ECOLOGY LETTERS

Ecology Letters, (2013) **16**: 252–260 doi: 10.1111/ele.12036

LETTER

Plant chemistry underlies herbivore-mediated inbreeding depression in nature

Stuart A. Campbell,[1,2]* Jennifer S. Thaler[1,2] and André Kessler[1]

[1]*Department of Ecology & Evolutionary Biology, Cornell University, Ithaca, New York, USA*
[2]*Department of Entomology, Cornell University, Ithaca, New York, USA*

**Correspondence: E-mail: sac73@cornell.edu*

Abstract

The cost of inbreeding (inbreeding depression, ID) is an important variable in the maintenance of reproductive variation. Ecological interactions such as herbivory could modulate this cost, provided that defence traits harbour deleterious mutations and herbivores are responsible for differences in fitness. In the field, we manipulated the presence of herbivores on experimentally inbred and outcrossed plants of *Solanum carolinense* (horsenettle) for three years. Damage was greater on inbred plants, and ID for growth and fitness was significantly greater under herbivory. Inbreeding reduced phenolic expression both qualitatively (phytochemical diversity) and quantitatively, indicating deleterious load at loci related to the biosynthesis of defence compounds. Our results indicate that inbreeding effects on plant–herbivore interactions are mediated by changes to functional plant metabolites, suggesting that variation in inbreeding could be a predictor of defence trait variation. The magnitude of herbivore-mediated, ecological ID indicates that herbivores could maintain outcrossing mating systems in nature.

Keywords

Ecological inbreeding depression, *Epitrix*, flea beetle, horsenettle, mating systems, phenolics, plant defence, secondary metabolites, Solanaceae, *Solanum carolinense*.

Ecology Letters (2013) **16**: 252–260

'I ought to have reflected that such elaborate provisions favouring cross fertilizations, as we see in innumerable plants, would not have been acquired for the sake of gaining a distant and slight advantage, or avoiding a distant and slight evil'. –C. Darwin (1876)

INTRODUCTION

Sexual reproduction is the dominant mode of reproduction for eukaryotes, but can be highly variable both across and within species, ranging from obligate outcrossing to self-fertilisation. Darwin was among the first to recognise that this variation can have profound consequences for fitness in the form of inbreeding depression, and postulated that this explained why so many species avoid incest and/or self-fertilisation (Darwin 1876; Barrett & Harder 1996). However, despite decades of research on the fitness costs of inbreeding (Husband & Schemske 1996), we still know little about the broader consequences of mating system variation, particularly in relation to the ecology and evolution of species interactions.

Inbreeding depression (ID), the reduction in fitness under inbreeding, arises due to the accumulation and expression of deleterious mutations (i.e. genetic load), and possibly overdominance effects, at loci linked to fitness (Charlesworth & Charlesworth 1999). Theoretical models of mating system evolution focus on ID as a primary impediment to the evolution of selfing, and typically predict the maintenance of outcrossing when the strength of inbreeding depression in populations, δ, exceeds 0.5; i.e. when the fitness of inbred offspring is less than half that of outbred offspring (Jarne & Charlesworth 1993). However, many species exhibit lower or higher ID than would be predicted by their ostensible mating system (Husband & Schemske 1996; Goodwillie *et al.* 2005). Studies attempting to resolve this paradox point out that inbreeding depression is usually measured in benign greenhouse or laboratory environments and that δ may be higher or more variable under natural stress (Armbruster & Reed 2005). Compared to greenhouse and lab studies, few studies have tested ID in nature, and fewer have explicitly compared the strength of ID in different environments (Dudash 1990; Armbruster *et al.* 2000). In general, stress appears to increase ID (Armbruster & Reed 2005; Fox & Reed 2011); however, stress can also reduce ID (Henry *et al.* 2003; Waller *et al.* 2008). Thus, understanding the ecological factors that modulate ID remains an important challenge for the study of mating systems (Cheptou & Donohue 2011).

Antagonistic species interactions are one class of ecological factors that have the potential to strongly influence the expression of ID. Interestingly, since Darwin's experiments showing differential ID as a function of competition (Darwin 1876), relatively few studies have examined these interactions in the context of mating systems. Inbreeding can influence competition (Schmitt & Ehrhardt 1990), parasitism (Ellison *et al.* 2011), disease (Stephenson *et al.* 2004), herbivory (see below) and possibly predation (Auld & Relyea 2010). However, evidence that antagonists could act as selective agents in mating system evolution is mixed. Several studies show inconsistent effects of antagonisms on mating systems (Carr *et al.* 2003; Puurtinen *et al.* 2004), whereas others suggest that the effects of inbreeding may be primarily contingent on genetic background (Ouborg *et al.* 2000), and not mating systems *per se*.

Interactions between mating and antagonisms are particularly relevant for plants, which are predominantly hermaphroditic, and must cope with local variation in pollinators and natural enemies such as herbivores. Herbivory is a ubiquitous antagonism faced by plants, and can alter plant phenotypes to influence mating systems. Herbivory can modify flower size/display (Strauss *et al.* 1996), pollen/nectar chemistry (Adler *et al.* 2006; Kessler & Halitschke 2009) and can cause selective abortion of selfed or outcrossed fruits (Steets

et al. 2006). Increases (Steets *et al.* 2006) but also decreases (Elle & Hare 2002) in selfing rates under herbivory have been reported, which may reflect selection for reproductive assurance, or alternatively, selection to increase genetic variation under stress.

Herbivores could also act as selective agents to determine the adaptive value of inbreeding and outcrossing. Several studies have tested whether herbivores can increase ID under controlled greenhouse settings. Herbivores have been shown to preferentially feed, or perform better, on inbred plants (Carr & Eubanks 2002; Leimu *et al.* 2008; Delphia *et al.* 2009a), although in several cases, the effect of inbreeding varied depending on population history (Carr & Eubanks 2002; Leimu *et al.* 2008). This variation is expected: in mixed-mating taxa, populations should vary widely in historic inbreeding rates, leading to variable purging of genetic load and population divergence in the expression of ID. Greenhouse studies have also shown the potential for herbivore-mediated inbreeding depression for growth (Carr & Eubanks 2002; Hull-Sanders & Eubanks 2005) and fitness correlates (Carr & Eubanks 2002) in the form of a significant breeding × herbivory interaction, but have also shown reductions of ID under damage (Leimu *et al.* 2008).

