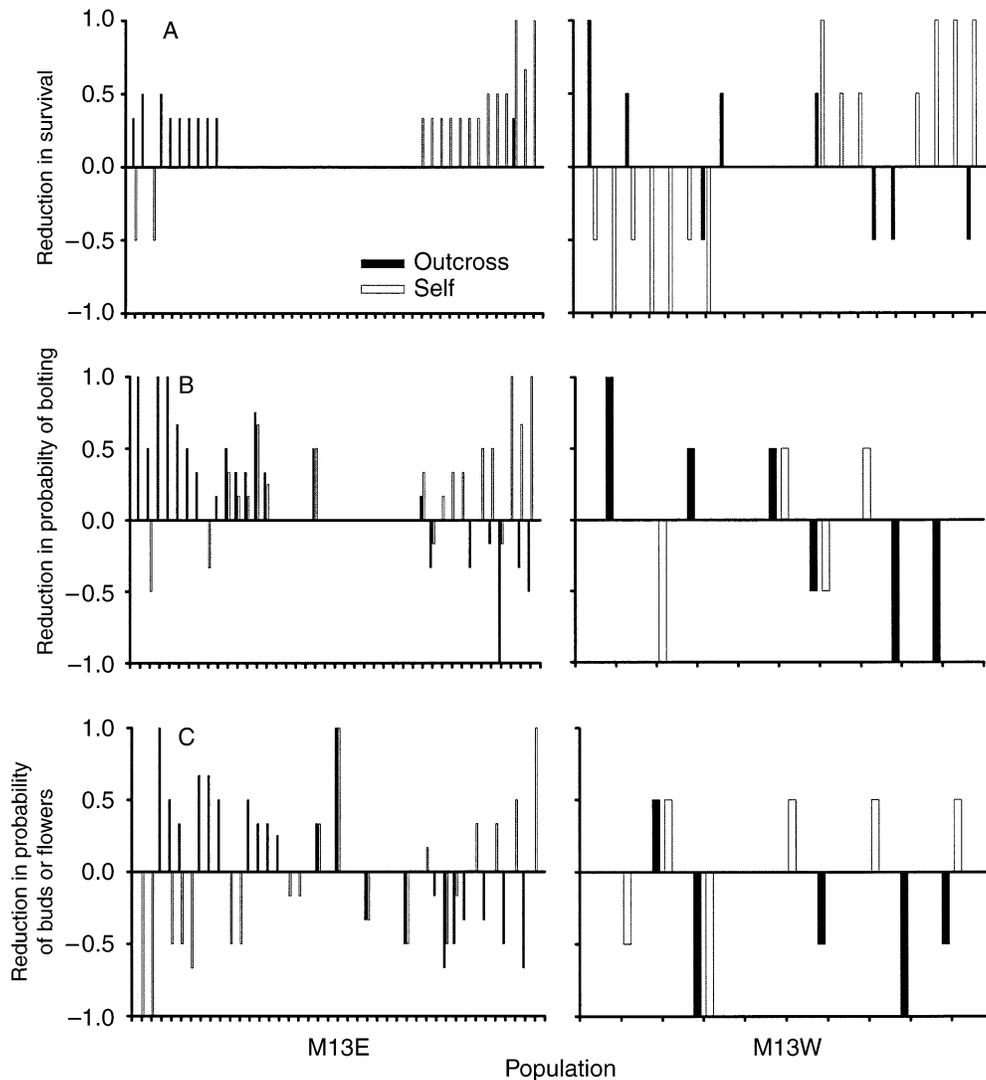


FIG. 2. Mean family-level reduction in (A) survival, (B) probability of bolting, and (C) probability of producing flowers or buds caused by spittlebug herbivores for outbred and inbred lines from four *Mimulus guttatus* populations growing in Napa County, California. Positive values indicate lower relative tolerance to herbivory (undercompensation), whereas negative values indicate higher tolerance to herbivory (overcompensation). Within each population, families are in rank order from those showing lower tolerance in outbred progeny than in self progeny (outbreeding depression for tolerance) to those with higher tolerance in outbred lines (inbreeding depression). Apparent gaps in data indicate families with mean reduction of zero (perfect compensation). Only families for which both self and outcross lines were present are plotted.

