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Reviewed work(s):

Source: *Ecology*, Vol. 87, No. 11 (Nov., 2006), pp. 2717-2727

Published by: [Ecological Society of America](#)

Stable URL: <http://www.jstor.org/stable/20069291>

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CONSEQUENCES OF VEGETATIVE HERBIVORY FOR MAINTENANCE OF INTERMEDIATE OUTCROSSING IN AN ANNUAL PLANT

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Abstract. Given the occurrence of mixed mating systems among plants, a general mechanism explaining the evolution and maintenance of this condition is needed. Although numerous theoretical models predict mixed mating to be evolutionarily stable, conditions favoring intermediate selfing are often stringent and have limited applicability. Here we investigated the role of vegetative herbivory, a ubiquitous biotic factor limiting plant reproduction, in the mating system expression of *Impatiens capensis* (Balsaminaceae), a species with an obligate mixed-mating system (individuals produce both selfing, cleistogamous, and facultatively outcrossing, chasmogamous flowers). Herbivory reduced proportional chasmogamous reproduction partially, but not entirely, through a reduction in plant size and the strength of this effect varied among replicates. Herbivory decreased geitonogamous selfing in chasmogamous flowers via several mechanisms including reduced chasmogamous flower display size and pollinator visitation rate and altered pollinator composition. Overall, herbivory caused a decrease in whole-plant outcrossing, indicating that the effects of herbivory on proportional chasmogamous reproduction, which favor selfing, outweigh the effects on chasmogamous outcrossing rate, which favor outcrossing. Not only do our findings unravel the mechanisms underlying herbivore-mediated changes in the mating system, but they also point to the role of natural enemies in contributing to the maintenance of a mixed mating system.

Key words: *Impatiens capensis*; inbreeding; mating system; outcrossing; plant–pollinator interactions; vegetative herbivory.

INTRODUCTION

Nearly 40% of angiosperms surveyed exhibit intermediate levels of outcrossing (Goodwillie et al. 2005). Understanding the ecological and genetic causes of this variation is a major research focus because changes in mating patterns have profound effects on individual fitness (Charlesworth and Charlesworth 1987), the genetic structure of populations (Hamrick and Godt 1990), as well as patterns of speciation (Barrett 1990). While several factors can influence selection for and the maintenance of mixed mating, most theoretical and empirical examinations have focused primarily on genetic aspects (e.g., Lande and Schemske 1985, Uyenoyama 1986, Charlesworth and Charlesworth 1990, Chang and Rausher 1999) or the role of pollination biology (e.g., Holsinger 1991, Schoen et al. 1996, Kalisz et al. 2004). However, the few models that have explicitly considered interactions between organisms and their natural enemies predict selection for intermediate selfing over much of the parameter space

(Lively and Howard 1994, Agrawal and Lively 2001). Despite these predictions, little empirical data exist regarding the effect of enemies on plant mating system (but see Levri and Real 1998, Elle and Hare 2002, Steets and Ashman 2004, Ivey and Carr 2005).

Herbivory is the primary antagonism limiting plant reproduction (Marquis 1992), and evidence is accumulating that it can alter mating system related traits (Levri and Real 1998, Elle and Hare 2002, Steets and Ashman 2004, Ivey and Carr 2005). However, we have little understanding of the mechanisms underlying herbivory-induced changes in mating system, although a number of possibilities exist. First, in plants that produce heteromorphic flowers—small, selfing (cleistogamous, CL) and large, open (chasmogamous, CH) flowers—leaf damage can alter allocation to flower types, reducing the proportional production of CH flowers (Steets and Ashman 2004, Steets et al. 2006). This change in relative heteromorphic flower production could have two causes: (1) it may be a passive response to reductions in plant size due to resource limitation with herbivory (van Kleunen and Fischer 2005), such that plants experiencing herbivory are unable to surpass a size threshold necessary for CH flower production (Schmitt et al. 1987) or (2) it may result from altered floral meristem fate, such that plants experiencing higher herbivory actively differentiate fewer of their primordia to CH flowers.

Manuscript received 21 October 2005; revised 24 March 2006; accepted 13 April 2006. Corresponding Editor: C. M. Herrera.

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Second, herbivory can cause reductions in floral attractive traits and floral display size (e.g., Strauss et al. 1996, Mothershead and Marquis 2000, Elle and Hare 2002, Steets and Ashman 2004), which in turn, may influence outcrossing by increasing autogamy (within-flower selfing) and/or decreasing geitonogamy (among-flower selfing). For example, Elle and Hare (2002) found that herbivory reduced floral display size of *Datura wrightii*, and plants with smaller displays had higher outcrossing rates. Third, pollinator abundance and faunal composition may change with herbivory-induced changes in flowering phenotype (Steets and Ashman 2004), which may have consequences for outcrossing rate if pollinating taxa differ in their propensity to visit multiple flowers on a plant. In addition to these mechanisms, herbivory may also influence post-pollination processes, and thus mating system (Levri and Real 1998). Given that many of the proposed mechanisms of herbivore-mediated changes in mating system oppose one another, it is crucial that we simultaneously study their combined effect on outcrossing, as this will provide insight to the potential role of herbivory in promoting mixed mating.

In this study, we manipulated levels of insect herbivory in natural *Impatiens capensis* populations to determine whether herbivory has consequences for outcrossing rate. Specifically, we addressed three main questions: (1) Does herbivory influence plant reproduction and reduce proportional CH reproduction? (2) Does herbivory alter the outcrossing rate of CH flowers? and (3) What are the combined effects of herbivory for whole-plant outcrossing rate? In addition, we examined the mechanisms by which herbivory changes the mating system of *I. capensis*. First, we investigated two mechanisms of herbivory-induced changes in heteromorphic flower production: plant size and meristem fate. Second, we investigated mechanisms by which herbivory alters the outcrossing rate of CH flowers: reductions in CH floral display or flower size and changes in pollinator abundance or composition.

