CHEMICAL ECOLOGY AND COEVOLUTION OF INTERACTIONS BETWEEN PLANT MATING AND DEFENCE STRATEGIES

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Sexual reproduction is ubiquitous and variable both within and among species. This variation has profound consequences (inbreeding depression, ID), which may explain why so many species avoid inbreeding. However, we know little about how such variation influences the ecology of antagonistic species interactions (predation, herbivory, parasitism). Consequently, we also know little about whether such interactions in turn play a significant role in the evolution of mating systems. I investigated how plant mating strategies (outcrossing and inbreeding) affect the chemical ecology and evolution of interactions with insect herbivores. At an intraspecific scale, I conducted experiments with wild horsenettle, *Solanum carolinense* (Solanaceae). Inbreeding deleteriously affected expression of defence-related secondary metabolites and resistance to insect herbivores in both the laboratory and field. Inbreeding disrupted phytohormone-mediated regulation of growth and defence responses, providing the first evidence of a hormonal basis to plant growth and defence responses to inbreeding. In the field, natural herbivory by flea beetles (*Epitrix* spp.) for three years led to significant ID for growth and fitness; in contrast, protection from insect herbivores almost completely alleviated the strength of ID. Thus, herbivore-mediated, ecological inbreeding depression could maintain outcrossing mating systems. I then examined mating system-defence interactions at a macroevolutionary scale. In the Solanaceae, outcrossing is enforced by a self-incompatibility mechanism that is ancestral, but has been irreversibly and repeatedly lost, allowing me to test the consequences of evolutionary mating system shifts for the macroevolution of defence. A phylogenetically-controlled study of constitutive and induced resistance in over 20 self-

incompatible and 30 self-compatible species, including petunia, tobacco, pepper, tomato and potato taxa, showed that mating and defence strategies have evolved in a correlated fashion: shifts from outcrossing to inbreeding mating systems are associated with reductions in constitutive resistance to *Manduca sexta* caterpillars, but strong and significant increases in inducibility of resistance traits. Across the family, mating systems exhibit divergent, but significant negative correlations between inducible and constitutive resistance strategies, providing the first robust phylogenetic evidence that these may be evolutionary alternatives. I conclude that the sex life of plants may be an important, unappreciated force in the ecology and evolution of insect-plant interactions.

BIOGRAPHICAL SKETCH

Stuart was born in Vancouver, B.C., Canada. He grew up in the smaller city of Port Coquitlam, once a bustling oolichan (oil fish) port on the Fraser River, though now reduced to the status of Vancouver suburb. Stuart's favourite time as child and teenager was spent at the family cabin on a small island in Howe Sound, spending most of his time exploring the island (laying down trails, building forts, combing beaches, and scrambling up rocky outcrops), swimming, snorkeling, fishing and hunting for crabs and canoeing with the seals – alone and with his brothers and sister. Thus, without realising it (and thanks to his parents), Stuart developed a love for the outdoors, nature and exploration.

Stuart attended Mary Hill Elementary and Junior High Schools, enrolling in the French Immersion program, and finally graduating from Terry Fox Senior Secondary School. He started at Simon Fraser University, Burnaby, B.C. in both English literature and something that might vaguely be construed as "pre-med". But two courses then changed everything: a course on algae and fungi with Jim Rahe, that had him out collecting samples and exploring the same intertidal that he had played in as a kid, and a course on animal behavior with Larry Dill, whose enthusiasm and intellect made behavioural ecology in particular, an exciting and challenging field. Stuart finished his BSc in biology, taking as many lab and field courses as were offered in ecology, behaviour and physiology. During his last year, he realized that his patchwork transcript and bimodal grade distribution were not going to be opening many doors for him, and so he started working as an assistant in the lab of Mark Winston (FRSC), assisting with lab apiculture and studies of bee behavior. He then moved down two lab benches to the lab of John Borden (FRSC), and worked with grad student Dezene Huber (now at the University of Northern B.C.). Working with Dezene introduced him to the fascinating world of chemical ecology and with Dezene's encouragement, Stuart approached John Borden about doing an honours project –which somehow turned into a MSc. After two years spent driving all over the province and playing in the woods with beetles, Stuart had learned just a little bit about science

and writing from John, as well as an appreciation for Chinese-Canadian fusion restaurants in small-town B.C.. Stuart then worked as a technician in the lab of John Klironomos, at UGuelph's Botany Department, and enjoyed learning about mycorrhizae, as well as plant physiological and evolutionary ecology from Hafiz Maherali and Christina Caruso (more or less respectively).

