THE EFFECTS OF FLORIVORY AND INBREEDING ON REPRODUCTION IN HERMAPHRODITES OF THE WILD STRAWBERRY, *FRAGARIA VIRGINIANA*

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Recently, the biotic context for sexual and mating system evolution in plants has received special attention, yet the significance of interactions with antagonists has only begun to be revealed. We investigated the effect of florivory on reproduction and inbreeding depression by simulating damage on selfed and outcrossed progeny of hermaphrodites of *Fragaria virginiana* and recording the response of reproduction, as well as measuring tolerance to florivory. While both florivory and inbreeding affected reproduction, their effects were independent with respect to sexual traits but not an asexual trait; inbreeding depression was florivory- and family-dependent, specifically, for plantlet production. Plants were intolerant to florivory in terms of flowers, moderately tolerant in terms of fruit, and most tolerant in terms of plantlets. However, only under severe damage was intolerance statistically significant. Inbreeding did little to change these patterns. Our findings suggest that florivory does not consistently influence inbreeding depression, but its indirect effects on plantlet production could lead to increased geitonogamous selfing. This, combined with previous work demonstrating increased autogamous selfing following weevil damage and knowledge of the mechanism of sex determination in this system, suggests that damage by weevils could contribute to the maintenance of hermaphrodites in gynodioecious *F. virginiana* populations.

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1.0   INTRODUCTION

The biotic context for sexual and mating system evolution in plants has received special attention in the last several years. While interactions with mutualists have long been recognized to be important in influencing key parameters of these evolutionary dynamics (Ashman 2000; Barrett 2003; Karron et al. 2004) the significance of interactions with antagonists has only just begun to be revealed (McCall and Irwin 2006; Ashman and Penet 2007; Steets et al. 2007; Wise and Cummins 2007). With respect to sexual system evolution, Ashman (2002; 2006) outlined the myriad ways that antagonists can affect the evolution of dioecy (separate males and females) from hermaphroditism (combined sexes). In particular, antagonists can affect the first step in this transition, that is, the evolution of gynodioecy (females and hermaphrodites) from hermaphroditism, via specific effects on relative seed production of the sex morphs, the selfing rates of hermaphrodites, and the expression of inbreeding depression in selfed offspring (Ashman 2002). While evidence for the effects of antagonists on seed production is substantial in hermaphroditic systems (e.g., Krupnick and Weis 1999; Mothershead and Marqui 2000; Sánchez -Lafuente 2007; Wise and Cummins 2007), and is steadily growing in gynodioecious ones (Puterbaugh 1998; Marshall and Ganders 2001; Collin et al. 2002; Ashman et al. 2004), only a few studies have addressed the effects of antagonists on the latter two components, and these studies were mostly conducted in hermaphroditic systems (Krupnick and Weis 1999; Juenger and Bergelson 2000a; Irwin 2003; but see Penet et al. 2008). Thus, whether plant-

antagonist interactions impact the first step in the evolution of dioecy via effects on mating system or inbreeding depression remains a largely open question.

Herbivores or florivores can affect the relative frequency of females and hermaphrodites in a gynodioecious population when their interaction with plants differs between sex morphs. In particular, if hermaphrodites are less resistant to or less tolerant of damage than females then females may more easily achieve the seed advantage needed for their maintenance and spread when herbivores are present (Ashman 2002; Cole and Ashman 2005). This may indeed be the case because herbivores preferentially attack hermaphrodite over female plants in many plant-herbivore systems (reviewed in Ashman 2002), and this is especially prominent in the case of damage to flowers or seeds, as seen in studies of flower-clipping weevils in *Fragaria virginiana* (Ashman et al. 2004), flower-visiting ants in *Eritrichium aretoide* (Puterbaugh 1998), and seed-consuming weevils in *Sidalcea hendersonii* (Marshall and Ganders 2001). Whether sex morph-differential damage leads to differences in seed production is in part due to sex differences in tolerance of damage (Cole and Ashman 2005), but little is known about the extent or cause of variation in tolerance in sexually dimorphic species (but see Ashman et al. 2004; Cole and Ashman 2005). Regardless, in a few cases damage has been demonstrated to increase females' contribution to the seed pool relative to hermaphrodites' (Puterbaugh 1998; Collin et al. 2002). In addition to the relative quantity of seeds produced by the sex morphs, the relative quality of the seeds is also an important contributor to sexual system evolution (reviewed in Charlesworth 1999). Herbivores may primarily affect seed quality by influencing the selfing rate of self-compatible hermaphrodites and the expression or magnitude of inbreeding depression of selfed offspring.

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Herbivore damage may modify hermaphrodite selfing rates by altering flowering characteristics such as display size or the proximity of anthers and stigmas within flowers and/or by influencing pollinator movements between or within flowers on a plant (Karron et al. 2004; Ivey and Carr 2005; Penet et al. 2008). Although not in all cases, selfing rate can increase with herbivore damage, e.g., in several hermaphrodite species (Ivey and Carr 2005; Steets et al. 2006; Schutzenhofer 2007) and one gynodioecious species (Penet et al. 2008). In the latter case, such an effect could contribute to the maintenance of females. In fact, antagonists may have their greatest impact on the first step in the evolution of dioecy if the antagonist-mediated increase in selfing is combined with an antagonist-mediated increase in the expression of inbreeding depression (Ashman 2002).

