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PLANT ANIMAL INTERACTIONS

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Inbreeding influences herbivory in *Cucurbita pepo* ssp. *texana* (Cucurbitaceae)

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Abstract In a series of field experiments *Diabrotica* beetle herbivory was found to influence the magnitude of inbreeding depression in *Cucurbita pepo* ssp. *texana*, an annual monoecious vine. Beetles damage flowers and fruits and chew dime-sized holes in leaf tissue between major veins. Inbred plants were found to be more likely to be damaged by beetles and to have more leaves damaged per plant than outcrossed plants. A positive linear association was found between the coefficient of inbreeding and the magnitude of leaf damage, whereas a negative association was found between coefficient of inbreeding and several male and female fitness traits. When pesticides were used to control beetle herbivory, the interaction between coefficient of inbreeding and pesticide treatment was significant for fruit production and marginally significant for pollen quantity per anther. Therefore, the magnitude of inbreeding depression in *C. pepo* ssp . *texana* varies depending on the severity of beetle herbivory.

Keywords *Diabrotica* · Female function · Inbreeding depression · Herbivory · Male function

Introduction

Insect herbivory constitutes one of the strongest environmental factors affecting plant fitness, resulting in a 10% or more annual loss of leaf material (Louda et al. 1989; Marquis 1992). Leaf damage has been shown to reduce vegetative growth as well as reproduction through both male and female function (e.g. Quesada et al. 1995; Mutikainen and Delph 1996; Strauss et al. 2001, 2002), and can induce costly chemical and physical defenses (Bergelson and Purrington 1996; Strauss et al. 2002). At the same time more than half of all species self-pollinate 20% or more of the time (Barrett and Eckert 1990), and almost all species show a loss of fitness as a result of inbreeding (Charlesworth and Charlesworth 1987; Husband and Schemske 1996; Crnokrak and Barrett 2002). The effects of herbivory in plant populations are well documented, as are the effects of inbreeding, but little theoretical or empirical work has been done on the interaction between these two ubiquitous forces (Strauss and Karban 1994; Matheson et al. 1995; Nunez-Farfan et al. 1996; Ouborg et al. 2000; Carr and Eubanks 2002). Furthermore, until recently many studies of both herbivory and inbreeding have concentrated on measures of fitness through female function, but even though most species are hermaphrodites and achieve about half of their fitness through male function, relatively little is known about how inbreeding and herbivory affect male function, particularly in concert.

Plants suffering from inbreeding depression may be less able to allocate resources to seeds and pollen and may be less buffered against stress conditions, such as leaf damage through herbivory. The severity of inbreeding depression has been shown to be greater under stress conditions than under more benign conditions (e.g. Ramsey and Vaughton 1998). Carr and Eubanks (2002) found that the presence of spittlebug nymphs increased the severity of inbreeding depression by a factor of three in one of the two populations that they studied. If inbred plants are less vigorous and grow more slowly than outcrossed plants, they may spend longer in vulnerable life history stages (e.g. seedling stage) or they may take longer to pass through vulnerable stages of organ development (e.g. leaf development). While herbivore damage may reduce the fitness of the host plant at any stage in the life cycle, the plant is most susceptible at the cotyledon stage when loss of tissue may reduce the growth rate and prolong the

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plant's susceptibility to herbivory (Tallamy and Krischik 1989).

To investigate the interaction between inbreeding and herbivory under natural conditions, we measured the amount of beetle damage and the severity of inbreeding depression for male and female fitness traits on inbred and outcrossed *Cucurbita pepo* ssp. *texana* plants. Beetles are known to feed on natural populations of *C. pepo* ssp. *texana* and may substantially reduce yield in cultivated species of *C. pepo* (Tallamy and Krischik 1989). In this study we asked the following questions: (1) Are inbred plants of *C. pepo* ssp. *texana* more likely to be damaged by beetles than outcrossed plants? (2) Do beetles damage a higher proportion of leaves on inbred plants relative to outcrossed plants? (3) Do beetles remove more area per leaf on inbred plants? (4) Does beetle damage differentially affect the severity of inbreeding depression for components of male and female function?