Upon arriving at Cornell, Stuart was overwhelmed by the abundance of interesting research, people and seminars, and spent the better part of his first year developing ideas and reading more than he was supposed to. He deeply enjoyed contributing to the Entomology Department as a member of *Jugatae*, particularly as a two-time coordinator of the department's Seminar Series. He also deeply enjoyed teaching numerous Entomology courses, and the Introductory Biology Laboratory. Stuart's projects had a rough period – voles, rabbits, mildew and horsenettle's aversion to incest all conspired to prevent him from fulfilling his desire of doing research, and there were many rough patches along the way. Eventually, things started to work, and work well, and he finished up his PhD in the lab of André Kessler, having learned a whole lot more about chemical and evolutionary ecology, and having been reminded by André that he might have a future in science after all.

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Second, I thank my committee members Monica Geber and Jennifer Thaler for the advice and input on these projects. Monica I thank in particular for being a source of inspiration, who with her opening question during my A-exam ("What do you think about sex?"), prompted a discussion of the coevolution of mating and defence that continues for me today. Monica has provided the most insightful (and at times incisive) comments during the formulation of my projects, and I am grateful to her for providing both sound advice, a voice of reason, and a sympathetic ear; and for being a role model for how to do evolutionary ecology. While no longer an official committee member, I also thank Anurag Agrawal. Jennifer and Anurag provided formative training on insect-plant interactions, and they provided a challenging, stimulating environment, particularly in the form of lab meetings, reading seminars and the plant-insect interactions group (PIG). I know I am a better scientist because of them. I'm grateful for their input and strong opinions, and hope they have come to appreciate mine.

I am grateful to numerous sources of funding that have supported my research. I thank the Natural Sciences and Engineering Research Council (Canada) for a three year post-graduate fellowship, and the Graduate School (particularly Sarah Hale) for supporting this fellowship

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Numerous undergraduates have assisted me with the unenviable task of working with a spiny *Solanum* (horsenettle). I thank them all for many hours spent extracting pollen for breeding, tending the plants, crushing tissue, counting feeding holes, weeding the field plot; and for all the sore (or missing) fingers and inhaled, dried leaf tissue ('death dust'). I owe you all: Sarah Moreno, Alice Combs, Rebecca Soloway, Ilana Goldowitz, Ordom Huot, Ellen Fagan, Micah Freedman, Leslie Decker, Kayleigh Chalkowski and David Dunstan. I especially thank: Leslie Decker, for many hours spent (re)weighing bits of horsenettle and many other Solanaceae, and doing everything else I asked (and more) with a smile; and Micah Freedman, who continues working on mating system/herbivory interactions, and who for three years has been an unfailing support to my different projects, particularly the comparative study.

Acquiring germplasm for these experiments was made possible through the help of numerous researchers and institutions around the world. I thank Tom Brown (Enfield, NY), Nancy Ostman, F. R. Wesley, Peter Marks and Drew Lewis (Cornell) for providing locations of, or access to, horsenettle populations around Ithaca, NY. I also thank Cris Kühlemeier (Berne) for *Petunia* seeds, Alice Stanford (University of the Virgin Islands) for *Solanum conocarpum* seeds,

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1. INTRODUCTION

A central goal in evolutionary biology is to link macroevolutionary patterns with microevolutionary processes such as natural selection (Simons 2002; Kutschera & Niklas 2004). Conversely, a current goal of ecology is to understand the consequences of phylogenetic history (macroevolution) for population-level ecological interactions such as mutualism or parasitism (Mitter *et al.* 1991). Few studies bridge these perspectives to understand the feedbacks between species-level variation and population-level interactions. Mating and reproductive strategies (e.g., sexual vs. asexual reproduction) provide a natural interface between evolution and ecology. Within species, mating strategies influence gene flow, recombination, and population structure (e.g., genetic variance) (Charlesworth & Charlesworth 1995; Hamrick & Godt 1996). Mating systems thereby influence interacting species by shaping the distribution of functional phenotypes, and can influence the response to natural selection by the local (a)biotic environment (Lande & Schemske 1985; Charlesworth 1992; Jarne & Charlesworth 1993; Charlesworth & Charlesworth 1995; Charlesworth 2003). Thus, macroevolutionary transitions in mating strategy can potentially have far-reaching consequences (Igić *et al.* 2008). Ecological interactions can feed back to drive the evolution of mating strategies. For example, classical theory on the evolution of sex suggests that antagonistic coevolution (e.g., between parasites and hosts) should favor sexual over asexual reproduction (van Valen 1973; Barton & Charlesworth 1998). However, few studies have tested how variation in sexual mating systems influences species interactions (and vice versa).