It is well known that the expression of inbreeding depression can vary with environmental conditions (Hauser and Loeschcke 1996; Cheptou et al. 2000; Steets et al. 2006; Botham et al. 2009), and the herbivore environment is no exception (Carr and Eubanks 2002; Ivey et al. 2004; Koslow and Clay 2007; Leimu et al. 2008); however, the direction of the effect can be quite variable. For instance, in *Lychnis flos-cuculi* inbreeding depression was reduced in the presence of snail herbivores because the snails depressed fruit production of outcrossed plants more than selfed plants (Leimu et al. 2008). In contrast, inbreeding depression in *Mimulus guttatus* increased in the presence of spittlebug herbivores. Spittlebugs increased (or had no effect on) flower production and/or aboveground biomass of outcrossed plants but reduced these traits in selfed plants (Carr and Eubanks 2002; Ivey et al. 2004). The mechanism(s) underlying such variable responses is not often known, but they could be a function of variation in inbreeding history of the population, individuals studied, or in the type of herbivore involved (e.g., Ivey et al. 2004; Leimu et al. 2008). In addition, since mating system can affect both plant

resistance to (Strauss and Karban 1994; Hayes et al. 2004; Du et al. 2008) and tolerance of herbivore damage (Carr and Eubanks 2002; Hull-Sanders and Eubanks 2005), either could underlie an herbivore-context dependency of inbreeding depression. A recent review (Núñez-Farfán et al. 2007), however, provides some insight into these two mechanisms and suggests that while inbreeding generally has mixed effects on resistance, it consistently has a negative effect on tolerance. However, because all the studies on the effects of herbivore context on inbreeding depression have been conducted in hermaphroditic species, we do not yet know whether such effects occur in gynodioecious species, nor if they occur in a direction that would compound the antagonist-mediated effects on selfing rate and thus, ultimately, could impact sexual system evolution.

In this study we aim to address this gap in our understanding of the role of the antagonist context in the evolution of gynodioecy by examining the effect of simulated weevil florivory on reproduction and the expression of inbreeding depression in *Fragaria virginiana*. The specific questions we addressed were: (1) Does florivory affect reproductive allocation of hermaphrodite plants and does the impact of florivory depend on inbreeding level, i.e. the cross type that produced the plant, or severity of damage? (2) Does the magnitude of inbreeding depression depend on florivore environment? And lastly, (3) does plant tolerance to florivory depend on the inbreeding level, severity of damage, or both?

2.0   MATERIALS AND METHODS

2.0.1   Study system

Fragaria virginiana is an herbaceous perennial native to the eastern United States (Staudt 1989) with a gynodioecious to subdioecious sexual system (Staudt 1989; Spigler et al. 2008). Thus, populations can contain females, hermaphrodites, males (pollen-bearing plants that produce no fruit), and occasional neuters (plants that produce neither pollen or fruit) (Stahler et al. 1995; Ashman 1999; Spigler et al. 2008). Recent work suggests that sex is determined by two linked loci (or gene regions) with major effects (Spigler et al. 2008), with limited recombination between the loci that can explain the variation in sexual phenotypes observed in wild populations.

In northwestern Pennsylvania plants flower between April and June, and produce approximately 12-14 flowers per ramet (Ashman 2003). Hermaphrodites produce perfect flowers and are self compatible. Both autogamous and geitonogamous self pollination are possible (Penet et al. 2008), and the rate of self fertilization for hermaphrodites in the source population of the material studied here ('PR' Ashman 1999) is 0.722 ± 0.004 (family-level selfing ranges from 0.167 to 1) (A. S. Rohde, C. L. Collin, L. Penet, A. Johnson and T-L. Ashman, unpublished data). Starting in the spring and continuing through the summer, *F. virginiana* also reproduces asexually via plantlets produced along stolons.

Anthonomus signatus (Coleoptera, Curculionidae), the strawberry bud-clipping weevil, is a significant florivore on *F. virginiana* (Kovach et al. 1999; Ashman and Penet 2007). *A. signatus* females oviposit in flower buds and then sever the pedicel, excising the bud. Larvae develop in the excised bud. Hermaphrodite plants can suffer consistent and high levels of damage by *A. signatus* (Ashman and Penet 2007). For instance, up to 70% of hermaphrodites in a population can be damaged, and on average these experience a damage intensity of 4.3 ± 0.4 buds clipped per plant (Penet et al. 2008). In extreme cases all flowers per plant can be clipped (Ashman et al. 2004; Ashman and Penet 2007). Previous experiments have confirmed that plants respond to simulated weevil clipping (i.e., clipping buds off with forceps) similarly to natural clipping by *A. signatus* (Ashman et al. 2004).