MATERIALS AND METHODS

Study system

Impatiens capensis Meerb. (Balsaminaceae) is a native annual that occurs throughout moist forests in eastern North America (Schemske 1978). It is suited to the objectives of this study as it exhibits an obligate mixed-mating system by producing heteromorphic flowers. Specifically, an individual can produce both closed, obligately selfing (CL) and facultatively outcrossing (CH) flowers. The CH flowers are not capable of autogamy because of strong temporal separation of anther and stigma maturation; however, selfing may occur due to geitonogamy. Further, previous studies have found broad variation in the outcrossing rate of CH flowers among populations (e.g., outcrossing rate [t_{CH}] = 0.29–0.71; Waller and Knight 1989).

Herbivory is common in *I. capensis* populations (see Schemske 1978), and the vegetative damage in the populations studied here was primarily caused by chrysomelid beetles, leaf miners, caterpillars, aphids, grasshoppers, and katydid (Steets 2005).

Consequences of herbivory for outcrossing rate

In three wild *I. capensis* populations in Crawford County, Pennsylvania near the Pymatuning Laboratory of Ecology (PLE, ~41°38' N, 80°26' W; see Steets 2005 for further information regarding populations L, T, W), we randomly selected six (W in 2002) or 14 1-m² plots (L, T, W in 2003). The populations were separated from each other by 2–10 km. Plots within populations were separated from one another by at least 5 m, and within each plot, 10 (2002) or seven (2003) individuals were tagged. Plots were assigned to one of two treatments: (1) reduced or (2) natural herbivory. Herbivory was reduced by applying two insecticides (Conserve [Dow Agro-Sciences, Indianapolis, Indiana, USA] and Endeavor [Syngenta Crop Protection, Greensboro, North Carolina, USA]) that reduce herbivory without affecting pollinator visitation to or growth or reproduction of *I. capensis* (Steets 2005: Appendix A), biweekly to plants in reduced herbivory plots. These insecticides reduce herbivory by the primary herbivores observed in populations in northwestern Pennsylvania (J. A. Steets, *personal observation*); Conserve inhibits feeding by chrysomelid beetles, leaf miners, and caterpillars whereas Endeavor inhibits feeding by aphids and whiteflies. Natural herbivory plots were sprayed with water at the same frequency to serve as a control. Insecticide applications began pre-flowering and continued until three weeks prior to a frost that killed the majority of plants. Damage levels were assessed one month prior to the end of the experiment by quantifying the number of leaves and the number of leaves with herbivore damage per plant.

To determine if herbivory causes a reduction in the proportional production of CH flowers, fruits, and seeds, we quantified CL and CH flower production on all tagged plants in each plot and estimated CL and CH fruit and seed production on these plants in 2002 and on three marked individuals per plot in 2003. We also collected up to 15 CL and 15 CH fruits from each tagged plant and calculated total CL and CH seed production as the product of CL and CH fruit production and plot mean CL and CH seed production per fruit, respectively. From these measures, we calculated the proportion CH flowers, fruits, and seeds. To determine whether herbivory affected CL or CH reproduction or proportion CH flowers, fruits, and seeds, we performed separate ANCOVAs (PROC GLM, SAS Institute) on the plot means of these traits. We considered each population–year combination a separate replicate in the ANCOVAs, and while this confounds the sources of site–year variation, we were most interested in controlling for spatiotemporal variation rather than exploring

the source of it. Herbivory treatment was designated a fixed effect and replicate and herbivory treatment \times replicate were random effects. To explore significant interaction terms further, we performed Tukey's multiple comparison tests. Initially we ran the full model. When the interaction term was nonsignificant (P to remove >0.15), we constructed and tested a reduced model by removing this term. Initial plant height (measured prior to first insecticide application) was included as a covariate to correct for any differences between treatments in initial plant size. Prior to analysis, proportion CH flowers, fruits, and seeds were arcsine-transformed to improve normality (Zar 1999).

To determine whether mating system of CH flowers is altered by herbivory, we estimated CH outcrossing rate using allozyme markers. We randomly selected 3–15 seeds (8.3 ± 0.57 progeny assayed/mother; 30.2 ± 5.04 seeds assayed/plot; mean \pm SE) from each tagged plant for allozyme analysis. In addition, electrophoretic phenotypes of maternal leaf tissue (2002) or five selfed CL seeds (2003) were used to infer the maternal phenotype. Enzymes were extracted from fresh or frozen (-70°C) tissues with the extraction buffers of Lu (1995) or Mitton et al. (1979), respectively. Extracts were absorbed through Miracloth (Calbiochem, La Jolla, California, USA) onto filter paper wicks and stored at -70°C until electrophoretic analysis. For plants sampled in 2002, we resolved four polymorphic loci on four one-locus systems (aspartate aminotransferase [*Aat*, EC 2.6.1.1], menadione reductase [*Mnr*, EC 1.6.99.2], phosphoglucosomerase [*Pgm*, EC 5.4.2.2], and uridine diphosphoglucose pyrophosphorylase [*Ugpp*, EC 2.7.7.9]). For plants sampled in 2003, we resolved two additional loci on one enzyme system, isocitrate dehydrogenase (*Idh*, EC 1.1.1.42). Further details of electrophoretic procedures are in Steets (2005). The differences in number of loci used between years did not affect the estimates of outcrossing rate (data not shown).