seven fixed effects: population, pollination treatment (self or outcross), and herbivore treatment (spittlebug or control), and all interactions. This model also had five random effects: block nested within population, family nested within population, pollination \times family within population, herbivore \times family within population, and pollination \times herbivore \times family within population. Because experimental treatment terms or their interactions were significant in this analysis (see *Re-*

sults), we fitted univariate mixed-model ANOVAs to these fitness components as well, using PROC MIXED (SAS Institute 2001). These models included the same fixed and random effects as above. A significant interaction between the pollination and herbivore treatments was interpreted as evidence for an effect of inbreeding on tolerance to herbivory. The significance of covariance components associated with random effects was tested hierarchically using likelihood ratio tests

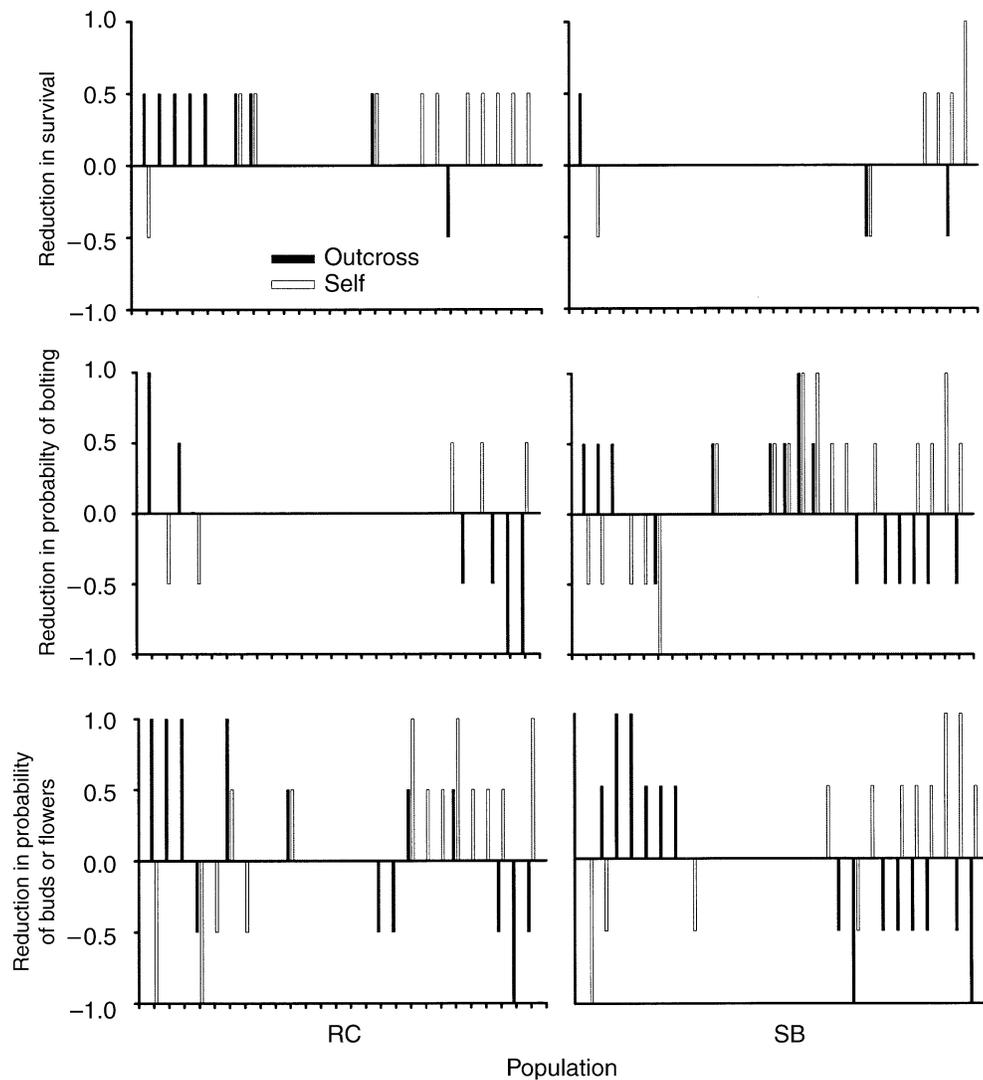


FIG. 2. Continued.