2.0.2 Experimental design

2.0.2.1 Production of experimental plants

Hermaphrodite genotypes that are the subject of this experiment were the same as those used in Botham et al. (2009) and thus the methods for creating them are described only briefly here; refer to Botham et al. (2009) for additional details. We selected several hermaphrodite *F. virginiana* that were known to be capable of producing fruit. These plants originated from a wild population ('PR', in Ashman 1999) but have been under greenhouse cultivation for several years. In the greenhouse at the University of Pittsburgh we emasculated flowers of each hermaphrodite prior to anthesis and conducted hand pollinations with either self pollen or a mixture of pollen from other pollen-bearing plants (i.e., hermaphrodites and males) from the same population of origin to produce plants of two 'inbreeding levels' (selfed and outcrossed). Hereafter, the outcrossed and selfed seeds of a given hermaphrodite genotype are referred to as a 'maternal

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family'. Individual seeds within maternal families are referred to as outcrossed or selfed 'progeny'. We chose eight maternal seed families from these crosses for this study based on two conditions: 1) they had enough seeds to conduct the study, and 2) they presented signs consistent with inbreeding depression at the seedling stage in a pilot study. While this may result in an over estimation of inbreeding depression for the population of origin, the main purpose of the current study was not to gain an estimate of the magnitude of inbreeding depression per se, but to determine whether florivory could alter its expression or if there was an effect of inbreeding on tolerance to florivory.

In July 2007 we randomly selected five selfed and five outcrossed progeny from each maternal family and generated nine clonal replicates from each (hereafter, 'clones'), for a total of 684 clones. Clones were initially grown in 4 cm tall pots of a 2:1 mix of Fafard #2 soil:sand. Approximately two months later these clones were transplanted into 10 cm tall pots of the same potting soil. After an additional two months of growth in the greenhouse under natural day lengths (9.5-11 hours) and temperatures of 7-20^0 C, we transferred plants to an outside plot for overwintering. In March 2008 we returned all clones to the greenhouse, where they were maintained under natural day lengths (12-14 hours) and temperatures of 10-20^0 C for the duration of the experiment. At this time, we randomly assigned each plant to one of three blocks and to one of three florivory treatments (described below). Each block contained one clone of each progeny genotype-by-florivory treatment. We watered plants daily and fertilized twice during the experiment, once in the Fall with an application of 50 ppm Plantex© (20-20-20 N-P-K) and once in the Spring with 0.104 g of 100-day release Nutricote© fertilizer beads (13-13-13 N-P-K). We hand-pollinated flowers three times a week with pollen collected from a pool of pollen donors to ensure full fruit set.

2.0.2.2 Florivory treatment

To create the florivory treatments we clipped buds from plants with forceps when buds were within two days of opening. To create the 'average' damage level we clipped four buds per clone, and to create the 'severe' damage level we clipped eight buds per clone. The 'none' plants receive no clipping (i.e., control). The average treatment is similar to the average damage observed in hermaphrodites in natural populations (Ashman et al. 2004), whereas the severe treatment reflects ~1 SD greater than the mean, still a natural level of damage (Ashman, personal observation).

2.0.2.3 Reproductive and vegetative traits scored

For each clone we recorded sexual (number of inflorescences, flowers, and fruits produced) and asexual (number of stolons and plantlets produced) traits and plant size. We used the product of the number of leaves and the diameter of the largest leaflet at the end of the experiment as an estimate of plant vegetative size. This value is a good estimate of above-ground biomass in *F. virginiana* (Ashman 1999). In addition, we produced an index of relative allocation to sexual vs. asexual reproduction (hereafter, 'RSAR'), based on meristem allocation. In strawberries, an axillary meristem can develop into an inflorescence or a stolon (Hancock 1999). For each plant we calculated the proportion of axillary meristems allocated to sexual reproduction as the number of inflorescences divided by the sum of the number of inflorescences and stolons. Thus, a value of 0.5 for RSAR indicates a plant allocated an equal proportion of its meristems to sexual and asexual reproduction, a value >0.5 indicates greater allocation of meristems to sexual reproduction, and a value <0.5 indicates greater allocation of meristems to asexual reproduction.

2.0.3 Analysis

2.0.3.1 Reproductive allocation

To determine whether florivory affects reproductive investment (flowers, fruits, plantlets) and allocation (RSAR) of hermaphrodite plants and whether this varies with inbreeding level we conducted a fixed-effects ANCOVA using PROC GLM in SAS (SAS 1996). Florivory treatment, inbreeding level, maternal family, and their interactions were fixed effects. We included maternal family in the design to account for the potential effects of inbreeding history, but maternal family was considered a fixed effect (because of the limited number of nonrandomly chosen families; Gotelli and Ellison 2004). In addition, the model included block and plant size to account for their effects on reproduction. All reproductive trait values except RSAR were transformed to conform to the assumptions of ANOVA (flower number and plant size were square-root transformed; number of inflorescences, fruits, and plantlets were natural log transformed). We present least squares means (controlling for plant size) on untransformed data in figures and tables. Analyses were performed with a sample size of 593 clones because 92 clones were excluded as they never flowered, died, or did not fully receive their assigned florivory treatment (see below). These exclusions were evenly distributed across experimental factors.