We used the Newton-Raphson procedure in Ritland's MLTR program (version 3.0; Ritland 2002) to estimate the multilocus t_{CH} and bootstrapped standard errors in the reduced and natural herbivory treatments of each replicate. Although outcrossing rate has parameter bounds of 0 and 1, with sampling error the maximum likelihood estimate of outcrossing rate can fall outside these bounds (Ritland 2002). The effect of herbivory on t_{CH} in each replicate was examined with a t test. We performed a weighted Z test (Whitlock 2005) to determine whether herbivory increased t_{CH} across all replicates. Because natural variation in damage level (see *Results*) existed among replicates, we additionally explored the relationship between damage on t_{CH} at the plot level using linear regression (PROC REG, SAS Institute). For this analysis we only included plots for which there were two or more plants to estimate t_{CH} .

We calculated the whole-plant outcrossing rate for each plot by taking the product of plot mean t_{CH} and plot mean proportional production of CH seeds per

plant. For plots in which we did not have adequate sample size to estimate t_{CH} we used the replicate-treatment mean t_{CH} in the previous calculation. The effect of herbivory on whole-plant outcrossing rate in each replicate was examined using t tests. We performed a weighted Z test (Whitlock 2005) to determine if herbivory affected whole-plant outcrossing rate across replicates.

Mechanisms of herbivory-induced change in heteromorphic flower production

Plant size mechanism.—If a height threshold must be reached to initiate CH flower production (Schmitt et al. 1987), then changes in proportional CH reproduction with herbivory could be a passive consequence of reductions in plant size. To test this hypothesis, we used a series of ANCOVAs to determine whether herbivory-mediated reductions in plant height led to reductions in proportional production of CH flowers, fruits, and seeds. First, we determined whether herbivory had a significant effect on final plant height. Then, in separate ANCOVAs for each of the three mating system metrics (proportion CH flowers, fruits, and seeds), we included final plant height, in addition to initial plant height, as a covariate. The mating system metrics were arcsine-transformed prior to analysis to improve normality (Zar 1999). For each mating system metric, we compared the proportion of variance explained (Gotelli and Ellison 2004) by herbivory treatment with and without final height in the ANCOVA.

Meristem fate mechanism.—To determine whether the change in proportion CH flowers, fruits, and seeds with herbivory was due to altered floral meristem fate (i.e., a floral meristem converting from CH to CL), we conducted a separate experiment in which one individual from each of 19 pairs of full-sib plants (from six populations) was assigned to either a reduced or natural herbivory treatment (as before). We then recorded the total number of nodes on the central axis and nodal location of the first CH flower. To determine if the node of the first CH flower differed with herbivory, we performed an ANOVA with herbivory treatment as a fixed effect and population and treatment \times population as random effects. The treatments did not differ in total node production ($P = 0.5$).

Mechanisms of herbivory-induced change in CH outcrossing rate

Floral display and flower size mechanism.—We quantified CH floral display size on three days during peak CH flowering on plants in the outcrossing rate experiment. To determine if herbivory reduces CH display size, we conducted mixed-model ANOVA (PROC GLM, SAS Institute) on plot mean CH display size as before. Outcrossing rate is expected to decrease in a nonlinear (i.e., decelerating exponential) manner as floral display size increases (Rademaker et al. 1999, Vrieling et al. 1999). To test this idea we determined if

CH display size was related to t_{CH} by performing nonlinear regression (PROC NLIN, SAS Institute) on plot means of these traits for 2003 plots. For these analyses we only included plots for which there were two or more plants to estimate t_{CH} .

To determine whether herbivory alters CH flower size, we also measured eight floral traits on one randomly selected female-phase flower per plot and performed a principal components analysis (PROC FACTOR, SAS Institute) following the procedure of Steets (2005). The first principal component explained 60% of the variance among plants in floral traits and all traits loaded positively (all loadings >0.46), indicating that it reflects overall flower size, so we explored the effect of herbivory on this principal component using mixed model ANOVA (as before). To determine if CH flower size is related to t_{CH} , we performed a linear regression (as before) between plot mean t_{CH} and CH flower size for plots in 2003. We expected that the larger-bodied bumble bees, that forage among flowers on a plant, might avoid small flowers and thus outcrossing might increase with a reduction in flower size.

Pollinator abundance and composition mechanism.—To determine whether herbivory alters pollinator abundance or composition, we observed visitation to flowers in the reduced and natural herbivory treatment of the W 2003 replicate of the outcrossing rate experiment. Specifically, across five days, we observed pollinator visitation in five reduced and three natural herbivory plots. On a given day, we recorded pollinator visitation to all open flowers in a plot for 15-min and rotated observation periods between herbivory treatments. In a total of 16 h of observation, we recorded 194 visits by bumble bees including *Bombus vagans* (Apidae), honey bees including *Apis mellifera* (Apidae), and small bees including *Augochlorella striata* (Halictidae). Pollinator identifications were made by Dr. John Rawlins at the Carnegie Museum of Natural History, and voucher specimens were filed at the Museum. We used log-likelihood G test (Zar 1999) to determine if pollinators undervisit flowers in natural herbivory plots relative to reduced herbivory plots. In addition, we determined whether the pattern of visitation (i.e., relative proportion of visits made) differed among pollinator types between herbivory treatments using a heterogeneity G test (Zar 1999). In both analyses flower visits were considered independent events and the unit of sampling was individual flowers within a plot.