The research described in this dissertation focuses on interactions between mating systems and plant-herbivore interactions as a model, not only for the broader study of mating system ecology, but also for understanding the conceptual links between functional traits, intraspecific/population ecology, and comparative biology. Part 1 is an experimental evaluation

of the effects of intraspecific mating system variation (inbreeding and outcrossing) for defence trait expression and resistance to insect herbivores; in addition, this paper demonstrates the strength of herbivore-mediated ecological inbreeding depression in the field. Part 2 is an analysis of how inbreeding affects two fundamental components of plant defence: inducible defence, and tolerance-related growth. Both types of traits can be considered as phenotypic plasticity in response to herbivory. In addition, Part 2 makes a significant contribution to the understanding of the mechanisms for plant responses under inbreeding by examining the effects of inbreeding on the expression of four key phytohormones that mediate growth and defence trait expression. Finally, Part 3 complements these intraspecific/microevolutionary approaches by testing for the consequences of mating system evolution for the macroevolution of defence strategies. The phylogenetic analyses in Part 3 contribute to our broader understanding of plant evolution, by showing how mating systems may coevolve with seemingly unrelated traits such as plant defence. Overall, these studies indicate that mating systems and plant defence may have influential reciprocal (i.e., coevolutionary) effects on each other at both chemical ecological and evolutionary scales.

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2. Plant chemistry links herbivory and the evolutionary ecology of plant mating systems

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ABSTRACT

Inbreeding reduces offspring fitness, and this cost (inbreeding depression, ID) should dictate mating system evolution. However, the ecological effects of inbreeding remain poorly understood. Ecological interactions such as herbivory should be affected by inbreeding if functional traits harbour deleterious load, and these interactions could thereby influence the magnitude of ID. We tested the effects of experimental inbreeding on the chemically-mediated interaction between *Solanum carolinense* (Solanaceae) and its native herbivores. In the field, we manipulated the presence of herbivores on experimentally inbred and outcrossed plants for three years. Damage was significantly greater on inbred plants, and inbreeding depression for growth, survival and reproduction was significantly greater under herbivory. The magnitude of ecologically-mediated ID was such that herbivores alone could maintain an outcrossing mating system. In a greenhouse experiment, we measured changes in constitutive and induced levels of defence-related secondary metabolites and found that inbreeding deleteriously altered phenolic expression both qualitatively (phytochemical diversity) and quantitatively, indicating deleterious load at loci related to the biosynthesis of defence compounds. Our results provide compelling evidence that inbreeding effects on plant-herbivore interactions are mediated by widespread changes to functional plant metabolites, suggesting that within- and among population variation in inbreeding could be a useful predictor of defence trait variation. We conclude that herbivory can be a potent agent of selection in the ecology and evolution of plant mating systems.

"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-fertilization (Jarne & Auld 2006; Barrett 2010). Darwin was among the first to recognise that this variation can have profound consequences for fitness in the form of inbreeding depression, and he postulated that this explained why so many species avoid incest and/or self-fertilization (Darwin 1876; Barrett & Harder 1996). However, despite decades of research on the fitness costs of inbreeding in both plants and animals (Husband & Schemske 1996; Crnokrak & Roff 1999), we still know little about the broader consequences of mating system variation (Keller & Waller 2002), 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 (and possibly overdominance effects) at loci linked to fitness (Dudash & Carr 1998; Charlesworth & Charlesworth 1999). Theoretical models of mating system evolution focus on ID as the primary impediment to the evolution of selfing (Jarne & Charlesworth 1993), and 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 (Lloyd 1979; Lande & Schemske 1985; Jarne & Charlesworth 1993). However, many species harbour lower or higher ID than would be predicted by their ostensible mating system (Husband & Schemske 1996; Johnston & Schoen 1996; Goodwillie *et al.* 2005; Winn *et al.* 2011). Studies attempting to resolve this paradox highlight the importance of factors such as biparental inbreeding (Uyenoyama 1986) and

selective interference (Lande *et al.* 1994; Winn *et al.* 2011), but also 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; Eckert & Barrett 1994; Armbruster *et al.* 2000). In general, stress appears to exacerbate ID (Armbruster & Reed 2005; Kristensen *et al.* 2008; Cheptou & Donohue 2011; Fox & Reed 2011); however, stress can also apparently ameliorate ID (Henry *et al.* 2003; Waller *et al.* 2008). Thus, understanding the ecological factors that modulate inbreeding depression remains an important challenge for the study of mating systems.