Materials and methods

Cucurbita pepo L. ssp. *texana* (A Gray) Filov (Cucurbitaceae) (Lira et al. 1995) (also known as *C. pepo* ssp. *ovifera* var. *texana*) is an annual, monoecious vine native to Texas and New Mexico and is thought to be the wild progenitor of cultivated squashes (Decker and Wilson 1987; Decker-Walters 1990; Decker-Walters et al. 2002). Growth is indeterminate and one staminate or pistillate flower is produced per node. Flowers are bee-pollinated and open in the morning and remain open and receptive for only 1 day.

Cucurbita pepo ssp. *texana* produces oxygenated tetracyclic triterpenes, known as cucurbitacins, which act to deter typical vertebrate and invertebrate herbivores due to the intensely bitter flavor, but *Diabrotica undecimpunctata howardi* Barber, the spotted cucumber beetle, and *Acalymma vittatum*, the striped cucumber beetle (Barber 1947), have been found to preferentially attack *Cucurbita* cotyledons with high cucurbitacin content, resulting in significantly reduced seedling biomass (Tallamy and Krischik 1989). Genotypes with low levels of cucurbitacin show less beetle damage (Chambliss and Jones 1966; Metcalf and Rhodes 1990), but were found to be more susceptible to spider mite infestation (Da Costa and Jones 1971), suggesting that optimal cucurbitacin concentration is a complex property.

A multi-year crossing design was used to produce plants having a range of coefficients of inbreeding. The coefficient of inbreeding (f) is a measure of the degree of relatedness between an individual's parents. A cross between unrelated individuals yields offspring with an f of 0, a cross between full sibs yields an f of 0.25, and self-pollination yields an f of 0.5. When self-pollinations are performed over a number of successive generations, f asymptotically approaches 1. Inbreeding reduces heterozygosity, leading to increased expression of deleterious recessive alleles and reduced contribution from overdominant loci. The resulting loss of fitness is known as inbreeding depression (Charlesworth and Charlesworth 1987). Prior to the start of the experiment seeds collected from plants growing in a natural population were grown in an experimental garden and outcrossed to produce five unrelated f=0 maternal lines as a base population, and in successive years, serial self and outcross pollinations were performed to produce seeds with coefficients of inbreeding ranging from 0 (outcrossed) to 0.875 (three generations of selfing).

To test the effects of inbreeding on herbivory by *Diabrotica* beetles, we grew 300 inbred and outcrossed *C. pepo* ssp. *texana* plants in the summer of 1998 under high and low herbivory conditions. We measured the amount of leaf damage due to herbivores and recorded various traits related to growth and reproductive output. Plants representing five families and three coefficients of inbreeding (0, 0.5, and 0.75) were germinated in a greenhouse and transplanted in a randomized block design into each of two adjacent 60 m ×60 m plots at 5 m intervals in an experimental garden at the Pennsylvania State University Agricultural Experimental Station at Rock Springs, Pennsylvania. All fields were fertilized with all essential micro and macro nutrients, including half the level of nitrogen, phosphorus, and potassium recommended for commercial squash production, which is sufficient for normal growth but unlikely to mask inbreeding depression. For the low herbivory treatment, one field was sprayed at bi-weekly intervals with Asana XL (Dupont), a contact pesticide, to kill *Diabrotica* beetles, and for the high herbivory treatment the other field was not sprayed. A water spray treatment was not used as a control due to the very small amount of liquid involved and the speed of evaporation, and additional experiments that did use a spray control showed the same results. Beetles cause a characteristic pattern of holes, usually 1.0–1.5 cm in diameter, in the portion of the leaves supplied by the smallest veins (Delph et al. 1997). Leaf damage was assessed by measuring the proportion of leaves on a plant demonstrating this pattern of damage. Data were analyzed with mixed model ANOVA with coefficient of inbreeding as a fixed effect and maternal family as a random effect.

In 1999, 200 *C. pepo* ssp. *texana* plants representing four coefficients of inbreeding (f=0, 0.25, 0.5, and 0.75) and five families were germinated in a greenhouse and transplanted into two adjacent 60 m ×60 m fields at 5 m intervals. Plants were screened 1 week after planting to determine whether inbred plants were more likely to be targets for beetle damage. A plant was scored as being damaged if any of its leaves were damaged by beetles. No insecticide was applied to the field prior to the screening. A χ^2-test was performed to test if beetle damage was independent of the coefficient of inbreeding.