To determine whether CH floral display and flower size affect pollinator abundance or composition in ways that could affect t_{CH} , we conducted two additional experiments. To determine if pollinator groups (bumble bees, honey bees, small bees) differ in their propensity to visit multiple open flowers on a plant (i.e., affect geitonogamous selfing), we set out arrays of four potted *I. capensis* plants with varying numbers of open CH flowers (18 arrays with 1, 2, 3, and 4 open flowers and six arrays with 2, 4, 6, and 8 open flowers) that span the

natural range of floral display size (0–10 open CH flowers, $N = 98$). During 30-min observation periods on seven days we recorded the total number of pollinator visits and geitonogamous visits by each pollinator group. We performed linear regressions (PROC REG, SAS Institute) between the mean number of geitonogamous visits and CH display size for each pollinator group.

To determine if the abundance of the different pollinating fauna is related to CH flower size, we transplanted 75 *I. capensis* seedlings from three populations into pots filled with Fafard number 4 soil (Conrad Fafard, Agawam, Massachusetts, USA). On three days during peak CH flowering, we quantified seven CH floral traits (lateral petal length and width, spur length, opening height, flower length, upper and lower petal length) using calipers and observed visitation by bumble bee, honey bee, and small bee pollinators to these flowers for 1 h (3 h total observation time). We performed a principal components analysis on the floral trait data (PROC FACTOR, SAS Institute) to estimate overall flower size via the first principal component, which explained 61% of the variance among plants in floral traits, and all traits loaded positively (all loadings >0.54). We performed linear regressions (PROC REG, SAS Institute) between visitation rate and CH flower size for each pollinator group (bumble bees, honey bees, and small bees).

RESULTS

Leaf damage

Overall, insecticide applications reduced herbivory from a mean of 35% to 16% leaf damage (mean reduction, 54%; $F_{1,38} = 11.3$, $P = 0.04$), but the size of the reduction varied among replicates (Fig. 1, $F_{3,38} = 4.5$, $P = 0.008$). W 2002 and W 2003 replicates had the highest natural levels of leaf damage, and thus showed the greatest reduction with insecticide application, i.e., herbivory was reduced by 70% and 61%, respectively. Whereas replicates with lower natural herbivory showed lower reductions, i.e., 52% in L and 20% in T (Fig. 1).

Does herbivory influence plant reproduction and reduce proportional CH reproduction?

Compared to reduced herbivory plants, plants experiencing natural herbivory had a 59–70% reduction in all components of CH reproduction (Table 1, Appendix) and a 16–23% reduction in CL reproduction (Table 1, Appendix); however the magnitude of effect depended upon replicate (Appendix, herbivory treatment \times replicate $P < 0.10$). The replicates with the greatest reductions in herbivory (i.e., W 2002 and W 2003) showed the greatest reductions in CH flower, fruit, and seed production, and CL flower production, most of which were individually significant (analysis not shown). Other replicates showed similar patterns but did not reach significance at $P < 0.05$. Combined, the differential effects of herbivory on CL and CH reproduction

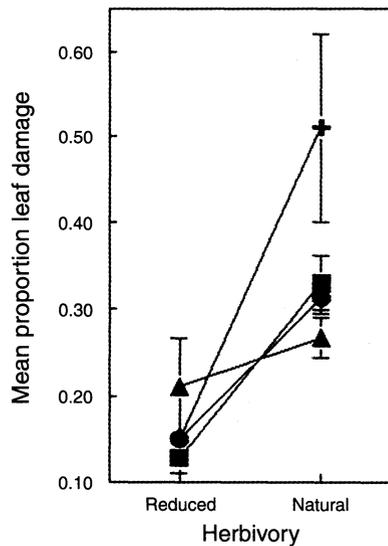


FIG. 1. Least-squares mean proportional leaf damage for *Impatiens capensis* individuals experiencing reduced or natural herbivory. Symbols represent different population replicates: "plus" symbol, W (2002); circle, L; triangle, T; square, W (2003). Error bars represent \pm SE.

resulted in a 42–48% reduction in the proportional production of CH flowers, fruits, and seeds with increasing herbivory (Fig. 2a, Table 1, Appendix, all herbivory treatment $P < 0.001$).

Does herbivory alter the outcrossing rate of CH flowers?

The replicate with the largest magnitude of change in herbivory between treatments showed a significant increase (25%) in t_{CH} with higher herbivory (W 2002: $t = 1.9$, $df = 11$, $P_{1-tail} = 0.04$). Another replicate showed a marginally significant increase (58%, T 2003: $t = 1.6$, $df = 15$, $P_{1-tail} = 0.06$) whereas the other two replicates showed slight (27% and 40%) increases, but these were not individually significant (both, $P_{1-tail} > 0.13$). The general pattern across all replicates was to increase t_{CH} with higher herbivory (mean increase 37%) and this was statistically significant (Fig. 2b, $Z = 2.25$, $P_{1-tail} = 0.01$). Regression analysis across all plots further demonstrates that as the magnitude of herbivory increases t_{CH} also increases (Fig. 2c, CH outcrossing rate = $1.69 \times$ leaf damage + 0.34, $F_{1,12} = 5.8$, $P = 0.03$).

What are the combined effects of herbivory for whole-plant outcrossing rate?

When we considered the consequence of herbivory for whole-plant outcrossing, one out of four replicates showed a significant reduction in whole-plant outcrossing rate (Fig. 2d, L 2003: $t = 2.2$, $df = 12$, $P = 0.05$), and the remaining three replicates showed no statistically significant effect ($P > 0.15$) indicating that in some cases the effects of herbivory on t_{CH} and proportional CH reproduction can negate one another. However, across replicates herbivory caused a significant decrease (aver-

age reduction 28%) in whole-plant outcrossing rate (Fig. 2d, $Z = 2.7$, $P = 0.007$), indicating that on average the effects of herbivory on t_{CH} , which favor outcrossing, are not strong enough to outweigh the effects on proportional chasmogamous reproduction, which favor selfing; thus, the overall effect of herbivory is to reduce outcrossing.