Antagonistic species interactions (competition, predation, herbivory, parasitism/disease) 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; Koslow & Clay 2007), herbivory (see next paragraph) and possibly predation (Hass 1989; Auld & Relyea 2010). However, despite acknowledgement that antagonists could act as selective agents in mating system evolution (Steets *et al.* 2007), evidence is mixed. Several studies show inconsistent effects of antagonisms on mating systems (Carr *et al.* 2003; Haag *et al.* 2003; Puurtinen *et al.* 2004), while others suggest that the effects of inbreeding may be primarily contingent on genetic background (Strauss & Karban 1994; Ouborg *et al.* 2000), and not mating systems per se.

We examined the interaction between plant mating systems and herbivory. Herbivory can alter plant phenotypes to influence several aspects of mating systems. For example, herbivory can

modify selfing/outcrossing rates by modifying flower size/display (Strauss *et al.* 1996), gender expression (Solomon 1985), pollen or nectar chemistry (Adler *et al.* 2006; Gegear *et al.* 2007; Kessler & Halitschke 2009) and also by causing selective abortion of selfed or outcrossed fruits (Steets *et al.* 2006). Increases (Ivey & Carr 2005; Steets *et al.* 2006) but also decreases (Levri & Real 1998; Elle & Hare 2002) in selfing rates under herbivory have been reported from different species, which may reflect selection for reproductive assurance, or alternatively, selection to increase genetic variation under stress.

In addition to inducing reproductive variation, herbivores may also act as selective agents to determine the adaptive value of inbreeding and outcrossing. A number of studies have tested whether herbivores can exacerbate ID, with variable results. In controlled laboratory and 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; Muola *et al.* 2011), although in several cases the effect of inbreeding was highly contingent on genetic background and/or 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. In addition, if populations are adapted to local herbivores, plants may exhibit outbreeding depression for resistance (Leimu & Fischer 2010). Greenhouse studies have also shown the potential for herbivore-mediated inbreeding depression for growth (Carr & Eubanks 2002; Ivey *et al.* 2004; Hull-Sanders & Eubanks 2005) and fitness correlates (Carr & Eubanks 2002) in the form of a significant breeding×herbivory interaction, but have also shown amelioration of ID under damage (Leimu *et al.* 2008).

In contrast, few studies have manipulated herbivory and inbreeding in the field, and none has shown 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), similar to studies of *Cucurbita pepo* (Cucurbitaceae) (Hayes *et al.* 2004; Stephenson *et al.* 2004). A field study of the effects of inbreeding on tolerance to herbivory in *Mimulus guttatus* (Phrymaceae) showed a marginally significant interaction only for growth (Ivey *et al.* 2004). Prior studies of *Solanum carolinense* (Solanaceae) have also found variable effects on herbivory on ID (Kariyat *et al.* 2011; Mena-Ali *et al.* 2008) though possibly as a result of variable genetic replication.

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. Alternatively, there may be deleterious load at defence trait loci that leads to an herbivore-specific component of ID, and an 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 which isolate the effects of herbivory in nature, and analyses of functional defence trait variation under inbreeding.

While studies have demonstrated effects of inbreeding on plant damage and resistance (herbivore performance), virtually nothing is known of the actual mechanisms underlying the effect of inbreeding on herbivory. Since 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 the expression of defence-related foliar secondary metabolites 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; DeRose & Roff 1999; Auld & Relyea 2010). Two studies have attempted to examine effects of inbreeding on plant volatile emission and have suggested that inbred plants may show reduced indirect defence (Delphia *et al.* 2009b; Kariyat *et al.* 2012). While suggestive, one of these studies may have confounded breeding with damage effects (Delphia *et al.* 2009b), and one was not replicated to appropriately test inbreeding effects (Kariyat *et al.* 2012). Thus, the mechanistic basis for inbreeding effects on defence remains unknown.