To test whether leaves from inbred plants were more severely damaged, in the summer of 2000, 100 outcrossed (f=0) and inbred (f=0.75) *C. pepo* ssp. *texana* plants from five families were germinated in a greenhouse and transplanted into a 60 m ×60 m plot at 5 m intervals in a randomized block design. To quantify the amount of leaf area removed, we collected the most basal leaf from each plant and imported each scanned leaf image into Corel PhotoPaint (Corel Corporation). The most basal leaf was selected because it was generally the first leaf to emerge on each plant, and each was approximately the same age and had been exposed to field conditions for approximately the same amount of time. We created two images for each leaf, one a solid monochrome image showing just the outline of the leaf and the other a monochrome image showing the interior outlines of the leaf area removed by beetles. For each leaf we divided the number of pixels in the first image by the number of pixels in the second image and subtracted from 1 to determine the proportion of leaf area removed. Data were analyzed with mixed model ANOVA with coefficient of inbreeding as a fixed effect and maternal family as a random effect.

To investigate the effect of differences in the degree of herbivory on the severity of inbreeding depression, in the summer of 2001, 200 *C. pepo* ssp . *texana* plants representing five coefficients of inbreeding (f=0, 0.25, 0.5, 0.75, and 0.875) and five families were germinated in a greenhouse and transplanted into two adjacent 0.4 ha fields at 5 m intervals in a randomized block design. Half of the plants were hand-sprayed with pesticides once per week to reduce beetle damage. The other plants served as controls and were untreated.

The number of new staminate and pistillate flowers was recorded each day to measure total flower production. In the first week in August staminate flower buds were lightly clamped with a twist-tie prior to opening to prevent pollen removal and then were collected the next day following anthesis. Anthers and loose pollen were removed and dried in scintillation vials in a drying oven at 45 °C for 2 weeks before being rehydrated for 1 day in a 0.5% NaCl solution and then sonicated for 15 min to dislodge pollen from anthers. Pollen number and size were determined using an ELZONE EX180 particle counter (Particle Data, Elmhurst, Ill., USA).

In early August, one staminate flower bud from each plant was clamped prior to anthesis and then the next day dehisced pollen from

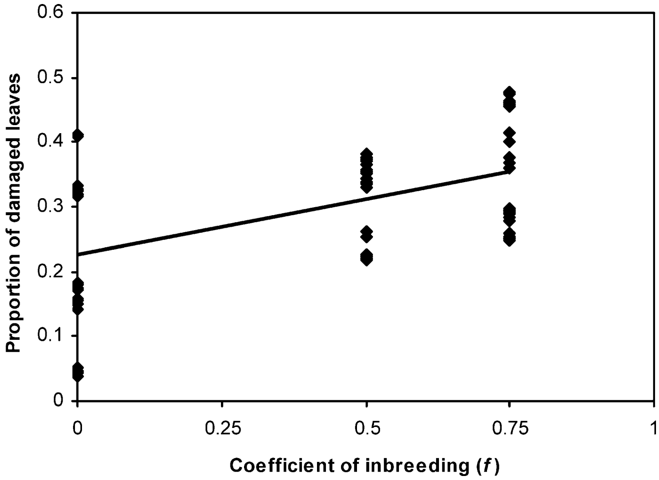

Fig. 1 Proportion of leaves on a plant showing beetle damage as a function of coefficient of inbreeding (f). The best fit line is plotted

each plant was collected and sprinkled onto Brewbaker and Kwack (1963) pollen-germination media and allowed to germinate and grow for 30 min, at which point 2 ml of 70% ethanol was applied to arrest growth. In vitro pollen tube growth rate was determined by averaging the lengths of 30 pollen tubes per plate measured using image analysis (Rich et al. 1989).

After the first lethal frost in September, the total number of mature fruits per plant was recorded and two fruits per plant were collected for seed extraction. Seed number was calculated by dividing the total seed mass by the mass of a random sample of 25 seed, and the mean from the two fruits was used in the analysis.