In this analysis we were particularly interested in the effects of florivory treatment and inbreeding level and their interaction. A significant effect of florivory treatment would indicate that plants allocate to reproduction differently in the face of florivore damage. A significant effect of inbreeding level would indicate that selfed and outcrossed plants differed in their reproductive investment or allocation, while a significant interaction between the two would indicate that the effects of florivory differ for selfed and outcrossed plants. An interaction of any

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of these with family would indicate that the maternal families in the experiment differed in their response to inbreeding or florivory or both. When a significant overall effect of a factor was found, we used Tukey's tests to identify the levels of the factor that were significantly different.

2.0.3.2 Inbreeding depression

A further examination of florivory and inbreeding level was conducted by comparing inbreeding depression in fruit, flower, and plantlet production, and RSAR expressed at the maternal family level within each of the three florivory treatments. To do this, we calculated means for selfed and outcrossed progeny for each family-florivory combination within each block. Then, we calculated inbreeding depression for each family and florivory level as $\delta = 1 - \dfrac{\bar{x}_s}{\bar{x}_o}$, where $\bar{x}_s$ is the family mean of selfed individuals and $\bar{x}_o$ is the family mean of outcrossed individuals (Johnston and Schoen 1994) under a given level of florivory within a block. This yielded 72 values of inbreeding depression for each trait. We determined whether maternal family or florivore treatment affected inbreeding depression expressed in each trait separately using a fixed-effects ANOVA with family, florivory, their interaction, and block as class variables. Prior to analysis, inbreeding depression for fruit, total flower, and plantlet production were square-root transformed to improve normality. When a significant overall effect of a factor was found, we used Tukey's tests to identify the levels of the factor that were significantly different.

2.0.3.3 Tolerance

To determine whether current(selfed or outcross pollination) or past (maternal family) inbreeding level affects plant tolerance to florivory, we calculated tolerance for selfed and outcrossed progeny under conditions of average and severe damage, separately. First, we calculated clone

mean values for each fitness component (flowers, fruits and plantlets) for each progeny-by-florivory treatment. Then we calculated tolerance as the difference in mean fitness of clones experiencing florivory and those not experiencing florivory divided by the severity of florivory (Strauss and Agrawal 1999; Wise and Carr 2008). Based on this calculation, a value of zero reflects full compensation (i.e., tolerance), whereas negative values reflect undercompensation and positive values reflect overcompensation. Because we were mainly interested in determining whether inbreeding level affected tolerance, we calculated mean tolerance for selfed and outcrossed progeny separately and tested each for a significant difference from 0 using *t*-tests (PROC TTEST; SAS 1996). We also determined whether tolerance of selfed progeny differed from that of outcrossed progeny using paired *t*-tests. Finally, we determined whether severity of florivory affected tolerance by testing whether the difference between tolerance under severe and average florivory was significantly different from 0 using *t*-tests. Bonferroni correction was applied to account for multiple tests.

3.0   RESULTS

3.0.1   Reproductive allocation

Simulated florivory reduced flower number (table 1). On average plants exposed to severe florivory produced the fewest flowers (mean $\pm$ SE: 13.6 $\pm$ 0.7), and those exposed to the average florivory level in turn produced significantly fewer flowers (15.7 $\pm$ 0.6) than undamaged plants (18.8 $\pm$ 0.6). In contrast, florivory did not significantly impact the average number of fruits, plantlets or RSAR (table 1). However, there was a significant family-by-florivory interaction for RSAR (table 1); as florivory severity increased, variation in RSAR among families was reduced and they converged on an RSAR that reflects equivalent allocation of meristems to sexual and asexual organs (fig. 1).

Inbreeding level significantly affected fruits, plantlets and RSAR, but not flower number (table 1). Moreover, the effect of inbreeding on these traits varied greatly among families. On average, selfed plants produced significantly more fruits (5.52 $\pm$ 0.26) than did outcrossed plants (3.32 $\pm$ 0.24), but this effect was most pronounced in three families (17, 254, and 425) (fig. 2B). Outcrossed plants produced significantly more plantlets (3.31 $\pm$ 0.10) than selfed plants (3. 01 $\pm$ 0.11), and this difference was quite extreme for one family (425; fig. 2C). Although the difference was small, selfed plants on average allocated a significantly greater fraction of meristems to sexual reproduction (0.52 $\pm$ 0.01) than did outcrossed plants (0.49 $\pm$ 0.01), but again two families (254, 425) had the most pronounced pattern (fig. 2D).

The only reproductive trait to show an interaction between inbreeding and florivory was plantlet production (table 1). Here, outcrossed plants produced ~15% more plantlets than selfed

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ones when damaged; this was significant under average damage, and the same pattern was present though non-significant under severe damage (fig. 3).