The goals of our study were two-fold: first, to test for herbivore-mediated, ecological ID under natural conditions using a three-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

Study system

Horsenettle, *Solanum carolinense* L. (Fig. 1), is a wild nightshade (Solanaceae) native to eastern North America. In the north-eastern United States (US), it is a short-lived, weakly andromonoecious and herbaceous perennial which thrives on nutrient-poor sites (Bassett & Munro 1986). Plants reproduce sexually (fruits) and asexually, through vegetative propagation from horizontal roots occasionally described as rhizomes. Individual genets (clones) comprise a variable number of ramets, and in competitive old-field habitats, asexual reproduction appears to be the dominant mode of reproduction. Plants emerge May to late June depending on the amount of ground cover, and produce racemose inflorescences August through October. Previous studies suggest that herbivory on *S. carolinense* alters gender expression (Solomon

1985) and there is correlational evidence that herbivory may influence natural selection for floral sex-ratios (Wise & Hebert 2010). Horsenettle possesses the gametophytic self-incompatibility system (GSI) typical of over one-third of the Solanaceae. GSI reduces inbreeding by preventing pollen tube growth when pollen and maternal plants share a common allele at the self-incompatibility locus (*S*-locus). This system should create a predominantly outcrossing sexual mating system. However, Travers et al. (Travers *et al.* 2004) have shown that horsenettle's SI system is plastic, and that RNases responsible for digesting the tubes of related pollen are less active early and late in floral development. This plasticity appears to be genetically based in certain 'leaky' *S*-alleles (Mena-Ali *et al.* 2009), and suggests that even SI taxa may exhibit variation in mating system. This plastic SI system makes horsenettle an ideal system in which to examine the effects of inbreeding, since inbreeding is natural but presumably rare enough to allow the accumulation of deleterious alleles at defence-related loci.

Horsenettle is host to a variety of insect herbivores, in addition to being occasionally grazed by rodents (rabbits and voles) and molluscs (snails and slugs). In upstate New York, the following insects consume horsenettle: Beetles – *Leptinotarsa* spp., *Epitrix* spp. (flea beetles, especially *E. fuscula*), *Plagiometriona clavata* (tortoise beetle) (Chrysomelidae) and *Trichobaris trinotata* (Curculionidae); Lepidoptera – larvae of several Sphingidae, including the Solanaceous specialist *Manduca sexta* (tobacco hornworm), as well as *Sesia rileyana* (Sesiidae), *Tildenia inconspicuella* (egg-plant leafminer) and *Frumenta nundinella* (leaf-roller and seed-predator) (Gelechiidae); Hemiptera – G*argarphia solani* (egg-plant lace bug) (Tingidae); *Poecilocapsus lineatus* (four-lined plant bug) (Miridae) (Somes 1916; Solomon 1981; Wise 2007, 2009). *Solanum carolinense* is defended against herbivores by a wide range of putative defence traits, including physical structures, such as stellate trichomes and spines, and defence-related secondary metabolites, particularly phenolics (caffeic acids and flavonoids), glycoalkaloids and also proteinase inhibitors (described in (Wise & Sacchi 1996; Cipollini *et al.* 2002). Allocation to

these defences appears to be under the influence of the abiotic environment (Cipollini *et al.* 2002), and is also sensitive to induction by herbivores (Walls *et al.* 2005).

Breeding protocol

Three populations in Tompkins County, New York were sampled: Dunlop Preserve (DP: 42° 23.1357' N; 76° 23.6198' W; 308m a.s.l.), Robert Treman State Park entrance (RT: 42° 24.1762' N; 76° 23.9332' W; 148m a.s.l.), Freese Road (FR: 42° 27.8603' N; 76° 26.6198' W; 320m a.s.l.). Distances between independent populations were: DP-RT, 12.5km; RT-FR, 11 km; FR-DP, 9.6 km. From each population, we sampled the fruits from 15-30 randomly selected maternal plants. Seeds from a single fruit from each plant were germinated on moist Metro-Mix 360 all-purpose potting soil (Scotts-Sierra Horticultural Products, Marysville, OH) and from each maternal plant, 3-7 individuals were transplanted to 6L pots, and maintained in a greenhouse environment under a 16hr light: 8hr dark schedule, watered *ad libitum*, and fertilized weekly (21-5-20 NPK, 150ppm). On plants from each original dam, flowers were assigned to receive pollen from (1) the same plant (self-fertilised), (2) a randomly selected sibling (sib-mated), and (3) a randomly selected sire from the same population (out-crossed) creating full-sib families in each breeding category. Where possible, pollen source was randomised on the maternal plants to avoid systematic bias in seed investment based on fruit position. We chose to group breeding treatments on the same plant so that maternal effects would be constant across the inbred and outbred seed families. All pollinations utilised pollen freshly collected using an electric tomato pollen extractor. Pollen was extracted from several open flowers on the sire, mixed, and gently applied to target stigmas ca. 1-2 days prior to anthesis using a clean toothpick, ensuring that the entire stigmatic surface was completely coated with pollen. These bud-pollinations effectively bypassed the SI system in horsenettle, allowing successful inbreeding, but also allowed us to pollinate at a consistent stage of flower development across breeding treatments. Over 60 dams were successfully pollinated in this way, producing 11851, 10560, and 12568 self-