(Littell et al. 1996). Reproductive effort and biomass were square-root transformed prior to analyses to better meet model assumptions.

We also considered three additional components of fitness: (3) survivorship (0 = dead, 1 = alive), (4) the probability of plants reaching the bolting stage (0 = rosette, 1 = bolting or flowering), and (5) the probability of plants producing flowers or buds (0 = rosette or bolting, 1 = with flowers or buds). A majority of experimental plants (68%) did not produce flowers or buds prior to harvest. Nonflowering plants in two populations (M13W and M13E) likely would never have produced flowers or buds due to the rapid seasonal decline in soil moisture that is characteristic of the region. Developmental speed leading to reproduction

(responses 4 and 5) is therefore an important fitness component for these annual plants. Because response variables (3), (4), and (5) were binomial, we modeled them using generalized linear mixed models (GLIMMIX macro of SAS: see Littell et al. 1996) and applied binomial errors to the models. Fixed and random effects were identical to the models already described.

RESULTS

The MANOVA comparing treatment effects on reproductive effort and biomass revealed significant effects of inbreeding (Appendix A, pollination effect, Wilks' lambda = 0.39, $P < 0.0001$), the pollination \times herbivore interaction (Appendix A, Wilks' lambda = 0.92, $P = 0.04$), the three-way interaction between pop-

ulation, pollination, and herbivore (Appendix A, Wilks' $\lambda = 0.96$, $P = 0.03$), and a marginally significant effect of herbivore (Appendix A, Wilks' $\lambda = 0.96$, $P = 0.09$). Therefore, we examined univariate responses to treatment effects as well to determine which of the responses might be driving these results. These results are summarized along with the results from the binomial GLIMMIX models below.

Main effect of pollination treatment.—Significant inbreeding depression was observed for biomass and survival. Across all populations, inbreeding in *M. guttatus* reduced plant biomass by nearly 30% (Fig. 1B, Appendix B, $F_{1,812} = 43.99$, $P < 0.0001$) and reduced the likelihood of surviving by 3.4% (Fig. 1C, Appendix B, $F_{1,468} = 7.29$, $P = 0.007$). Inbreeding had no significant overall effect on reproductive effort or development (Appendix B). The reduction in biomass due to inbreeding ranged from 17% to 37% among populations, although the population \times pollination treatment interaction was only marginally significant (Fig. 1B, Appendix B, $F_{3,797} = 2.29$, $P = 0.08$).

Main effect of spittlebug herbivores.—Spittlebug damage to plants resembled what has been observed in greenhouse experiments (Carr and Eubanks 2002); leaves were misshapen and curled, and stems were curled or internodes shortened where feeding sites were established. Spittlebug nymphs caused a marginally significant reduction in biomass of 11% across all populations (Fig. 1B, Appendix B, $F_{1,328} = 3.28$, $P = 0.07$). In addition, they reduced the likelihood of survival by 4.2% (Fig. 1C, Appendix B, $F_{1,487} = 14.10$, $P = 0.0002$) and reduced the likelihood of bolting by 15% (Fig. 1D, Appendix B, $F_{1,293} = 6.62$, $P = 0.01$). The impact of spittlebugs on survival varied significantly among populations, ranging from a 0.1% increase in SB to an 8.4% decline in RC (Fig. 1C, Appendix B, $F_{3,436} = 5.48$, $P = 0.001$).

Pollination \times herbivore interaction.—Inbreeding significantly reduced tolerance to spittlebug herbivores with respect to biomass; across all populations, outcrossed plants with spittlebugs had a 2.5% increase in biomass, whereas the biomass of selfed plants with spittlebugs was reduced 25% relative to controls (Fig. 1B, Appendix B, $F_{1,812} = 7.18$, $P = 0.008$). Inbreeding had no significant overall effect on tolerance with respect to reproductive effort, survival, or development (Appendix B). We found marginally significant variation among populations in the effect of inbreeding on tolerance for two fitness traits; the biomass data, for example, indicated only moderate effects of inbreeding on tolerance in populations M13E and RC, whereas inbred plants in populations M13W and SB had large reductions in tolerance (Fig. 1B, Appendix B, $F_{3,797} = 2.29$, $P = 0.07$). Similarly, inbred plants with spittlebugs had an increased probability of producing buds

or flowers in M13E, whereas inbreeding reduced tolerance to spittlebugs in SB (Fig. 1E, Appendix B, $F_{3,156} = 2.44$, $P = 0.07$).