Table 1. ANCOVAs for reproductive traits in hermaphrodite *Fragaria virginiana*

	Flowers		Fruits		Plantlets		RSAR	
Factor	df	F	df	F	df	F	df	F
FLOR	2	20.46****	2	2.06	2	0.03	2	0.51
INB	1	0.12	1	28.49****	1	4.51**	1	6.40**
MFAM	7	1.72	7	26.59****	7	11.28****	7	7.71****
FLOR x INB	2	0.35	2	0.19	2	4.35***	2	0.69
MFAM x INB	7	1.84	7	9.57****	7	4.50****	7	7.15****
FLOR x MFAM	14	0.85	14	0.70	14	0.72	14	2.27***
FLOR x MFAM x INB	14	0.77	14	0.62	14	1.68*	14	0.85
Plant size	1	58.40****	1	12.94****	1	15.79****	1	0.97
Block	2	0.42	2	0.84	2	0.71	2	0.18

Note. Inbreeding level (INB), florivory treatment (FLOR), maternal family (MFAM), and block are fixed effects. RSAR = relative allocation to sexual versus asexual reproduction.

* $P < 0.06$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$.
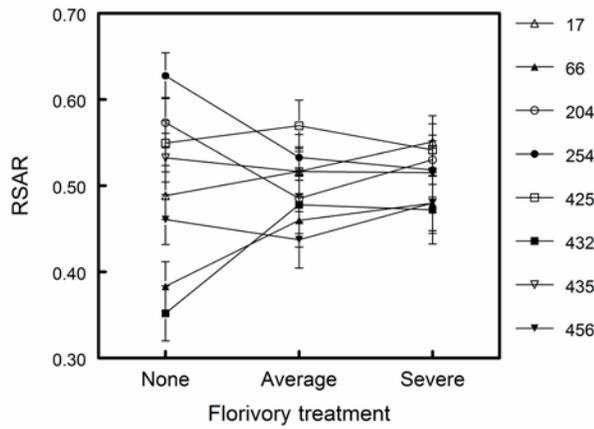
Figure 1. Least squares means (±SE) for relative meristem allocation to sexual and asexual reproduction (RSAR) under three florivory treatments (none, average, and severe) in eight maternal families of *Fragaria virginiana*.
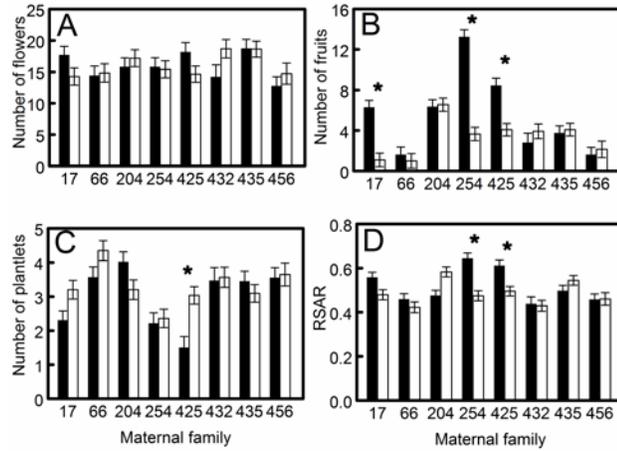
Figure 2. Least squares means (±SE) of flower production, fruit production, plantlet production, and relative meristem allocation to sexual and asexual reproduction (RSAR) for selfed (black bars) and outcrossed (white bars) progeny for eight maternal families of *Fragaria virginiana*. Significant differences (P < 0.05) between selfed and outcrossed progeny within a given maternal family are indicated by an asterisk above the columns.
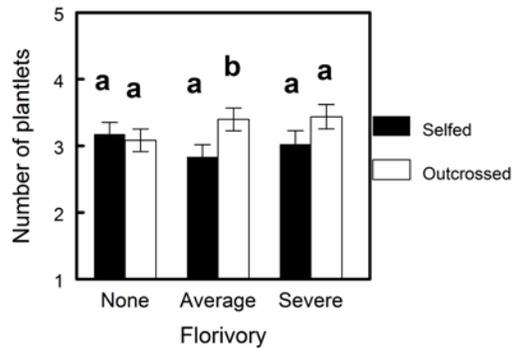
Figure 3. Plantlet production (least squares means ±SE) of selfed and outcrossed *Fragaria virginiana* under three levels of florivory. Within a treatment level, selfed and outcrossed means that do not share a letter are significantly different at P < 0.05.

3.0.2 Inbreeding depression

Interestingly, there was no significant overall effect of florivory treatment on the expression of inbreeding depression (table 2), but there was a maternal family-by-florivory interaction for inbreeding depression in plantlets (table 2; fig. 4). In response to increasing florivory, two families expressed an increase in inbreeding depression (425, marginally significant; 435) while another changed from an expression of inbreeding depression under average florivory to outbreeding depression under severe florivory (254) (fig. 4). Complex patterns prevailed in most families (fig. 4). In fact, maternal families varied significantly for inbreeding depression in all traits except flower number (table 2). Three families (17, 254, and 425) exhibited strong outbreeding depression in fruit production (fig. 5B); two of those families (17 and 425) also

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exhibited moderate inbreeding depression in the number of plantlets produced (fig. 5C). Both

plantlet production and RSAR also showed maternal family-variation ranging from inbreeding to

outbreeding depression (fig. 5C and 5D).