Mechanisms of herbivory-induced change in heteromorphic flower production

Plant size mechanism.—Herbivory reduced final plant height by 10%; however, the magnitude of effect depended upon replicate (Table 1, Appendix, herbivory treatment \times replicate $P < 0.01$). Herbivory significantly reduced plant height in the replicate that experienced the greatest change in herbivory levels among treatments (W 2002, Tukey, $P = 0.04$). When final plant height was included in the analyses of proportion CH reproduction, the proportion of variance explained by herbivory treatment was reduced by 24–29% (proportion CH flowers, from 25.8% to 19.6%; proportion CH fruits, from 23.8% to 17.3%; proportion CH seeds, from 23.7% to 16.9%). These results demonstrate that the herbivory-induced change in mating system is only partially due to the effect of damage on plant height.

Meristem fate mechanism.—Natural herbivory plants tended to produce their first CH flower at an earlier node than reduced herbivory plants, indicating that the fate of floral meristems also changes with herbivory, however this was not statistically significant (least square means \pm SE: 11.8 ± 1.01 vs. 14.1 ± 1.01 ; $F_{1,26} = 3.71$, $P = 0.11$).

Mechanisms of herbivory-induced change in CH outcrossing rate

Floral display and flower size mechanism.—Herbivory significantly reduced CH flower size (PC1 \pm SE: -0.41 ± 0.29 vs. 0.46 ± 0.24 , Appendix, herbivory treatment $P < 0.05$), and tended to reduce CH display size (0.36 ± 0.16 vs. 1.01 ± 0.18 open flowers/plant, Appendix, herbivory treatment $P = 0.2$); however the effect of herbivory on the latter trait varied with replicate

TABLE 1. Least-squares means (\pm SE) of *Impatiens capensis* vegetative and reproductive traits by herbivory treatment (reduced, natural).

Trait	Herbivory treatment	
	Reduced	Natural
Height (cm)	62.1 \pm 1.38	56.0 \pm 1.15
No. CL flowers	23.1 \pm 1.17	19.3 \pm 0.98
No. CL fruits	17.3 \pm 1.55	13.3 \pm 1.35
No. CL seeds	20.1 \pm 2.02	15.9 \pm 1.76
No. CH flowers	13.5 \pm 1.33	5.5 \pm 1.12
No. CH fruits	10.4 \pm 1.31	3.4 \pm 1.10
No. CH seeds	22.9 \pm 3.89	6.7 \pm 3.27
Proportion CH flowers	0.26 \pm 0.02	0.15 \pm 0.02
Proportion CH fruits	0.27 \pm 0.03	0.14 \pm 0.03
Proportion CH seeds	0.34 \pm 0.04	0.18 \pm 0.03

Note: CL, cleistogamous; CH, chasmogamous.

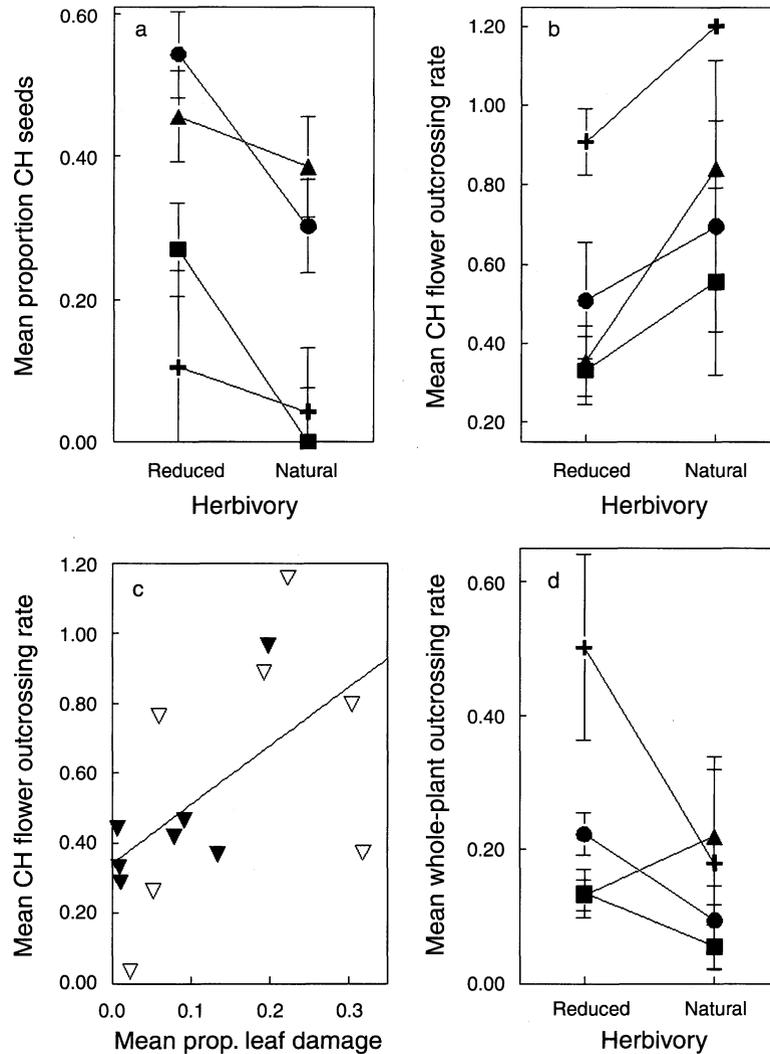


FIG. 2. (a) Least-squares mean proportional production of chasmogamous (CH) seeds per plant and (b) mean CH flower outcrossing rate for *Impatiens capensis* individuals experiencing reduced or natural herbivory. Symbols represent different population replicates: "plus" symbol, W (2002); circle, L; triangle, T; square, W (2003). (c) Plot mean CH flower outcrossing rate as a function of plot mean proportion leaf damage (CH outcrossing rate = $1.69 \times$ leaf damage + 0.34, $F_{1,12} = 5.8$, $P = 0.03$). Symbols represent different herbivory treatments: open triangles, natural herbivory; solid triangles, reduced herbivory. (d) Mean whole-plant outcrossing rate for *I. capensis* individuals experiencing reduced or natural herbivory. Symbols are as in Fig. 2a. Error bars represent \pm SE. Refer to *Results* and *Appendix* for statistics.