Variation among plant families.—Some of the strongest effects that we observed involved the family term (Appendix B). Plant families varied significantly in reproductive effort (Appendix B, $\chi^2 = 29.45$, $P < 0.0001$) and in the probability of bolting (Appendix B, $\chi^2 = 33.29$, $P < 0.0001$). Families varied in the effect of inbreeding on reproductive effort (Appendix B, $\chi^2 = 3.98$, $P = 0.02$), survival ($\chi^2 = 21.81$, $P < 0.0001$), the likelihood of producing flowers or buds ($\chi^2 = 19.22$, $P < 0.0001$), and in the probability of bolting ($\chi^2 = 16.83$, $P < 0.0001$). The effect of spittlebugs also varied among families with respect to plant biomass (Appendix B, $\chi^2 = 9.86$, $P = 0.0008$), and the probability of producing flowers or buds ($\chi^2 = 10.56$, $P = 0.0006$). Finally, we found significant three-way (pollination \times herbivore \times family) interactions for survival (Fig. 2A, Appendix B, $\chi^2 = 195.55$, $P < 0.0001$), the likelihood of bolting (Fig. 2B, Appendix B, $\chi^2 = 96.52$, $P < 0.0001$), and the probability of producing buds or flowers (Fig. 2C, Appendix B, $\chi^2 = 19.44$, $P < 0.0001$). These three-way interactions indicate that the effect of inbreeding on tolerance to herbivory varied among plant families. Wide variation in tolerance among families (Fig. 2) may have limited our ability to detect significant trends in main effects of inbreeding on tolerance for these traits (Fig. 1, Appendix B).

DISCUSSION

There are three primary results from our study. First, we found that inbreeding can alter the outcome of plant–insect interactions, including the expression of tolerance to herbivory, in natural settings. We observed variation among traits for this effect, but this result remains significant as a demonstration that inbreeding, which is common in *M. guttatus* as well as many other plants (Vogler and Kalisz 2001), can alter ecological interactions between plants and herbivores in nature. Second, we found significant variation among plant families in the effect of inbreeding on tolerance to herbivory. This variation establishes the opportunity for natural selection to improve population-level tolerance to herbivory in an inbreeding population. Finally, our results indicate that herbivores can alter the expression of inbreeding depression in plants, which implicates a possible role for herbivores in selection for plant mating systems (Ashman 2002).

We observed considerable variation in the effects of inbreeding on tolerance to herbivory. Previous studies have also reported variable effects of inbreeding on interactions with natural enemies (Núñez-Farfán et al. 1996, Ouborg et al. 2000, Carr and Eubanks 2002, Carr et al. 2003). The effects of inbreeding in a population

can depend on its inbreeding history (Husband and Schemske 1996) or the history of its selection regime. Variation among family lines in inbreeding effects can also result from inbreeding history (Uyenoyama and Waller 1991) or from differences among lines in the frequency of deleterious mutations (Schultz and Willis 1995). Finally, for any additive trait, some family-level variation from inbreeding is expected simply due to variation in breeding values among genotypes, which can be observed in the fitness of the self-fertilized progeny (Schultz and Willis 1995). Thus, the variation in inbreeding effects on tolerance that we observed was not altogether unexpected.