Data were analyzed using a mixed model analysis of covariance with coefficient of inbreeding and seed weight as covariates, and family and the interaction between family and the coefficient of inbreeding as random effects. Plants in the sprayed and unsprayed treatments were analyzed separately.

Results

In the first experiment in 1998, the proportion of leaves damaged by beetles 7 days after planting increased significantly with coefficient of inbreeding (ANCOVA $P=0.046$, Fig. 1), even though the total number of leaves did not differ significantly. Neither the proportion of damaged leaves nor leaf number per plant varied significantly among families. In the second experiment, we found that beetle preference was not independent of coefficient of inbreeding ($\chi^2=10.1$, $df=3$, $P=0.018$), and the proportion of damaged plants increased monotonically with coefficient of inbreeding, ranging from 42% of outcrossed ($f=0$) plants to 75% of inbred ($f=0.75$) plants. In the third experiment in 2000, beetles removed about 1.1 ±0.3% of the leaf area from the most basal leaf on outcrossed plants and 1.6±0.4% on inbred plants, but this difference was not significant.

In the fourth experiment, several aspects of both male and female fitness decreased with increasing level of inbreeding. Among male fitness traits, staminate flower number decreased with increasing coefficient of inbreeding in both sprayed and unsprayed plants, but in sprayed plants pollen number per flower was also found to decrease with increasing coefficient of inbreeding (Table 1, Fig. 2). Among female fitness traits, pistillate flower number, seed number, and seed mass each decreased with increasing coefficient of inbreeding in both sprayed and unsprayed plants, but in unsprayed plants fruit number per plant was also found to decrease with increasing coefficient of inbreeding (Table 2, Fig. 3). The number of days before emergence of the first flower increased linearly with coefficient of inbreeding ($P=0.001$) (Fig. 4). No effect of family was found for any trait (Tables 1 and 2), although there was a significant interaction between family and coefficient of inbreeding for pollen diameter (Table 1).

The magnitude of inbreeding depression was lower for most traits when pesticides were applied, although inbreeding depression was much higher for pollen number per flower when treated with pesticides (Table 3). An informal paired t-test comparing inbreeding depression on sprayed versus unsprayed plants paired by trait was marginally insignificant ($t=-1.83$, df=8, $P=0.052$). The strongest inbreeding depression occurred for male function in unsprayed plants, and the weakest inbreeding depression occurred for female function in sprayed plants (Table 4). Generally inbreeding depression was more severe for male traits than female traits under both treatments.

Table 1 Mixed model analysis of variance for male fitness traits in sprayed and unsprayed fields in 2001. *Treatment* refers to whether or not plants were sprayed weekly with pesticides, f is the coefficient of inbreeding (0, 0.25, 0.5, 0.75, and 0.875), *Family* refers to the five maternal lines, *Seed weight* refers to the weight of seeds before planting, and *Field* is a blocking factor referring to the two adjacent plots. Spray treatment was treated as a fixed effect, family and field were random effects, and f and seed weight were covariates

	Treatment	N	F	Family	f × Family	Seed weight	Field
Staminate flowers	Sprayed	74	0.017**	0.450	0.719	0.324	0.253
	Not sprayed	71	0.009***	0.076	0.269	0.006***	0.350
Pollen number per flower	Sprayed	55	0.003**	0.407	0.230	0.507	0.003***
	Not sprayed	41	0.630	0.866	0.374	0.283	0.113
Pollen diameter	Sprayed	55	0.403	0.096	0.021*	0.774	0.089[a]
	Not sprayed	41	0.814	0.675	0.746	0.683	0.982
Pollen tube growth rate in vitro	Sprayed	10	0.708	0.080[a]	0.535	0.981	
	Not sprayed	10	0.702	0.764	0.600	0.875	

[a] $0.10>P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$

Table 2 Analysis of variance for female fitness traits in sprayed and unsprayed fields. Terms are as in Table 1