Table 2. ANOVAs testing for the effects of florivory treatment and maternal family on inbreeding depression in

sexual and asexual reproductive traits of *Fragaria virginiana*

	Flowers		Fruits		Plantlets		RSAR	
Factor	df	F	df	F	df	F	df	F
FLOR	2	0.34	2	0.73	2	1.61	2	1.23
MFAM	7	1.3	7	13.05***	7	3.88**	7	5.5***
FLOR x MFAM	14	0.44	14	0.64	14	2.22*	14	0.95
Block	2	0.21	2	0.55	2	0.34	2	0.12

Note. Florivory treatment (FLOR), maternal family (MFAM), and block are fixed effects.

RSAR = relative allocation to sexual versus asexual reproduction.

* P < 0.05; ** P < 0.01; *** P < 0.001.
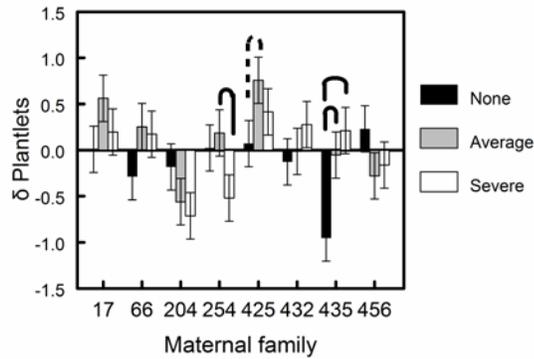
Figure 4. Mean (±SE) inbreeding depression (δ) in plantlet production for *Fragaria virginiana* hermaphrodites by maternal family under three levels (none, average, or severe) of simulated florivory. Treatments within a family that were significantly different are connected by curved solid lines (P < 0.05) or curved dashed lines (P = 0.059).
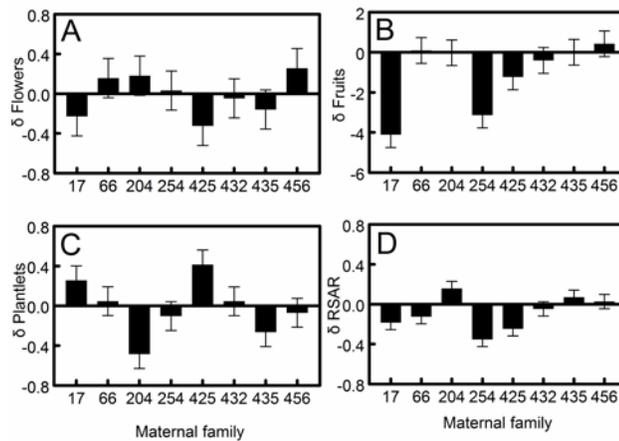
Figure 5. Mean (±SE) inbreeding depression (δ) in eight maternal families of *Fragaria virginiana*. Variation among families was significant for fruits and plantlets but not for flowers or relative allocation to sexual versus asexual reproduction (RSAR; table 2). Note that Y-axis scaling for fruits is different from the rest.

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3.0.3 Tolerance

Plants showed variable patterns of tolerance, ranging from an almost complete lack of compensation (flowers) to slight overcompensation (plantlets) (fig. 6). Under average florivory, indices of tolerance were generally negative, reflecting undercompensation, but none were significantly different from 0. However, under conditions of severe florivory, indices of tolerance for flowers (fig. 6A) and fruits (fig. 6B) were significantly negative, reflecting a lack of tolerance. Tolerance indices did not, however, differ between severe and average damage (all $|t|$ < 1.06; P> 0.32; df= 7), suggesting that the difference in statistical significance was largely due to a reduction in variance in tolerance under severe damage. Likewise, in no case was tolerance of selfed and outcrossed plants significantly different (all $|t|$ < 1.96; P> 0.09; df= 7), although one needs to keep in mind that these t-tests had limited power.
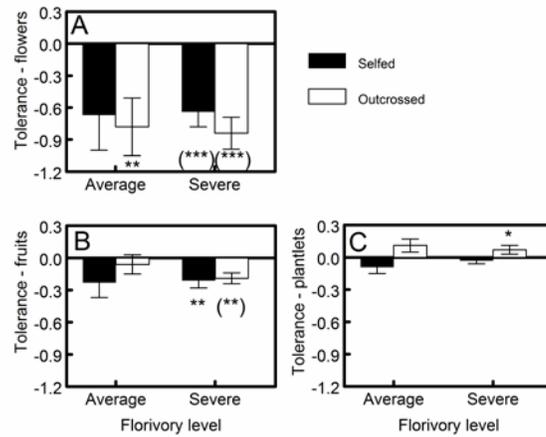
Figure 6. Mean (±SE) tolerance of selfed and outcrossed *Fragaria virginiana* exposed to average and severe simulated florivory. Tolerance indexes are in the units of fitness components per bud removed (see "Material and Methods" for details); those that are significantly different from 0 based on *t*-tests are noted as follows: one asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.01$, and three asterisks indicate $P < 0.001$. Tests that retained significance after Bonferroni correction are indicated in parentheses.