Nonetheless, this variation is significant for the ecology and evolution of plant–herbivore interactions. Tolerance decreased with inbreeding in 14–43% of families within populations with respect to survival, whereas tolerance increased or remained constant with inbreeding among the remaining families (Fig. 2). Similar variation was observed for other fitness traits such as the probability of producing buds or flowers (23–41% of families decreased in tolerance with inbreeding) and the probability of bolting (0–42% of families decreased in tolerance with inbreeding). The alleles represented in family lines with decreased tolerance are therefore less likely to be represented in subsequent generations in an inbreeding population infested by herbivores. Over time, average population-level tolerance would be expected to increase under these circumstances. To the extent that herbivore performance is also determined by plant inbreeding, the demography of plants and herbivores could also be impacted (Stinchcombe 2002). Although we were unable to measure herbivore performance in our study, a greenhouse study by Carr and Eubanks (2002) found a significant decrease in mean host plant quality of M13W plants with inbreeding, which suggests that herbivore populations may also decline with plant inbreeding (Awamack and Leather 2002). The wide variation among families, including increased tolerance with inbreeding, implicates a role for deleterious recessive alleles in the genetic control of tolerance (see also Ouborg et al. 2000, Carr et al. 2003). This contrasts with most previous studies of vertebrates, which show more consistent declines in defense against natural enemies with inbreeding (e.g., Arkush et al. 2002), possibly due to the importance of variation at major histocompatibility complex (MHC) loci for defense.

Our results indicate that spittlebugs altered the expression of inbreeding depression in *M. guttatus*. Inbreeding depression for biomass (calculated as $\delta = [w_o - w_s]/w_o$, where w_o and w_s are outcross and self mean fitness, respectively) more than doubled in plants with spittlebugs (Fig. 1). The effect of spittlebugs on inbreeding depression also varied significantly among

plant families for three traits (Fig. 2). Interestingly, most of our estimates of inbreeding depression were lower than previous estimates of inbreeding depression for similar traits in *Mimulus guttatus* (Willis 1993, Carr and Dudash 1996, Carr and Eubanks 2002), even though most previous studies were conducted in greenhouses. One study (Willis 1993) explicitly compared *M. guttatus* families grown in the greenhouse and field and found that inbreeding depression generally increased in the field (7–19% for survival, 12% increase for flower number in one population, 5% decrease in a second). Other studies have also commonly found inbreeding depression to be higher in the field (Keller and Waller 2002). In contrast, we found inbreeding depression for biomass in M13W in the field to decrease 56% (spittlebug plants) and 91% (control plants) relative to what was observed by Carr and Eubanks (2002) in greenhouse studies of this population. Inbreeding depression for reproductive effort in M13W in the field decreased as well relative to that observed for flower number in the greenhouse (Carr and Eubanks 2002). Perhaps more significantly, we found the variance among families in inbreeding depression for M13W to increase in the field both for biomass (a 44% increase for control plants, 83% for spittlebug plants) and reproductive effort (250% for control plants, 370% for spittlebug plants) relative to the greenhouse study of Carr and Eubanks (2002). This increase in variance may indicate that natural environments, and especially spittlebugs on plants in natural environments, provide greater opportunities for selection among lines for traits influenced by inbreeding depression, such as those associated with, for example, mating systems (Crow and Kimura 1970). This result has broader implications for the evolution of plant mating systems; if herbivores magnify variation among family lines in inbreeding depression in natural settings, herbivores may change the persistence or spread of self-fertile mutants in populations (Ashman 2002, Carr and Eubanks 2002).

To our knowledge, this study marks the first attempt to examine the impacts of inbreeding on plant tolerance to herbivory in a natural setting. We found that inbreeding can change the tolerance of plants to herbivory, that this effect can vary significantly among family lines, and that herbivores can change the expression of inbreeding depression. These findings underscore the importance of monitoring inbreeding risk in small populations under conservation because of the potential for added vulnerability due to interactions with natural enemies. Inbreeding stands out as an important phenomenon in natural settings not only because of its direct effects on fitness, but also because of its indirect fitness effects via interactions with natural enemies.

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APPENDIX A

Results from a MANOVA testing the effects of experimental inbreeding on tolerance to herbivory by the spittlebug *Philaenus spumarius* in four Napa County (California, USA) field populations of *Mimulus guttatus* are available in ESA's Electronic Data Archive: *Ecological Archives* E085-013-A1.

APPENDIX B

Results of univariate mixed-model ANOVA testing the effect of experimental inbreeding on tolerance to herbivory by *Philaenus spumarius* in four Napa County (California, USA) field *Mimulus guttatus* populations are available in ESA's Electronic Data Archive: *Ecological Archives* E085-013-A2.