In contrast, fewer studies have manipulated herbivory and inbreeding in the field, and have yielded conflicting evidence for a significant herbivory × breeding interaction indicative of an herbivore- or defence trait-specific component of ID. Field studies of *Datura stramonium* (Solanaceae) have found contrasting effects of inbreeding on damage among years (Bello-Bedoy & Núñez-Farfán 2010 vs. Núñez-Farfán *et al.* 1996), and no significant interaction for fitness when herbivory was manipulated (Bello-Bedoy & Núñez-Farfán 2011). Prior studies of *Solanum carolinense* (Solanaceae) have also found contrasting effects of herbivory on ID (Kariyat *et al.* 2011 vs. Mena-Ali *et al.* 2008), although possibly as a result of variable replication (see also Stephenson *et al.* 2004 vs. Hayes *et al.* 2004). A field study of the effects of inbreeding on tolerance to herbivory in *Mimulus guttatus* (Phrymaceae) showed a significant interaction for growth, but not other fitness traits (Ivey *et al.* 2004).

In part due to the variable results from these few field studies, prior research has not distinguished two competing hypotheses for the evolutionary ecology of inbreeding–herbivory interactions. On the one hand, herbivory may simply impose an additional, non-specific stress on plants, leading to additive fitness costs of inbreeding and damage. Alternatively, there may be deleterious mutations at defence trait loci that lead to an herbivore-specific component of ID, and non-additive fitness costs of inbreeding and herbivory, (i.e. herbivory × breeding interaction). Distinguishing these two hypotheses ('non-specific stress' vs. 'defence trait depression') would improve our ability to predict when inbreeding should be relevant for herbivory (and vice versa), and requires field experiments to isolate the effects of herbivory in nature, and analyses of functional defence trait variation under inbreeding.

As any functional trait could harbour deleterious mutations, inbreeding could affect a multitude of quantitative traits underlying defence against herbivory. We are not aware of any study examining inbreeding effects on foliar defence traits; in fact, relatively few studies have examined the effects of inbreeding on non-reproductive traits (e.g. morphology, behaviour or physiology) in any taxon (Norman *et al.* 1995; Auld & Relyea 2010). Two studies have examined effects of inbreeding on volatile emission from inbred clones, tentatively suggesting that inbreeding reduces indirect defence (Delphia *et al.* 2009b; Kariyat *et al.* 2012). The mechanistic basis for inbreeding effects on direct defence remains unknown.

The goals of our study were two-fold: first, to test for herbivore-mediated, ecological ID under natural conditions using a 3-year manipulative field experiment and replicate genetic families; second, to test for the effects of inbreeding on the expression of defence-related secondary metabolites.

METHODS

Field experiment

We conducted experiments using horsenettle, *S. carolinense* L. (Solanaceae), that we experimentally mated to create replicate families of self-fertilised, sibling-mated and outcrossed progeny (See Appendix S1 in Supporting Information for details of study system and breeding protocol). From three populations, we selected 73 selfed, sib-mated and outcrossed full-sib seed families from 29 dams (i.e. 29 paired sets of selfed and outcrossed families, with 15 triplets including families of sib-mated plants). Seeds were germinated on Metro-Mix potting soil (Metro-Mix 360, Sun-Gro Horticulture Ltd., Vancouver, BC, Canada) in 27-well flats. We used seed-grown plants to capture critical variation in mortality and performance early in development, rather than using clones from fully established plants. In June 2009, after 1 month of growth, seedlings were transplanted to the field site and arranged in a nested design in sprayed and unsprayed main blocks ($n = 8$), with individual selfed, outcrossed (and sib-mated plants where applicable) from each family grouped together at a random position within each main block. Plants were *c.* 1 m apart in rows. This design was chosen to facilitate herbivore exclusion (which is more effective at the plot, rather than individual plant level), and allow inbred and outcrossed representatives from each grandmaternal family to experience similar conditions. At transplant, plants were comparable in size, and similar to local *S. carolinense*. Plants were watered daily for 1 week after transplanting, and herbivore-exclusion plants were sprayed approximately weekly (depending on local precipitation) with a 0.0003% solution of esfenvalerate (Ortho® Bug-B-Gone spray, The Scotts Company, Marysville OH, USA), which is effective at eliminating insect herbivores without changing plant growth (Carson & Root 2000). Surrounding vegetation was manually cropped on a biennial basis. The experiment was maintained for three seasons (June-September, 2009–2011). Plants were censused for foliar damage and growth characteristics in August 2009, and for growth and fitness data in October 2009, 2010 and 2011. We report reproduction and growth from 2011, as this was the first year the plants flowered (typical for horsenettle plants grown from seed in the field), and as these data represent fitness after 3 years of cumulative herbivore exposure over the course of the experiment. Damage due to herbivore exposure (almost exclusively by *Epitrix* flea beetles) was assessed as the number of feeding holes on every leaf; for *Epitrix* damage, the number of feeding holes and the area removed are strongly correlated ($R^2 = 0.793$, $P < 0.0001$). Fewer than 5% of plants in the experiment were attacked by other herbivores, typical for NY. We do not report subsequent estimates of foliar damage, as an unknown component of flea beetle herbivory following year 1 would have occurred belowground through larval feeding. We measured survival, aboveground end-of-season biomass (dry mass), asexual reproduc-

tion (vegetative ramet number) and sexual reproduction (whether plants fruited, and fruit number) as measures of fitness.