	Treatment	*N*	*f*	Family	Family	Seed weight	Field
Pistillate flowers	Sprayed	74	0.002**	0.602	0.490	0.220	0.929
	Not sprayed	71	0.006**	0.078	0.614	0.094[a]	0.797
Fruits	Sprayed	72	0.076[a]	0.659	0.137	0.748	0.206
	Not sprayed	67	0.047*	0.539	0.624	0.617	0.393
Seed number per fruit	Sprayed	64	0.025*	0.489	0.228	0.728	0.542
	Not sprayed	60	<0.001***	0.086[a]	0.190	0.937	0.404
Total seed mass per fruit	Sprayed	64	0.012**	0.404	0.430	0.339	0.022*
	Not sprayed	60	<0.000***	0.400	0.534	0.618	0.087[a]
Seed germination rate	Sprayed	72	0.359	0.552	0.652	0.522	0.008**
	Not sprayed	67	0.071	0.435	0.786	0.061	0.147

[a] $0.10>P>0.05$, $*P<0.05$, $**P<0.01$, $***P<0.001$

Discussion

This study suggests that beetle feeding behavior is not independent of coefficient of inbreeding, and that inbred plants are damaged to a greater extent than outcrossed plants. Our evidence indicates that beetles preferentially attack inbred seedlings. Previous *Cucurbita* studies have shown that *Diabrotica* beetles can discriminate among genotypes and tend to preferentially attack seedlings with high cucurbitacin content, significantly reducing seedling biomass (Tallamy and Krischik 1989), but in this study no consistent differences were found among families, suggesting that variation among genotypes based on coefficient of inbreeding (and hence homozygosity) is a better predictor of beetle preference than maternal line. Because beetle dispersal tends to be limited once established on plants (Bach 1989), small initial differences in likelihood of establishment may result in compounded fitness costs throughout the life cycle of the plant.

At a time shortly after transplanting, no difference in the number of leaves per plant was detected among outcrossed and inbred individuals, but outcrossed plants began producing flowers significantly earlier than inbred plants, suggesting that outcrossed plants recover from transplant shock more quickly and are generally more vigorous than inbred plants. Following transplantation, plants typically grow slowly for several weeks while developing extensive underground root systems before beginning to produce