4.0   DISCUSSION

By exposing outcrossed and selfed clones of *F. virginiana* hermaphrodites to simulated weevil florivory, we demonstrated that both damage and inbreeding affect reproduction, but their effects are largely independent with respect to the sexual traits examined.   Interestingly, this independence was not seen for the asexual reproductive trait, plantlet production. Thus, it was only for this trait that inbreeding depression was florivory-dependent, and even here the pattern varied among families. In fact, inbreeding depression was maternal family-dependent for the majority of traits examined. Lastly, while plants largely did not compensate for florivory this was only significant under severe damage, and inbreeding level did little to change this pattern. In the following paragraphs we interpret these results in light of other studies of antagonist-dependent inbreeding depression and their potential role in sexual system evolution.

4.0.1   Independent vs. interactive effects of inbreeding and florivory

A particularly interesting outcome of the work presented here is that florivory and inbreeding level had largely independent effects. This was because each affected different sexual traits. Florivory reduced flower number but had no effect on fruit production, whereas inbreeding increased fruit production but had no effect on flower number. The absence of an inbreeding effect on flower number could be due to an absence of genetic variation in this trait or its tolerance to florivory. While flower number can be a highly heritable trait (Hof et al. 1999; Ashman 2003; Caruso 2004), the limited sampling of genotypes used in this study may have restricted genetic variation. However, Ashman and colleagues (2004), using a larger set of maternal genotypes, also did not detect genetic variation in tolerance to florivory with respect to

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flower number, suggesting a paucity of variation for tolerance to florivory is not uncommon. This might not be surprising given that traits like tolerance, which may be subject to strong selection and are tightly linked to fitness, are expected to have low genetic variation (Geber and Griffen 2003; Blows and Hoffmann 2005). Other studies, however, have found significant genetic variation in tolerance (Shen and Bach 1997; Stinchcombe and Rausher 2002; Fornoni et al. 2003), including one which examined tolerance to florivory (Wise et al. 2008) (but see Juenger and Bergelson 2000b; Ivey et al. 2009), indicating that the magnitude of variation may depend heavily on the system.

The absence of a florivory effect on fruit number is in line with the finding of Ashman et al. (2004) for hermaphrodite genotypes, indicating that hermaphrodites are generally tolerant to average levels of damage for this fitness component, although they may be intolerant at severe damage levels (fig. 6). The finding of an effect of inbreeding on fruit number, however, is novel, particularly given the surprising direction of this effect, which is particularly pronounced in three families (fig. 2B). For these families, selfed progeny had higher fruit production than outcrossed progeny, a finding consistent with outbreeding depression for fruit production. This result may best be explained by considering the genetics underlying fruit production in this gyno(sub)dioecious species. Recently, Spigler et al. (2008) demonstrated that two linked gene regions are responsible for sexual phenotype. Their model postulates that female fertility (fruit production) is conferred by an allele **G** that is dominant (or co-dominant) to an allele **g** that codes for female sterility, such that hermaphrodite individuals are **GG** or **Gg** at this 'locus' whereas **gg** individuals produce no fruit (i.e., are males) (Spigler et al. 2008). Given this simple model, selfed progeny of a **GG** hermaphrodite will also be homozygous **GG** whereas outcrossed progeny will be a mix of **GG** and **Gg**, given that pollen from the outcross pollen pool would

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contain either **G** or **g** alleles. If **G** is codominant then the mean fruit setting ability of the outcrossed progeny would be lower than the selfed progeny. (In contrast, if **G** is dominant then in these families there would be no difference between selfed and outcrossed progeny in fruit production.) On the other hand, if a hermaphrodite is **Gg** then its selfed progeny will be 25% **GG,** 50% **Gg** and 25% **gg.** If the frequency of **g** in the outcross pollen pool was greater than 50% (a possibility given that **gg** plants were included as outcross pollen donors) then outcrossed progeny would be more likely to be **gg** than selfed progeny, and as a consequence on average outcrossed progeny would have lower fruit production than selfed progeny, under either dominance or codominance of **G**. While we do not yet know which scenario was the case, controlled crosses to evaluate the putative genotypes at the **G** locus for the maternal hermaphrodites used in this study and, thus, to test this hypothesis, are underway. Regardless, this finding reveals a particularly intriguing 'cost' to outcrossing for hermaphrodite genotypes in a subdioecious species, and suggests that there could be selection for autonomous selfing to maintain them.

Plantlet production was the only trait for which inbreeding and florivory had interactive effects, and thus inbreeding depression was florivory-dependent, although the pattern varied among families (fig. 4). Selfed progeny produced fewer plantlets than outcrossed progeny under florivory, significantly so under average damage levels (fig. 3). The higher plantlet production under florivory may reflect resource redistribution from the combination of reduced fruit production of outcrossed plants (see above) and lower investment in flower expansion and maintenance when buds are lost to florivory. Such an interpretation is supported most clearly by patterns seen in two families (17 and 425) which both showed outbreeding depression in fruits and inbreeding depression in plantlets that increased with severity of florivory. Such results lend

credence to the idea that the presence of antagonists can lead to different fitness outcomes for a given reproductive strategy (i.e., that a shift from sexual to asexual reproduction may be favored when florivores are present).