Defence trait experiments

Field measurements of defence traits confound intrinsic (constitutive) differences among genetic families with differences due to herbivore preference and induced responses. To provide estimates of phytochemical traits under controlled conditions, we conducted greenhouse experiments that tested the effects of artificial herbivory on inbred and outcrossed horsenettle families. This approach allowed us to independently evaluate chemical ecological mechanisms for the effects of inbreeding on herbivory and fitness. From the original pool of families, a subset (n = 14) was selected at random, and clonal replicates of two to five selfed and outcrossed offspring per family were randomly assigned to receive one of three damage levels: 0, 10 and 20% (n = 234 plants). Damage levels were chosen based on field observations of New York horsenettle populations, in which damage rarely exceeds 20–25%. Clones were created by subdividing main roots into 1.5 g segments, and placing segments in a 1 : 1 : 1 mixture of potting soil (Metro-Mix 360) : vermiculite : perlite in 27-well flats to sprout. We chose to use cuttings in the greenhouse experiments because this approach allowed us to maximise power to detect differences among damage treatments. Plants were grown in the greenhouse for 2 weeks, transplanted to 355 mL pots, and grown for 3 weeks, as described above, with trays randomised to minimise position effects. Damage levels were imposed using a paper hole punch to every leaf, with punch holes evenly distributed over the entire leaf and care taken to avoid the midrib. While mechanical wounding does not elicit the same plant responses as real feeding or the application of a biochemical elicitor (e.g. jasmonic acid), this approach carefully controls the amount of damage and avoids the potential for inbreeding effects on feeding rate or the response to exogenous elicitors. One week later, the fourth to eighth fully expanded leaf from each plant was excised, and 100 mg of fresh tissue (excluding midvein) was sampled for secondary metabolite analysis. Samples were weighed, flash frozen in liquid N_2 and stored at −80 °C. Samples were simultaneously homogenised and extracted using a FastPrep® tissue homogeniser (MP Biomedicals® LLC, Santa Ana, CA, USA) at 6 m/s for 90 s using 0.9-g grinding beads (Zirconia/Silica 2.3 mm, Biospec® Products Inc., Bartlesville, OK, USA) and 1 mL of an ice-cold 40% methanol, 0.5% acetic acid solvent. A 15-μL aliquot of supernatant was analysed for secondary metabolites by high-performance liquid chromatography (HPLC) on an Agilent® 1100 series HPLC equipped with a Gemini C18 reverse-phase column (3 μm, 150 × 4.6 mm, Phenomenex Inc., Torrance, CA, USA) using a standard method targeted at phenolic compounds, particularly hydroxycinnamic acids and flavonoids (Keinanen *et al.* 2001). Several of these compounds are negatively genetically correlated with damage by *Epitrix* flea beetles in the field (e.g. chlorogenic acid, R^2 = 0.24; P = 0.0088, Campbell *et al. unpublished*), strongly implicating them as defence-related secondary metabolites in this system. We quantified the amounts of all phenolic peaks with identifiable UV spectra using peak area, normalised by the fresh mass of the sample. A second subsample of these families (n = 10) was grown under identical conditions, and replicate (n = 4–6) undamaged inbred and outbred plants were sampled and analysed using the same protocol. These plants were used to estimate changes to the diversity in secondary metabolite production under inbreeding. Using chromatograms from each sample at λ = 320 nm (hydroxycinnamic acid derivatives) and 360 nm (flavonoids), we counted the number of compounds (peaks) to calculate 'peak richness' as an estimate of compound diversity. Peaks were counted if they were quantifiable (i.e. when they possessed a clear UV spectrum and exceeded the baseline noise of the chromatograms) (Keinanen *et al.* 2001).

We grew a third set of plants from the greenhouse populations to conduct a bioassay of constitutive variation in resistance between mating systems using *Manduca sexta* L. (Lepidoptera: Sphingidae). From the youngest fully expanded leaf of each plant, we took 2.5-cm^2 leaf discs (average of two discs per plant). Discs were mounted on a pin over moist filter paper (to facilitate larval preference for feeding on leaf undersides), and neonate *M. sexta* larvae were added and allowed to feed for 2.7 days. Larvae did not run out of plant material during this period. Following removal, larvae were allowed to clear their gut contents for *c.* 12 h and were then weighed. Discs were scanned and analysed for the amount of tissue consumption using ImageJ® (U. S. National Institutes of Health, Bethesda, MD, USA).

Analyses

Field experiment data were analysed as a standard multifactor linear model using JMP® v9 (α = 0.05). We analysed data directly to avoid the asymmetry of conventional ratio indices of ID (δ) (Keller & Waller 2002). Variables were checked for consistency with model assumptions where applicable (homoscedasticity among treatment variables and normality), and fruit number, biomass and ramet number were log transformed to improve ANOVA assumptions. Survival and probability of fruiting were tested against the binomial distribution by likelihood ratio tests; fruit counts were tested against a Poisson distribution. Model terms included population, maternal family, breeding treatment and herbivory treatment. Terms involving population and family were specified as random. Our data set was unbalanced with respect to genetic family, and thus we specified two- and three- way interactions for only the population, breeding and herbivory terms (Littell *et al.* 2002). Because the herbivory manipulation was at the level of the block, a herbivory × block term was specified as the error term for the test of the significance of herbivory (i.e. a split-plot design). Field damage, bioassay data (larval growth and amount of leaf consumption) and phenolic diversity were analysed using a matched-pairs approach, by calculating the global average of the least-squares mean contrasts between inbred and outcrossed plants within each family; for the majority of families, we used the contrast of self-fertilised and outcrossed plants (n = 21), while the contrast of sib-mated and outcrossed plants was used in the case of missing selfed plants (n = 5). Confidence intervals (95%) were used to determine whether this difference (Inbred–Outcrossed) was significant based on whether the interval included zero. Metabolite data were log transformed and analysed similarly to the fitness data, with maternal family, herbivory, breeding treatment and their interactions as model terms. Family terms were specified as random. While we analysed our fitness data directly, we also derived estimates of the magnitude of the family-wise inbreeding load, for one fitness trait (ramet production) to graphically illustrate genetic variation for inbreeding effects in the presence and absence of herbivores. We focussed on asexual reproduction as it is likely

the primary mode of reproduction for established populations (SAC *pers obs*). We used inbreeding load, or decline in log fitness (W) as a function of the inbreeding level (F), $d(W)/d(F)$, as $B = \ln(W_o + 0.1) - \ln(W_i + 0.1)$, (Keller & Waller 2002), where W_o and W_i are the mean relative fitnesses of outcrossed and selfed progeny, respectively, and 0.1 corrects for zero fitness (Escobar *et al.* 2008). As in Escobar *et al.* (2008), we note that B is not equivalent to a simple logarithm of δ.

RESULTS

In the bioassay, herbivore growth and consumption (both in absolute and proportional terms) were greater on inbred plants (Fig. 1a, b) (95% confidence intervals for the genetically controlled contrast between inbred and outbred plants do not include zero). In the field, while the proportion of plants attacked did not vary by breeding treatment ($\chi^2 = 0.213$; $P = 0.899$), the amount of damage received by selfed and sib-mated plants was on average 62 and 134% greater, respectively, compared with outcrossed plants (Fig. 1c). There was considerable variation among maternal families in the magnitude of this damage, as indicated by the contrast between selfed and outcrossed progeny (Fig. 1d); however, there was significantly greater damage on inbred plants after controlling for genetic background (Fig. 1d).