(Appendix, herbivory treatment \times replicate $P < 0.05$). Herbivory significantly reduced the CH display size of the W 2002 replicate 86% (Tukey, $P = 0.04$). In addition, there was a significant nonlinear negative relationship between t_{CH} and CH display size (Fig. 3a, $t_{CH} = 0.79 \exp(-0.47 \times \text{CH display size})$, $F_{2,12} = 24.3$, $P < 0.0001$), demonstrating that geitonogamy is reduced in plants with smaller displays. CH outcrossing rate was not significantly affected by CH flower size (Fig. 3b, CH outcrossing rate = $-0.14 \times \text{CH flower size} + 0.6$, $F_{1,11} = 1.5$, $P = 0.25$).

Pollinator composition and abundance mechanism.—Pollinators visited flowers in reduced herbivory plots nearly five times as frequently as those of natural

herbivory plots (1.64 ± 0.24 vs. 0.36 ± 0.16 visits/flower/h, $G = 66.7$, $df = 1$, $P < 0.0001$). Furthermore, bumble bee visitation was depressed more by herbivory than visitation by small bees and honey bees (Fig. 4a, $G_H = 6.21$, $df = 2$, $P < 0.05$). This result is corroborated by the fact that bumble bees respond positively to CH flower size (Fig. 4b, bumble bee visitation = $0.81 + 0.33 \times \text{CH flower size}$, $F_{1,35} = 16.4$, $P = 0.0003$), whereas small bees and honey bees do not (Fig. 4c and d, both $P > 0.6$). The change observed in pollinator composition with herbivory (Fig. 4a) has consequences for CH outcrossing rate because the pollinating taxa differ in their geitonogamous foraging behavior. Bumble bees increase the number of geitonogamous visits with CH

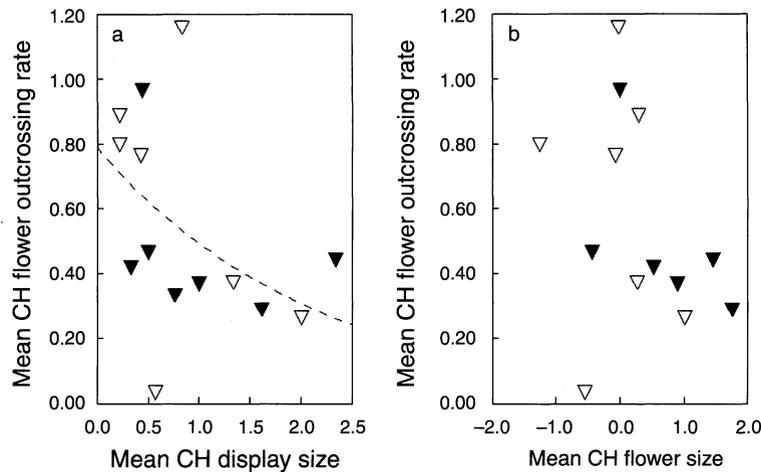


FIG. 3. Relationship between plot mean CH flower outcrossing rate and (a) plot mean CH display size (CH outcrossing rate = $0.79 \exp(-0.47 \times \text{CH display size})$, $F_{2,12} = 24.3$, $P < 0.0001$) or (b) CH flower size (CH outcrossing rate = $-0.14[\text{CH flower size}] + 0.6$, $F_{1,11} = 1.5$, $P = 0.25$). The dashed line represents significant regression. Symbols represent different herbivory treatments: open triangles, natural herbivory; solid triangles, reduced herbivory.

display size (Fig. 4e, number of geitonogamous bumble bee visits = $-0.35 + 0.24 \times \text{CH display size}$, $F_{1,4} = 144.9$, $P = 0.0003$), but small bees and honey bees do not (Fig. 4e, both $P > 0.75$).

DISCUSSION

In this study, we report significant effects of herbivory for plant mating system. First, we found that overall herbivory increased outcrossing of CH flowers due to effects on both CH flowering display and pollinator abundance and composition. Second, we found that herbivory increased selfing at the level of relative heteromorphic flower production due to changes in plant size and floral meristem fate. We expand on these findings next and discuss the implications of our results for the role of natural enemies in maintaining mixed mating.

Effects of herbivory on CH outcrossing

This study demonstrates that herbivory causes a significant increase in the outcrossing rate of CH flowers (Fig. 2b). As selfing in CH flowers of *I. capensis* can only occur via geitonogamy, the increase in CH outcrossing with herbivory was due to a decrease in this mode of selfing. The degree to which geitonogamy was reduced by herbivory depended upon the magnitude of leaf damage experienced by plants (Fig. 2c), with the replicate (W 2002) that experienced the largest change in leaf damage between treatments also showing a significant increase in CH outcrossing rate (Fig. 2b). Our results reveal a few mechanisms for the change in CH outcrossing with herbivory. First, herbivory tended to reduce CH flowering display size, resulting in highly damaged plants receiving fewer geitonogamous pollinator visits relative to those experiencing less damage. Herbivory-induced changes in CH display led to an overall reduction in pollinator visitation and a change in

the composition of visitors. Natural herbivory plants were visited more by small solitary bees and honey bees and less by bumble bees relative to reduced herbivory plants (Fig. 4a). Given that honey and small bees tend to visit only a single open flower on a plant, whereas bumble bees forage in a pattern that promotes geitonogamy (Fig. 4e), the change in pollinator composition with herbivory also contributes to increased CH outcrossing. These findings highlight the need for more detailed studies of the effect of different pollinator species on mating system expression as well as how enemies or other ecological conditions may alter pollinator fauna, and thus the mating system.