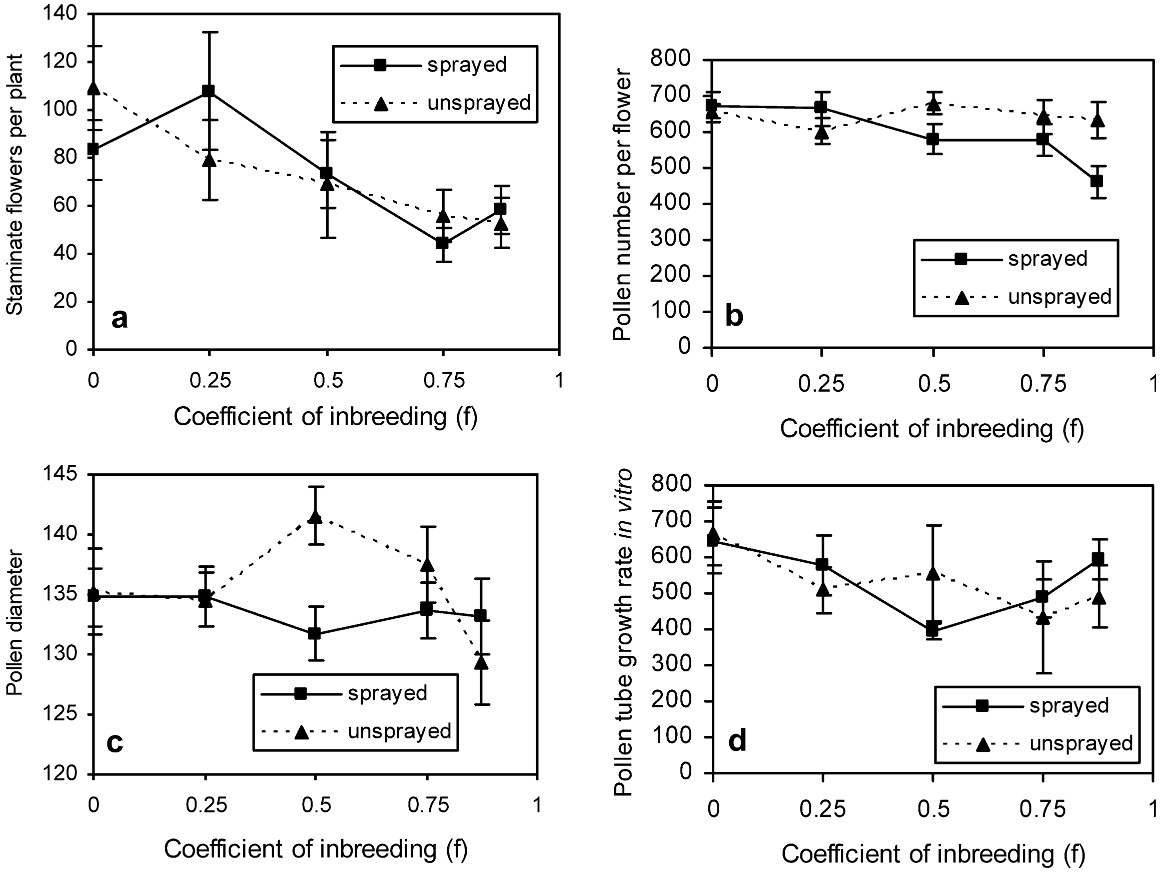

Fig. 2 Means ± SE for male fitness traits for sprayed and unsprayed plants plotted against coefficient of inbreeding (*f*)