Analyses of fitness and growth in the field experiment show minor effects of inbreeding in the herbivore-exclusion treatment, but significant reductions due to inbreeding in four of five growth

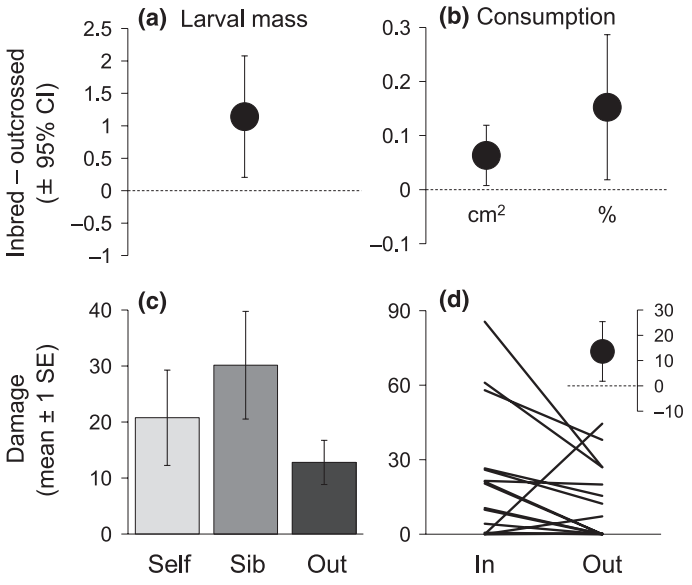

Figure 1 Laboratory and field measurements of resistance and herbivory on inbred and outcrossed progeny of *Solanum carolinense*. Top panels show the average of the contrasts between inbred and outbred progeny of each maternal family, ± 95% confidence intervals, for (a) growth of herbivores (mg FW) and (b) absolute and proportional tissue consumption. (c) Absolute damage on field-grown plants (number of feeding holes per plant). (d) Reaction norm plot of damage in inbred and outbred families, illustrating genetic variation in the effect of inbreeding on damage. Inset is the average of the contrasts between inbred and outbred progeny of each family, ± 95% confidence interval. Confidence intervals do not include zero, indicating significantly greater performance, consumption and damage on inbred relative to outcrossed plants.

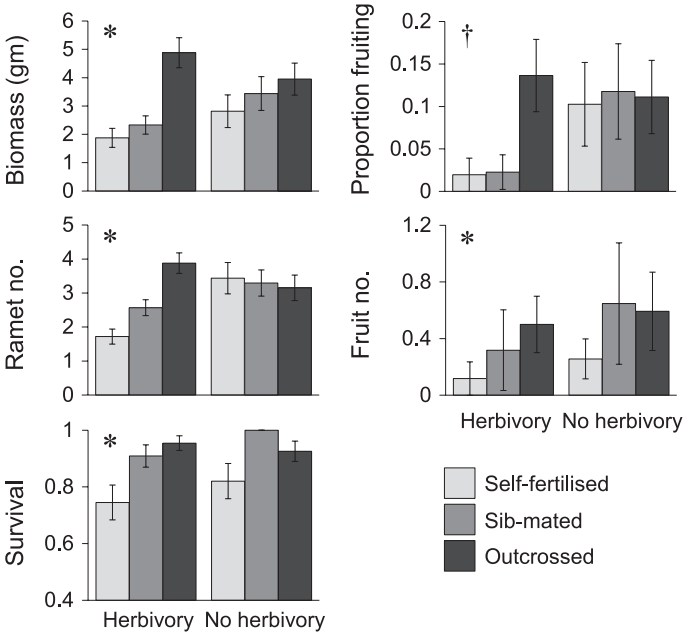

Figure 2 Growth (aboveground biomass) and fitness components (survival, asexual and sexual reproduction) of self-fertilised, sib-mated and outcrossed *Solanum carolinense* in the field, with and without herbivores. Data are means ± 1 SE. Interaction between herbivory and breeding treatments (Table S1) denoted by * ($P < 0.05$) and † ($P < 0.06$).

and fitness traits when plants were exposed to herbivores (Fig. 2). Biomass reductions due to selfing and biparental inbreeding were 40 and 15%, respectively, when plants were protected from herbivory, but were 160 and 110%, respectively, under herbivory. Similarly, the reduction in asexual reproduction due to selfing was 125% under herbivory, but only 57% under herbivore exclusion, and the reduction in survivorship in selfed vs. outcrossed plants was 21 and 11% in herbivore and herbivore-exclusion treatments respectively. In 2011, *c.* 15% of plants had fruited , and the proportion of fruiting plants was seven-fold higher in outcrossed relative to inbred plants under ambient herbivory, with only slight differences when herbivores were excluded (Fig 2). A similar pattern was found for fruit number, and although fruit production was variable, there were also significant overall reductions due to herbivory and inbreeding (Table S1). For most growth and fitness traits, ANOVA accordingly showed significant herbivory × breeding interaction terms (Table S1), in addition to significant family effects.

Variation among families (Fig 1d, Table S1) prompted us to calculate inbreeding load for asexual reproduction in each family to examine genetic variation in ID with and without herbivores. Inbreeding load varied among families, but was 0.93 ± 0.20 under herbivory, (mean ± 1 SE) and 0.07 ± 0.29 in the absence of herbivores; i.e. not different from zero (Fig. 3).

There were widespread changes to defence-related metabolites as a function of simulated herbivory and inbreeding. Six phenolics (hydroxycinnamic acid derivatives) were consistently detectable in all family, breeding and herbivory treatments, and are the focus of our quantitative analysis (Fig. 4 for representative chromatograms). Mechanical damage successfully induced plants: three of six phenolics were significantly upregulated under simulated herbivory (Fig 5,

Figure 3 Field measurements of genetic variation in inbreeding load in a fitness correlate (asexual reproduction), when protected from herbivory (white bars) and when exposed to herbivory (black bars). Inbreeding load (B) was calculated for each family as $B = (\ln W_o - \ln W_i)$, where W_o and W_i are the mean fitnesses of inbred and outcrossed progeny respectively. Dashed line denotes theoretical minimum (corresponding to a conventional depression of $\delta = 0.5$) at which an outcrossing mating strategy is favoured over inbreeding. Families with very low values of B appear as almost absent.

Table S2). In two cases (Fig. 5a,b), this relationship appeared linear. In one case (Fig 5e), induction was only apparent at 20% damage, and only in outbred plants. Simulated herbivory caused apparent downregulation in inbred plants in some compounds: compared with controls, inbred plants with 20% damage showed 47 and 52% reductions in the amounts of compounds D and F respectively. The two compounds with the most pronounced reductions due to inbreeding (Fig. 5c,f) appeared to be relatively invariant in response to the damage treatment; compound C (tentatively identified as chlorogenic acid), and compound F were, respectively, reduced 64 and 52% as a result of inbreeding. In total, outcrossed plants had 47% greater phenolic investment compared with inbred plants, with greater expression for five of the six individual compounds we analysed. Outcrossed plants also produced a greater diversity of phenolics: the number of hydroxycinnamic acid peaks was reduced under inbreeding by 35% (Fig 6).