Only a few other studies have investigated the effects of herbivory on outcrossing of open-pollinated flowers (Elle and Hare 2002, Ivey and Carr 2005). Elle and Hare (2002) report findings similar to ours; herbivory reduced the floral display of *Datura wrightii*, and plants with smaller displays had higher outcrossing rates. On the other hand, Ivey and Carr (2005) found that herbivory reduced outcrossing in *Mimulus guttatus*, but the mechanism was unclear. To gain a more general understanding of the role of herbivores in plant mating system expression, future work should be designed to determine the relative importance of the various potential mechanisms.

Effects of herbivory on heteromorphic flowering

Although herbivory increased CH outcrossing, at the same time it shifted the mating system toward selfing in terms of relative heteromorphic seed production. The reduction in the proportion of CH seeds was primarily due to herbivory reducing CH reproduction more than CL reproduction (Table 1), a pattern that is also seen in response to other antagonists, e.g., competitors (Steets et al. 2006). Other studies have also demonstrated that stressful abiotic conditions can lead to reduced produc-

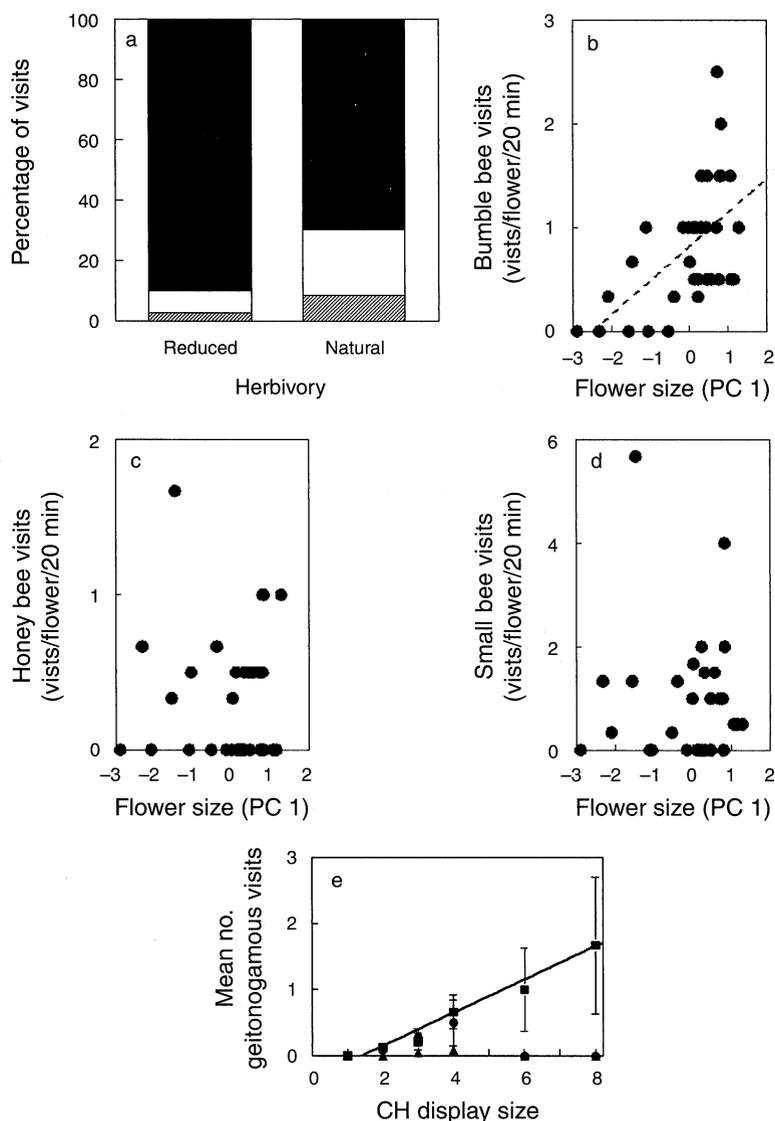


FIG. 4. (a) Percentage of pollinator visits to reduced and natural herbivory plants in the W (2003) population. Shading represents different pollinator classes: solid bar, bumble bees; open bar, honey bees; hatched bar, small bees. (b) Bumble bee visits, (c) honey bee visits, and (d) small bee visits as a function of CH flower size (principal component 1). The dashed line represents significant linear regression between bumble bee visitation and CH flower size (bumble bee visitation = $0.81 + 0.33 \times \text{CH flower size}$; $F_{1,35} = 16.4$, $P = 0.0003$). (e) Geitonogamous pollinator visits made by bumble bees (squares), honey bees (triangles), and small bees (circles) to plants of varying display sizes. The solid line represents the significant linear relationship between geitonogamous bumble bee visitation and CH display size (geitonogamous bumble bee visits = $-0.35 + 0.24 \times \text{CH display size}$; $F_{1,4} = 144.9$, $P = 0.0003$). Error bars represent \pm se.