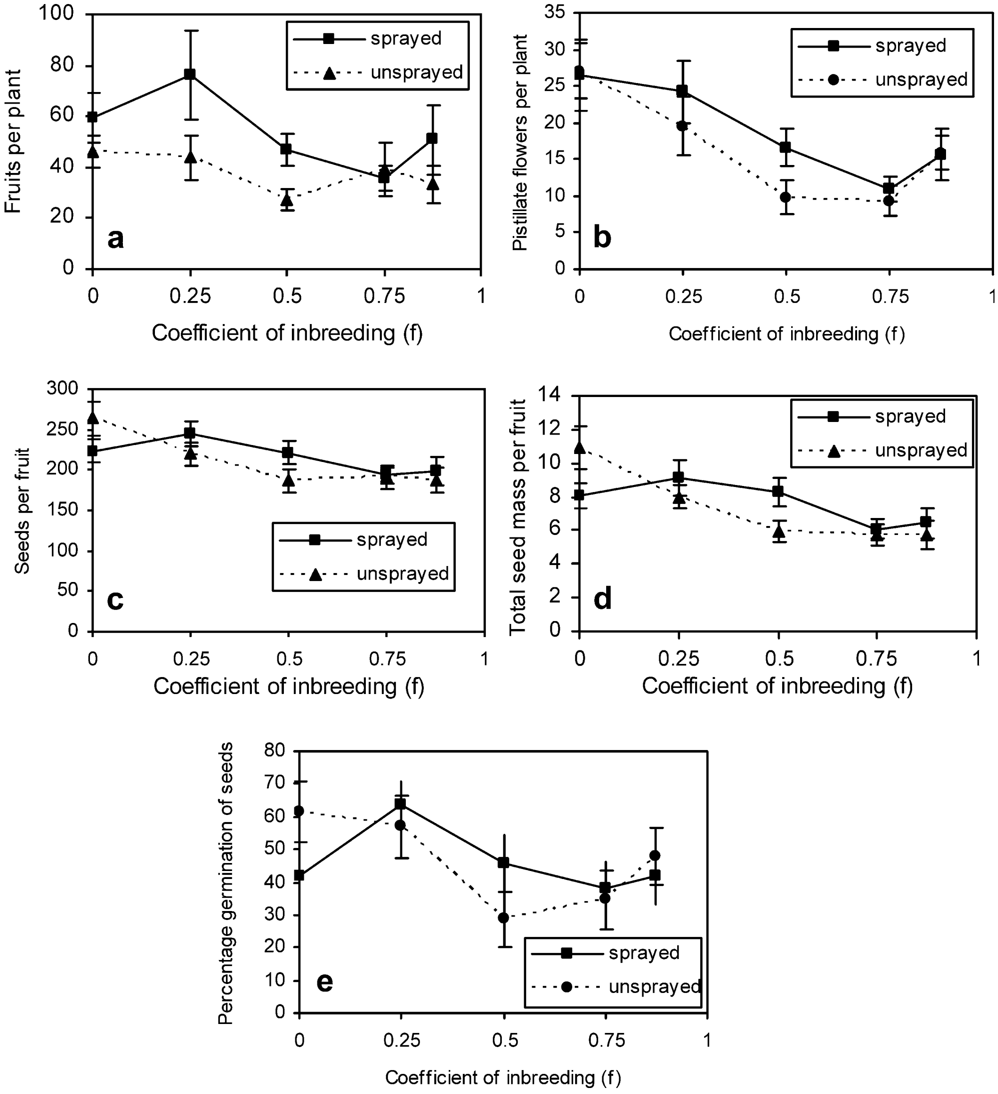

Fig. 3 Means ± SE for female fitness traits for sprayed and unsprayed plants plotted against coefficient of inbreeding (*f*)

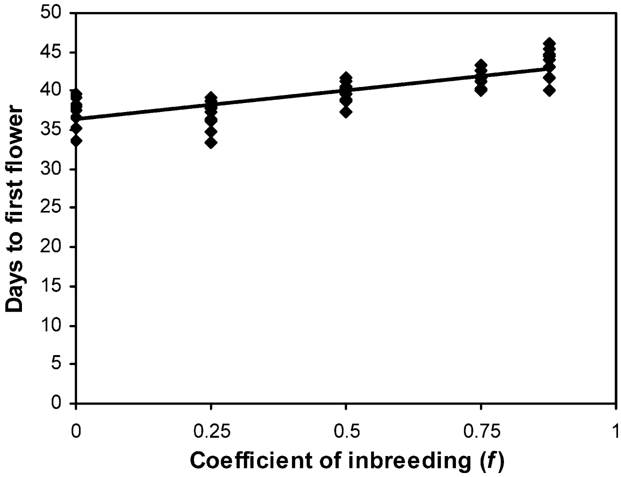

Fig. 4 The number of days to first flower plotted against coefficient of inbreeding (*f*). The best fit line is plotted

flowers (Avila-Sakar et al. 2001). While beetle damage may reduce fitness at any stage of the life cycle, the plant is most vulnerable at the cotyledon stage (Tallamy and Krischik 1989). Beetle damage at this stage may reduce resources available for both root and shoot growth and shift allocation of resources to repair and defense, reducing the growth rate and prolonging the amount of time the plant remains in this vulnerable stage (Tallamy and Krischik 1989).

Plants are at greater risk from beetles at the cotyledon stage because cotyledons tend to have higher concentrations of cucurbitacins than later leaves and have relatively high nutrition and little mechanical defense against herbivory (Tallamy and Krischik 1989). Cucurbitacin concentration can change rapidly during seedling development (Jaworski et al. 1985), and slight levels of mechanical stress to leaves may cause cucurbitacin levels in leaves to rise dramatically within minutes (Tallamy and Krischik 1989). Because inbred plants may experience a greater level of environmental stress than outcrossed plants under the same conditions, inbred plants may tend to produce higher levels of cucurbitacins, making them more attractive to beetles (Tallamy and Krischik 1989). By providing a more attractive target for beetles and by remaining in this early vegetative growth phase longer, the

Table 3 Inbreeding depression for four male and five female fitness traits

Function	Trait	Sprayed	Unsprayed
Male components	Staminate flowers per plant	0.30	0.52
	Pollen number per flower	0.32	0.03
	Pollen diameter	0.01	0.04
	Pollen tube growth rate in vitro	0.08	0.26
Female components	Pistillate flowers per plant	0.41	0.41
	Fruits per plant	0.14	0.28
	Seeds per fruit	0.11	0.29
	Total seed mass per fruit	0.20	0.47
	Percentage germination of seeds	0.00	0.22

Table 4 Multiplicative inbreeding depression for male and female fitness in sprayed and control herbivory treatments. *Male* 1-(relative fitness for male flowers × pollen count × pollen tube growth rate), *Female* 1-(relative fitness for fruits × seeds per fruit × seed mass)

Function	Sprayed	Unsprayed
Male components	0.56	0.66
Female components	0.24	0.60

amount of beetle damage inflicted on inbred plants may be compounded.