DISCUSSION

Our results provide strong evidence that herbivory can be an agent of selection favouring outcrossing in this species. This study is apparently among the first to demonstrate significant herbivory × breeding interactions for fitness and growth traits using replicate genetic families under natural environmental conditions (Ivey *et al.* 2004). These interactive effects were most apparent for the likelihood of fruiting, fruit number, asexual reproduction and biomass, but were also evident in survivorship. In the presence of herbivores, outcrossed plants consistently had twice the growth and fitness of inbreds (Fig. 2), which suggests that outcrossing populations of *S. carolinense* experiencing herbivory (i.e. most horsenettle populations) should be highly resistant to the establishment of selfing alleles, thereby maintaining an outcrossing mating system. Moreover, our data suggest that populations that escape herbivory would be susceptible to invasion by alleles conferring increased selfing. Small populations often suffer mate limitation (Sexton *et al.* 2009), but can also escape herbivores (Kery *et al.* 2001), and these two conditions may collectively influence the evolution of mating system transitions among populations; conversely, high herbivory in small, inbreeding populations could contribute to local extinction. A phylogenetic analysis of the Solanaceae demonstrated that mating system transitions from outcrossing to inbreeding have influenced the evolution of defence strategies (Campbell and Kessler *unpublished data*); taken together, these results suggest coevolutionary relationships among population size, plant defence and sexual mating systems at both macro- and microevolutionary scales.

Studies have suggested that ID should generally be greater under the stress of field conditions (Armbruster & Reed 2005). However, an interesting result from our study is a lack of inbreeding effects

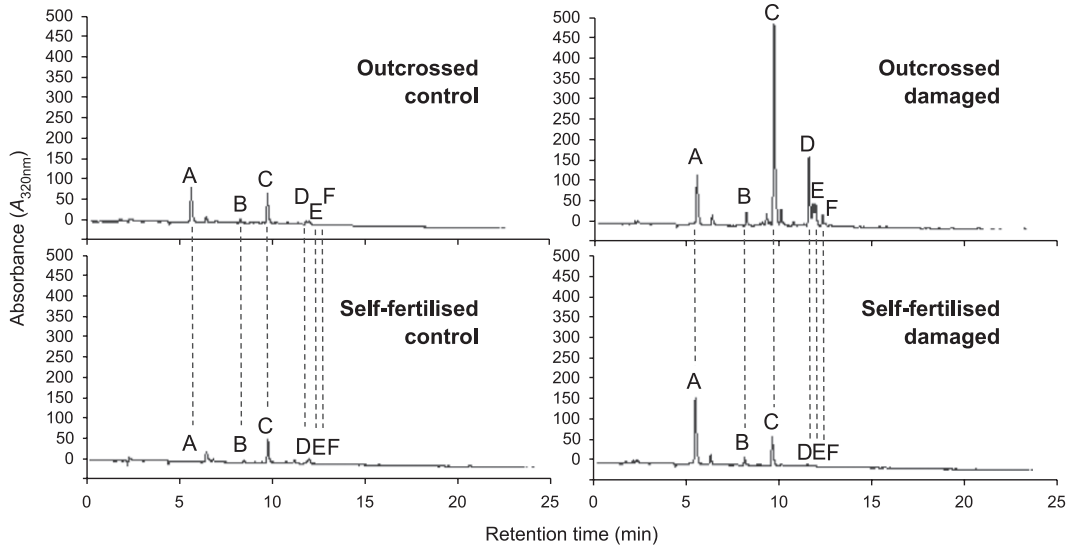

Figure 4 Representative HPLC chromatograms of inbred (self-fertilised) and outcrossed *Solanum carolinense* that had either received 20% manual damage, or been left undamaged (control). Letters denote different caffeic acid-based phenolic compounds (unidentified), as ascertained by retention times and UV_{320} spectra in comparison with an authentic chlorogenic acid standard. C = chlorogenic acid (tentative). Note that some compounds were undetectable in both control and damaged selfed plants.

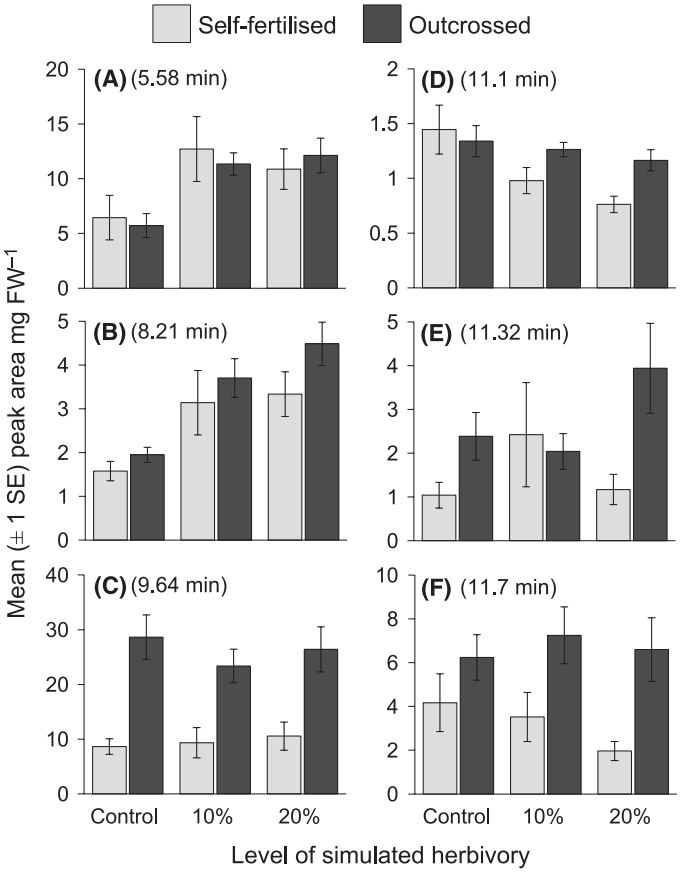

Figure 5 Amounts of six defence-related phenolics (hydroxycinnamic acid derivatives) in inbred and outcrossed *Solanum carolinense* that were exposed to 0, 10 and 20% simulated herbivory. Letters (retention times) correspond with peaks in Figure 4.