tion of energetically expensive CH flowers and seeds (Schemske 1978, Waller 1980, Bell and Quinn 1987, Le Corff 1993, Lu 2000). However, prior to the current study, the mechanism for change in relative heteromorphic flower production with stress was unclear. We found that changes in the mating system were due to both passive and active processes. In accord with a previous study on *I. capensis* demonstrating that CH flower production is size dependent (Schmitt et al. 1987), we found that herbivory tended to reduce plant height, which in turn reduced CH flower production. However,

when we controlled for final plant height in the analyses, there was still an $\sim 40\%$ difference between the herbivory treatments in proportional CH reproduction, indicating that other mechanisms also play a role. Specifically, natural herbivory plants tended to produce their first CH flower at an earlier node but produced fewer total CH flowers than reduced herbivory plants. Herbivory treatments did not differ in total node production (data not shown, $P > 0.05$), indicating that natural herbivory plants also stopped CH flower production at an earlier node than reduced herbivory plants. These results

support the idea that floral meristem fate is altered by herbivore damage.

Consequences for the evolution and maintenance of intermediate outcrossing

Three lines of evidence indicate that herbivores may contribute to the maintenance of mixed mating: (1) herbivory has a balancing effect on *I. capensis* whole-plant outcrossing rate, (2) heterogeneity in herbivore pressure may set up conditions thought to select for mixed mating, and (3) herbivory influences parameters thought to stabilize mixed mating. We expand on these ideas next.

As both inbreeding (Waller 1984, Mitchell-Olds and Waller 1985, Schmitt and Ehrhardt 1990, Lu 2002) and outbreeding depression (McCall et al. 1991) can manifest in *I. capensis* populations, it is likely that there exists an intermediate level of outcrossing that maximizes fitness (Waser and Price 1989, Waser 1993). Indeed one study found evidence for intermediate outcrossing distances as an optimal mating system in one *I. capensis* population (McCall et al. 1991). Given that genetic similarity often decreases with distance, it is likely that this optimal intermediate outcrossing distance will translate into an optimal intermediate outcrossing rate (Waser and Price 1989). Our finding that herbivory caused an increase in *I. capensis* selfing at the level of heteromorphic flowering but also a reduction in selfing at the level of CH outcrossing resulting in no significant difference in whole-plant outcrossing between herbivory environments in three replicates (T, W 2002, W 2003), is also suggestive of an optimal outcrossing rate in *I. capensis* populations that is achieved by plastic responses to changing environmental conditions. If CH floral display and relative heteromorphic flower production act in opposing directions to maintain an optimum outcrossing rate, we would expect stabilizing selection for intermediate outcrossing rates, but the actual value might vary with environment. Experiments that determine whether stabilizing selection exists on outcrossing rate, and whether the plastic mating system response seen here is adaptive are needed.

Spatial and temporal variation in the intensity of herbivory (e.g., Louda 1989, Rand 2002, Steets and Ashman 2004) may select for a mixed mating strategy. For example, models that focus on the role of ecological condition in mating system evolution find that resource limitation (e.g., Schoen and Lloyd 1984, Iwasa 1990) or variable pollinator visitation (e.g., Schoen et al. 1996) select for mixed mating. Because herbivory can affect both plant resource status (reviewed in Crawley 1989) and pollination environment (e.g., Strauss et al. 1996, Steets and Ashman 2004; see *Results*) and both can vary spatiotemporally (Louda 1989, Rand 2002, Steets and Ashman 2004; see *Results*), we suggest that this antagonism may drive heterogeneity or stochasticity in resource and pollination environments, and consequently select for mixed mating.

Last, our study indicates that herbivory can influence parameters important to the stability of mixed mating systems. For example, Masuda et al. (2001) modeled the conditions necessary for the evolution of heteromorphic flower production (a mixed mating strategy), and concluded that when geitonogamy in CH flowers increases with increasing floral display size, the outcrossing advantage of CH reproduction is reduced resulting in mixed mating (via heteromorphic flowering) as an evolutionary stable strategy. Our results support this aspect of the model but additional parameters, such as inbreeding depression, must also be quantified to fully parameterize the model and to determine whether herbivory contributes to the maintenance of heteromorphic flower production in *I. capensis*.

Conclusions

Overall, our study sheds new light on whether antagonists play a role in the expression and evolution of mixed mating. We have shown that herbivory alters several key components of mating system, and when coupled with knowledge that herbivore pressure is very common (Marquis 1992) and often heterogeneous (Louda 1989, Rand 2002, Steets and Ashman 2004), our results suggest that enemies contribute to the maintenance of intermediate outcrossing in plants. Empirical studies measuring selection on the mating system in different herbivory environments would greatly add to our understanding of the role of natural enemies in the evolution of mixed mating.

ACKNOWLEDGMENTS

We thank J. Byrnes, D. Rosenberger, and members of the Ashman lab group for field assistance; C. Dean, E. Gonzales, K. Hanley, D. Trapnell, and other members of the Hamrick lab for assistance with allozyme work; E. York for greenhouse assistance; the staff of PLE for logistical support; and D. Carr, A. Case, J. Chase, S. Kalisz, T. Knight, J. Lawrence, S. Tonsor, two anonymous reviewers, and members of the PEER group for discussions and/or comments that improved the quality of this work. This research was supported by a John Sidney Karling award, the McKinley-Darbaker fund, an Andrew Mellon Predoctoral Fellowship, the Association for Women in Science, and the NSF (grants DEB-0108099 and DEB-0412120). This is contribution number 181 to the Pymatuning Laboratory of Ecology.

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APPENDIX

The effect of herbivory treatment, replicate, and their interaction on vegetative and reproductive traits of *Impatiens capensis* (*Ecological Archives* E087-164-A1).