Maternal family variation in inbreeding depression was seen for plantlets (table 2) and was also evident from an interaction between maternal family and inbreeding level for fruits and RSAR in the ANCOVA (table 1). Variation among families in inbreeding depression is a common phenomenon that could reflect several causes. First, family variation in inbreeding depression has been linked to among-lineage variation in selfing rate and subsequent purging of deleterious recessive alleles (Lande and Schemske 1985). However, some systems have shown no consistent relationship between inbreeding history and inbreeding depression (Carr et al. 1997; Stone and Motten 2002). Second, Dudash et al. (1997) suggested that family differences may be due to the nature of deleterious mutations carried by individuals that undergo selfing, i.e., if traits are affected by deleterious recessive alleles, overdominance, or epistatic interactions. Third, family variation in inbreeding depression in response to a stressor could result from family variation in the magnitude of phenotypic variation, where families with higher phenotypic variance for a given trait have a higher likelihood of displaying inbreeding depression (Waller et al. 2008). Both variation in inbreeding history and extent of phenotypic variation are likely causes for the patterns seen here because, 1) although the selfing rate of maternal hermaphrodites used in this study are not known, selfing rates of hermaphrodites from the source population are known to vary widely (range: 0.167 to 1; unpublished data), and 2) families varied two-fold in coefficient of variation for plantlet, RSAR and fruit production (unpublished data).

4.0.2 Tolerance and inbreeding depression

Our study joins a small but growing number that have examined the effects of inbreeding on tolerance (reviewed in Núñez-Farfán et al. 2007). We found tolerance varied with trait, and plants were significantly intolerant under severe damage levels, but that inbreeding had little effect on tolerance. This latter result contrasts with studies showing negative effects of inbreeding on tolerance (Carr and Eubanks 2002; Ivey et al. 2004; Hull-Sanders and Eubanks 2005), but this could be due to many factors, including the type of damage inflicted, the fitness traits measured, amount of genetic variation for tolerance, or experimental sample size. Given the few studies conducted, that no prior studies have examined inbreeding's effects on tolerance to florivory, and the variability of responses demonstrated thus far, it may be premature to draw conclusions about the prevalence of negative effects of inbreeding on tolerance; instead it underscores the need for studies of inbreeding and tolerance to be conducted in more systems, with more types of antagonists and response variables.

4.0.3 Implications for sexual system evolution

Florivory may affect sexual system evolution if it affects the relative seed production of hermaphrodites and females, the selfing rate of hermaphrodites, and/or inbreeding depression (Ashman 2002; 2006). Ashman et al. (2004) addressed the first of these mechanisms for the strawberry-bud clipping weevil system and found that weevil damage did not significantly alter relative seed production. Our current results inform directly on the last mechanism and indirectly on the second mechanism; we address each of these in turn.

As described above, florivory did not, in general, affect inbreeding depression -- only for plantlet production was there a significant increase in inbreeding depression in the 'presence' of florivores. However, the effect of increased plantlet production by outcrossed plants under florivory could feed back and indirectly increase hermaphrodite selfing rate. Specifically, because selfing can occur via pollen movement both within flowers (autogamous) or between flowers (geitonogamous) in strawberry, increased plantlet production could result in an increase in geitonogamous selfing among plantlets of a clone. In fact, selfing rate of focal fruits on hermaphrodites increases with an increase in the proportion of local flowering ramets that are clones of the focal genet in wild strawberry populations (L. Penet, C. L.Collin, and T-L. Ashman, unpublished ms.). Weevil damage is patchy but persistent across years (Ashman and Penet 2007), so even small differences in clonal growth could be magnified over time. Such an effect, however, could potentially be modified by the negative effect of outcrossing on fruit production. As described above, in some (but not all) maternal families outcrossed progeny had lower fruit production than selfed ones; this could mitigate the potential increase in geitonogamous pollination. It is also important to recall that weevil damage has been seen to increase autogamous selfing through reduced floral display (Penet et al. 2008), so the net effect of weevil damage on the selfing rate would be to increase total selfing via both mechanisms. Although it is difficult to predict the net effect on selfing, the fitness consequence of such selfing ultimately depends on the severity of inbreeding depression—which from accounts here (e.g., fig. 5) and in Botham et al. (2009) suggests that it is highly variable but on average less than 0.5. Taken at face value these findings suggest that weevil damage may not influence population sex ratio in *F. virginiana* through its effects on inbreeding depression, but rather via affects on selfing rate.

If in general weevil damage increases selfing rate then it could impact the sex ratio by increasing the production of **GG** hermaphrodites. More **GG** hermaphrodites would be produced under selfing than outcross or panmictic pollination because the preponderance of males (**gg**) and low fruiting (putative **Gg**) hermaphrodites in wild populations (Ashman 1999; R. Spigler and T-L. Ashman unpublished data) leads to more **g** than **G** pollen in the xenogomous pool. In addition, there are several lines of evidence suggesting that **G** pollen may have lower siring success than **g** pollen (T-L. Ashman and M. Harbist, unpublished data). A test of this possibility requires an understanding not just of variation in phenotypic gender and its association with sex ratio but also an understanding of the frequency of genotypes at the female function locus and its association with sex ratio and weevil damage.

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