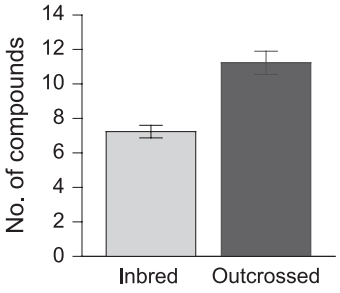

Figure 6 Average diversity (± 1 SE) of defence-related phenolics in undamaged inbred and outcrossed *Solanum carolinense* ($P = 0.01$).

when plants were protected from herbivory (Fig. 2). In other words, field conditions other than herbivory (e.g. ambient nutrients, moisture, pH, light and competition with surrounding vegetation) were associated with a lack of ID, and if anything, may alleviate its expression relative to the magnitude of ID found in a greenhouse study (Mena-Ali *et al.* 2008). This result is inconsistent with some (Fox & Reed 2011), but not all (Waller *et al.* 2008) prior empirical studies. The relationship between ID and stress may be more complex than usually thought, and specific to particular stressors, although few studies have explicitly examined environment-specific ID (Cheptou & Donohue 2011). In our study, herbivory accounted for a majority of the observed inbreeding depression, with inbreeding loads for asexual reproduction being consistently high across most families under herbivory, but variable, and indistinguishable from zero overall, when protected (Fig 3). In the absence of strong intrinsic effects of inbreeding in the herbivore-exclusion treatment or strong herbivore effects overall, we conclude that inbreeding depression in the field may primarily be ecologically mediated for this species, at least for the 3 years of this study. One implication of this ecologically mediated ID is that purging of genetic load for fitness-related traits would be much more rapid under herbivory, suggesting a role for herbivores in the coevolution of inbreeding and ID.

We found significant population and genetic variation for fitness, growth and damage. This variation did not override the interactive effects of herbivory and inbreeding, in contrast to other studies that have found highly divergent, or even opposing effects of inbreeding among populations (Ouborg *et al.* 2000; Carr & Eubanks 2002). One reason for this difference may simply be the fact that *S. carolinense* is predominantly outcrossing (although see Travers *et al.* 2004), and populations may carry greater deleterious load on average than more inbreeding taxa, particularly given the opportunity for that load to be sheltered by periods of facultative asexual reproduction. The strength of ecological ID may be greater in outcrossing taxa and populations, suggesting that SI status (or another direct mating system correlate) could be used to predict the effects of inbreeding on antagonistic species interactions.

The mechanism for ecological ID in our system appears to be decreased resistance in inbred horsenettle plants (Fig 1; see also Delphia *et al.* 2009a). Our analysis of foliar secondary metabolites suggests that this is at least partly driven by reductions in secondary metabolites under inbreeding. In addition to reducing the number of compounds produced by 35%, overall investment in phenolic expression was quantitatively reduced by 47% by inbreeding (Table S2). Phenolics are ubiquitous defence-related secondary metabolites in many Solanaceae (Friedman 1997), and are significantly negatively correlated with flea beetle damage in the field in *S. carolinense* (Campbell *et al. unpublished*), implicating them as defensive metabolites.

The interactive (non-additive) fitness effects of herbivory and inbreeding, and our chemical analysis, collectively support the hypothesis that there is deleterious load associated with defence traits (defence depression hypothesis), rather than the hypothesis that herbivory is simply an additional, non-specific stress. Under a functional trait depression hypothesis, we predict the effects of inbreeding on herbivory to be specific to herbivore identity, trait variation and the efficiency of load purging, all of which vary among populations. Thus, this hypothesis may be consistent with variable effects among families, populations and herbivores found in prior studies (Carr & Eubanks 2002; Hull-Sanders & Eubanks 2005; Leimu *et al.* 2008). This hypothesis could be used as a framework for predicting inbreeding effects on defence. For example, herbivore species that differed in their tolerance of plant traits affected by inbreeding would be predicted to generate herbivore-specific selection on mating systems, and could lead to divergent communities on inbred and outbred plants. Moreover, we would predict greater selection against inbreds by generalist herbivores,

rather than specialist herbivores, if the latter are less sensitive to host plant defence traits. These hypotheses remain to be tested; one prior laboratory study has explicitly compared the performance of generalist and specialist herbivores on inbred and outcrossed plants, but found *outbreeding* depression for resistance to two specialist beetles and one generalist moth, and inbreeding depression only for resistance to a generalist aphid (Hull-Sanders & Eubanks 2005); in contrast, our study examined two Solanaceous specialists (flea beetles and *M. sexta*). A non-specific stress hypothesis, under which the additive effects of inbreeding and herbivory should covary primarily with herbivore abundance or impact, could be used as an alternative hypothesis in this framework, and this hypothesis also has some support (Leimu *et al.* 2008).

The quantitative and qualitative reductions in defence-related compounds under inbreeding also raise interesting questions on the location of genetic load in defence trait expression and the connection between homozygosity and phenotypic diversity. Secondary metabolite expression can be broken down into three stages: (1) Acquisition/processing of resources during primary metabolism, (2) allocation of precursors from primary metabolism (e.g. amino acids) and (3) biosynthesis of secondary metabolites from these precursors. In this simplistic model, each stage could harbour deleterious mutations for key enzymes, and/or for regulatory sequences or signalling molecules. Distinguishing the relative contributions of deleterious load at each level is difficult as pathways for the biosynthesis of defence molecules operate by feedback/signalling mechanisms that may themselves harbour genetic load, making it difficult to differentiate upstream regulatory/signalling mutations from downstream enzymatic mutations. Nevertheless, our results allow us to begin to tease apart the source of inbreeding effects on defence traits.

First, if mutations at the level of resource acquisition or primary metabolism were primarily responsible, we would predict correlations between growth and defence. However, intrinsic differences in growth due to inbreeding (in the absence of herbivory) were minor in the field (Fig 2), and similarly, inbreeding has relatively minor effects on growth in greenhouse experiments (Mena-Ali *et al.* 2008), leading us to tentatively reject resource limitation. Mutations in allocation would be predicted to affect a set of related pathways similarly, e.g. all compounds produced from a common precursor. However, the phenolics we measured are all products of the phenylpropanoid pathway, and derived from phenylalanine (Petersen *et al.* 2010), yet exhibited diverse responses to inbreeding. This suggests that the load associated with defence trait expression in this species may be predominantly localised at the later stages of biosynthesis. This is consistent with the finding that no compound was upregulated under inbreeding, which might be expected if mutations occurred early in biosynthesis when plants could still reallocate precursors. Our findings suggest that this load is variable, with reductions in biosynthetic efficiency for some compounds, but loss-of-function mutations for others. This produced an apparent correlation between presumed allelic diversity (increased homozygosity under inbreeding) and chemical phenotypic diversity (Fig 6) that may be of broader significance for studies of ID and species interactions, particularly as studies have suggested that phytochemical diversity may play an important role in defence (Berenbaum *et al.* 1991). However, we have no way of distinguishing the relative importance of the qualitative and quantitative changes to defensive chemistry due to inbreeding in *S. carolinense* at this time.

Finally, our results also suggest that signalling/regulatory machinery involved in plant responses to herbivory (e.g. the jasmonic acid pathway) were affected by inbreeding. In two phenolics, a reduction due to inbreeding was only apparent under high (20%) damage, indicating reduced upregulation in inbred plants (Fig 5b,f). Moreover, inbred plants exhibited apparent downregulation of two compounds in response to herbivory (Fig 5d,f). These results suggest deleterious mutations at loci involved in the regulation of the defence response both in terms of ramping up defence-related metabolites, and maintaining production of others, although we note that the damage × breeding interaction was only significant for one compound (Table S2). These findings lead us to hypothesise that the production of phytohormones involved in trait regulation (e.g. jasmonic acid) was disrupted by inbreeding, although this hypothesis remains to be tested.