In addition to being more likely to be attacked by beetles, inbred plants were also more severely damaged than outcrossed plants. Beetles damaged a larger proportion of the leaves on inbred plants than on outcrossed plants, although no difference was found in the percent of leaf area removed per leaf. However, only one leaf per plant was collected in this study, and only 1–2% of the leaf area had been removed on most leaves. Because leaves from inbred plants showed a slight but nonsignificant tendency to have more leaf area removed than leaves from outcrossed plants, it is possible that this study lacked the statistical power to detect differences in leaf area removed. It should be noted that we harvested and analyzed young leaves. If these leaves were not harvested, they would continue to accumulate beetle damage until they senesced much later in the season. Because the effect of beetle damage is localized, and flowers in the axils of damaged leaves are more affected by leaf damage than flowers elsewhere on the plant (Quesada et al. 1995), the cumulative effect of slight differences in leaf area removal and proportion of damaged leaves may disproportionately affect cumulative fitness over the life cycle of the plant.

While the loss of leaf area as a result of beetle damage reduces photosynthetic capacity and results in loss of stored resources (Quesada et al. 1995), beetles may also act as vectors for bacterial wilt and other pathogens (Agrios 1978; Hoffman et al. 1996). Variation in susceptibility and tolerance to herbivory is often genetically based (Simms and Rausher 1987, 1989; Kennedy and Barbour 1992), and evidence from inbred crop lines suggests that some mechanisms of herbivore resistance involve dominance relationships (Smith 1989; Kennedy and Barbour 1992). Inbreeding increases the frequency of homozygous loci in individuals, increasing the expression of deleterious recessive alleles and reducing the number of overdominant loci. Because inbreeding affects dominance relationships, inbred plants may be less resistant to herbivores than more heterozygous individuals and may be more vulnerable targets for herbivory. Therefore, increased contact with beetles may increase the likelihood of exposure and infection by pathogens. Additional studies are underway to investigate the interaction between inbreeding and disease in *C. pepo* ssp. *texana*.

Reducing the presence of beetles by spraying pesticides reduced the severity of inbreeding depression slightly for male function and greatly for female function. A number of studies have shown that the severity of inbreeding depression varies with environmental stress (Pedersen 1968; McCall et al. 1989; Dudash 1990; Schmitt and Ehrhardt 1990; Schmitt and Gamble 1990; Wolfe 1993; Carr and Dudash 1995; Hauser and Loeschcke 1996; Roff 1997; Cheptou et al. 2000), and recently herbivory has been shown to exacerbate the severity of inbreeding depression as well (Carr and Eubanks 2002; but see Nunez-Farfan 1996). Therefore inbred plants may both be more likely to be targeted by herbivores and then to suffer disproportionately when exposed.

While inbreeding depression was found for both male and female traits, and while inbreeding depression was generally less severe for both male and female traits when plants were treated with pesticides, the spray treatment dramatically improved female fitness, suggesting that herbivory affects female function more strongly than male function (Hendrix and Trapp 1981, 1989; Allison 1990; but see Snyder 1993). Leaf damage has been shown to decrease several aspects of female function, including pistillate flower production, seed production, seed size, and seed viability (Stephenson 1982, 1984; Roach and Wulff 1987; Lee 1988; Marquis 1984, 1992; Obeso 1993; Quesada et al. 1995), as well as several aspects of male function including staminate flower production, pollen production, pollen provisioning, pollen tube growth rate, and siring success (Stephenson et al. 1992; Quesada et al. 1995; Strauss et al. 1996; Mutikainen and Delph 1996; Aizen and Raffaele 1998). Leaf damage may also reduce growth and survival and cause reallocation of resources to defensive compounds instead of reproductive output (Quesada et al. 1995). However, when herbivore pressure is reduced and additional resources are available for reproduction, they may be allocated to the relatively more expensive female function, whereas when herbivory limits the availability of resources for reproduction, it may be more efficient to avoid the high per-unit fitness costs associated with female function in favor of the less costly male function (Lloyd and Webb 1997).

Future studies should further examine the role of cucurbitacin in beetle preference to determine if preference for inbred plants is due to higher cucurbitacin content, weaker defenses, or a combination of factors. Because beetles preferentially damage inbred plants and because the presence of beetles influences the magnitude of inbreeding depression, it is likely that *Diabrotica* beetles have played and will continue to play a role in the evolution of the mating system in *C. pepo* ssp. *texana*.

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