In conclusion, our study (based on replicate genetic families from multiple populations) shows strong, herbivore-mediated, ecological inbreeding depression in nature. The strength of ID, in concert with a range of additional factors (Goodwillie *et al.* 2005) is considered a primary impediment to selfing (Husband & Schemske 1996), and thus our results indicate that herbivory may be a significant factor in mating system evolution in this species. Increased ID under herbivory may be mediated in part by the significant qualitative and quantitative reductions in defence-related secondary metabolites, and alterations to the signalling and regulatory machinery governing expression of those compounds. These findings suggest new avenues of investigation in this emerging field of research.

ACKNOWLEDGEMENTS

We thank R. Halitschke (Cornell) for assistance with phenolic analysis; M. del Campo (Cornell) for providing *Manduca sexta* larvae; P. Cooper (Cornell) for assistance with plant maintenance; N. Ostman, F. R. Wesley, P. Marks, D. Lewis (Cornell) and T. Brown (Enfield, NY) for assistance locating and accessing horsenettle populations; S. Moreno, A. Combs, O. Huot and M. Freedman for assistance with plant breeding; E. Fagan, M. Freedman, L. Decker, K. Chalkowski, D. Dunstan (Cornell) and particularly R. Halitschke and S. Cardinal (Canadian National Insect Collection, Ottawa, ON), for assistance in the field and lab; the Natural Sciences and Engineering Research Council (Canada) (NSERC PGS-D fellowship to SAC), the National Science Foundation (USA) (NSF-IOS 0950225 to AK), the United States Department of Agriculture National Research Initiative (USDA grant 2006-35302-17431 to JST), the Grace Griswold Graduate Research Endowment (Cornell Entomology Grant to SAC), the Andrew Mellon Foundation (Cornell Graduate Research Grants to SAC) and the College of Agriculture and Life Sciences (Cornell University) for funding; M. Geber, A. Agrawal, A. Parachnowitsch, P. Marks, R. Raguso, R. Halitschke, S. Cardinal, S. Deleon, K. Morrell, B. Lazzaro, the Cornell Plant-Insect Interactions Group (PIG), and three anonymous reviewers for feedback on this project and/or drafts of this manuscript. The authors affirm they have no conflicts of interest. Journal restrictions caused us to exclude numerous citations, particularly on inbreeding effects in animals, and we apologise to those authors whose work we were unable to cite.

AUTHORSHIP

SAC conceptualised the research; SAC designed the research with AK and JST; SAC conducted the experiments and collected the data; SAC analysed the data with AK; SAC wrote the manuscript with AK and JST.

REFERENCES

Adler, L.S., Wink, M., Distl, M. & Lentz, A.J. (2006). Leaf herbivory and nutrients increase nectar alkaloids. *Ecol. Lett.*, 9, 960–967.

Armbruster, P. & Reed, D.H. (2005). Inbreeding depression in benign and stressful environments. *Heredity*, 95, 235–242.

Armbruster, P., Hutchinson, R.A. & Linvell, T. (2000). Equivalent inbreeding depression under laboratory and field conditions in a tree-hole-breeding mosquito. *Proc. R. Soc. Lond. B Biol. Sci.*, 267, 1939–1945.

Auld, J.R. & Relyea, R.A. (2010). Inbreeding depression in adaptive plasticity under predation risk in a freshwater snail. *Biol. Lett.*, 6, 222–224.

Barrett, S.C.H. & Harder, L.D. (1996). Ecology and evolution of plant mating. *Trends Ecol. Evol.*, 11, A73–A79.

Bello-Bedoy, R. & Núñez-Farfán, J. (2010). Cost of inbreeding in resistance to herbivores in *Datura stramonium*. *Ann. Bot.*, 105, 747–753.

Bello-Bedoy, R. & Núñez-Farfán, J. (2011). The effect of inbreeding on defence against multiple enemies in *Datura stramonium*. *J. Evol. Biol.*, 24, 518–530.

Berenbaum, M.R., Nitao, J.K. & Zangerl, A.R. (1991). Adaptive significance of furanocoumarin diversity in *Pastinaca sativa* (Apiaceae). *J. Chem. Ecol.*, 17, 207–215.

Carr, D.E. & Eubanks, M.D. (2002). Inbreeding alters resistance to insect herbivory and host plant quality in *Mimulus guttatus* (Scrophulariaceae). *Evolution*, 56, 22–30.

Carr, D.E., Murphy, J.F. & Eubanks, M.D. (2003). The susceptibility and response of inbred and outbred *Mimulus guttatus* to infection by Cucumber mosaic virus. *Evol. Ecol.*, 17, 85–103.

Carson, W.P. & Root, R.B. (2000). Herbivory and plant species coexistence: community regulation by an outbreaking phytophagous insect. *Ecol. Monogr.*, 70, 73–99.

Charlesworth, B. & Charlesworth, D. (1999). The genetic basis of inbreeding depression. *Genet. Res.*, 74, 329–340.

Cheptou, P.O. & Donohue, K. (2011). Environment-dependent inbreeding depression: its ecological and evolutionary significance. *New Phytol.*, 189, 395–407.

Darwin, C. (1876). *The Effects of Cross and Self Fertilisation in the Vegetable Kingdom*. J. Murray, London.

Delphia, C.M., De Moraes, C.M., Stephenson, A.G. & Mescher, M.C. (2009a). Inbreeding in horsenettle influences herbivore resistance. *Ecol. Entomol.*, 34, 513–519.

Delphia, C.M., Rohr, J.R., Stephenson, A.G., De Moraes, C.M. & Mescher, M.C. (2009b). Effects of genetic variation and inbreeding on volatile production in a field population of horsenettle. *Int. J. Plant Sci.*, 170, 12–20.

Dudash, M.R. (1990). Relative fitness of selfed and outcrossed progent in a self-compatible, protandrous species, *Sabatia angularis* L (Gentianaceae): a comparison in 3 environments. *Evolution*, 44, 1129–1139.

Elle, E. & Hare, J.D. (2002). Environmentally induced variation in floral traits affects the mating system in *Datura wrightii*. *Funct. Ecol.*, 16, 79–88.

Ellison, A., Cable, J. & Consuegra, S. (2011). Best of both worlds? Association between outcrossing and parasite loads in a selfing fish. *Evolution*, 65, 3021–3026.

Escobar, J.S., Nicot, A. & David, P. (2008). The Different Sources of Variation in Inbreeding Depression, Heterosis and Outbreeding Depression in a Metapopulation of Physa acuta. *Genetics*, 180, 1593–1608.

Fox, C.W. & Reed, D.H. (2011). Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution*, 65, 246–258.

Friedman, M. (1997). Chemistry, biochemistry, and dietary role of potato polyphenols. A review. *J. Agric. Food Chem.*, 45, 1523–1540.

Goodwillie, C., Kalisz, S. & Eckert, C.G. (2005). The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annu. Rev. Ecol. Evol. Syst.*, 36, 47–79.

Hayes, C.N., Winsor, J.A. & Stephenson, A.G. (2004). Inbreeding influences herbivory in *Cucurbita pepo* ssp *texana* (Cucurbitaceae). *Oecologia*, 140, 601–608.

Henry, P.Y., Pradel, R. & Jarne, P. (2003). Environment-dependent inbreeding depression in a hermaphroditic freshwater snail. *J. Evol. Biol.*, 16, 1211–1222.

Hull-Sanders, H.M. & Eubanks, M.D. (2005). Plant defense theory provides insight into interactions involving inbred plants and insect herbivores. *Ecology*, 86, 897–904.

Husband, B.C. & Schemske, D.W. (1996). Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution*, 50, 54–70.

Ivey, C.T., Carr, D.E. & Eubanks, M.D. (2004). Effects of inbreeding in *Mimulus guttatus* on tolerance to herbivory in natural environments. *Ecology*, 85, 567–574.

Jarne, P. & Charlesworth, D. (1993). The evolution of the selfing rate in functionally hermaphrodite plants and animals. *Annu. Rev. Ecol. Syst.*, 24, 441–466.

Kariyat, R.R., Scanlon, S.R., Mescher, M.C., De Moraes, C.M. & Stephenson, A. G. (2011). Inbreeding depression in *Solanum carolinense* (Solanaceae) under field conditions and implications for mating system evolution. *PLoS ONE*, 6, 7.

Kariyat, R.R., Mauck, K.E., De Moraes, C.M., Stephenson, A.G. & Mescher, M.C. (2012). Inbreeding alters volatile signalling phenotypes and influences tri-trophic interactions in horsenettle (*Solanum carolinense* L.). *Ecol. Lett.*, 15, 301–309.

Keinanen, M., Oldham, N.J. & Baldwin, I.T. (2001). Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. *J. Agric. Food Chem.*, 49, 3553–3558.

Keller, L.F. & Waller, D.M. (2002). Inbreeding effects in wild populations. *Trends Ecol. Evol.*, 17, 230–241.

Kery, M., Matthies, D. & Fischer, M. (2001). The effect of plant population size on the interactions between the rare plant *Gentiana cruciata* and its specialized herbivore *Maculinea rebeli*. *J. Ecol.*, 89, 418–427.

Kessler, A. & Halitschke, R. (2009). Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: predictions and case study. *Funct. Ecol.*, 23, 901–912.

Leimu, R., Kloss, L. & Fischer, M. (2008). Effects of experimental inbreeding on herbivore resistance and plant fitness: the role of history of inbreeding, herbivory and abiotic factors. *Ecol. Lett.*, 11, 1101–1110.

Littell, R.C., Stroup, W.W. & Freund, R.J. (eds.) (2002). *SAS® System for Linear Models*. SAS Institute Inc, Cary, NC.

Mena-Ali, J.I., Keser, L.H. & Stephenson, A.G. (2008). Inbreeding depression in *Solanum carolinense* (Solanaceae), a species with a plastic self-incompatibility response. *BMC Evol. Biol.*, 8, 10.

Norman, J.K., Sakai, A.K., Weller, S.G. & Dawson, T.E. (1995). Inbreeding depression in morphological and physiological traits of *Schiedea lydgatei* (Caryophyllaceae) in two environments. *Evolution*, 49, 297–306.

Núñez-Farfán, J., Cabrales-Vargas, R.A. & Dirzo, R. (1996). Mating system consequences on resistance to herbivory and life history traits in *Datura stramonium*. *Am. J. Bot.*, 83, 1041–1049.

Ouborg, N.J., Biere, A. & Mudde, C.L. (2000). Inbreeding effects on resistance and transmission-related traits in the Silene-Microbotryum pathosystem. *Ecology*, 81, 520–531.

Petersen, M., Hans, J. & Matern, U. (2010). Biosynthesis of phenylpropanoids and related compounds. In: *Annual Plant Reviews* (ed Wink, M.). Blackwell Publishing, Oxford, pp. 182–257.

Puurtinen, M., Hytonen, M., Knott, K.E., Taskinen, J., Nissinen, K. & Kaitala, V. (2004). The effects of mating system and genetic variability on susceptibility to trematode parasites in a freshwater snail, *Lymnaea stagnalis*. *Evolution*, 58, 2747–2753.

Schmitt, J. & Ehrhardt, D.W. (1990). Enhancement of inbreeding depression by dominance and suppression in *Impatiens capensis*. *Evolution*, 44, 269–278.

Sexton, J.P., McIntyre, P.J., Angert, A.L. & Rice, K.J. (2009). Evolution and ecology of species range limits. *Annu. Rev. Ecol. Evol. Syst.*, 40, 415–436.

Steets, J.A., Salla, R. & Ashman, T.L. (2006). Herbivory and competition interact to affect reproductive traits and mating system expression in *Impatiens capensis*. *Am. Nat.*, 167, 591–600.

Stephenson, A.G., Leyshon, B., Travers, S.E., Hayes, C.N. & Winsor, J.A. (2004). Interrelationships among inbreeding, herbivory, and disease on reproduction in a wild gourd. *Ecology*, 85, 3023–3034.

Strauss, S.Y., Conner, J.K. & Rush, S.L. (1996). Foliar herbivory affects floral characters and plant attractiveness to pollinators: implications for male and female plant fitness. *Am. Nat.*, 147, 1098–1107.

Travers, S.E., Mena-Ali, J. & Stephenson, A.G. (2004). Plasticity in the self-incompatibility system of *Solanum carolinense*. *Plant Species Biol.*, 19, 127–135.

Waller, D.M., Dole, J. & Bersch, A.J. (2008). Effects of stress and phenotypic variation on inbreeding depression in *Brassica rapa*. *Evolution*, 62, 917–931.

SUPPORTING INFORMATION

Additional Supporting Information may be downloaded via the online version of this article at Wiley Online Library (www.ecologyletters.com).

Editor, Ted Turlings
Manuscript received 1 May 2012
First decision made 13 June 2012
Second decision made 27 September 2012
Manuscript accepted 18 October 2012