fertilised, sibling-mated, and outcrossed seeds, respectively. Seeds were extracted from fruits, washed in 3% hypochlorite to prevent mould, and dried for storage. Full-sibling seed families were then randomly selected from grand-maternal family for subsequent experiments (depending on availability of sufficient seeds).

Field experiment

A fourth population, approximately equidistant from the source populations, was selected for the site of a common garden experiment: Sally Dunn Pasture/Turkey Hill Road (SD/TH: 42° 22.3890' N; 76° 25.5660' W; 290m a.s.l.). The SD/TH population was selected for the common garden experiment because it was large for this species in this area (200-400 genets), it harboured an abundance of *Epitrix fuscula* beetles (a dominant herbivore of horsenettle at this latitude), and because a large area adjacent to it was identified as being free of horsenettle plants and therefore suitable for the experiment. Seventy-three selfed, sib-mated and outcrossed full-sib seed families from 29 dams were selected (i.e., 29 paired sets of selfed and outcrossed families, with 15 triplet sets also including families of sib-mated plants), and seeds were germinated on moist Metro-Mix potting soil in 27-well flats to generate replicate seedlings of approximately equal size. We chose to use seed-grown plants in order to capture critical variation in mortality and performance early in development, rather than using clones from fully-established plants. In late June 2009, after one month of growth, seedlings were transplanted to the mown fieldsite and randomly assigned one of two treatments (i.e., a split-family design): no herbivory and ambient herbivory. Plants were 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. All plants were approximately 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 abiotic

and biotic conditions. At the time of transplant, plants were of a comparable size, and were similar in size to local sprouting *S. carolinense*, although early season ramet size in local horsenettle populations is highly contingent on surrounding vegetation. Plants were watered daily for the first week after transplanting, using water from an adjacent creek, and sprayed approximately weekly (depending on local precipitation patterns) with a 0.0003% solution of esfenvalerate (Ortho® Bug-B-Gone™ multi-purpose spray), which is effective at eliminating and deterring insect herbivores, but does not change plant growth (Carson & Root 2000); A. Agrawal, *pers comm*). Surrounding vegetation was cropped by hand on a biennial basis. The experiment was maintained for three seasons (June-September, 2009-2011). Plants were censused for damage and growth characteristics in August 2009, and for growth and fitness data in October 2009, 2010 and 2011. We report here reproduction and growth from 2011, since these data represent fitness after three years of cumulative herbivore exposure over the course of the experiment. Damage due to herbivore exposure was assessed as the number of shot-holes on every leaf, since for *Epitrix* damage on horsenettle, the number of feeding holes and the area removed are strongly and significantly correlated ($R^2 = 0.793$, $P < 0.0001$). Fewer than 5% of plants in the experiment were attacked by other herbivores, which is typical for New York state populations. Growth characteristics measured included leaf number, ramet number, ramet height, the size of three randomly selected, fully expanded leaves from each individual and aboveground end-of-season biomass (dry mass); fitness data included asexual reproduction (vegetative ramet number) and sexual reproduction (whether plants fruited, and fruit number).

Defence trait experiments

Field measurements of plant defence-related traits confound intrinsic (constitutive) differences among genetic families with differences due to variable herbivore preference and variable induced responses to those herbivores. To avoid this, and 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=20) were selected at random, and clonal replicates of 2-5 selfed and outcrossed offspring per family were randomly assigned to receive one of three damage levels: 0%, 10%, and 20%. 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 1.5g 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 (N=530) were grown in the greenhouse for two-weeks, transplanted to 355mL (four-inch) pots filled with soil, and grown for an additional three weeks, as described above, with trays randomized to minimise greenhouse position effects. Damage levels (based on visual estimation) were imposed using a standard paper hole-punch to every leaf. One week after damage, a single leaf from each plant was excised with a razor blade, and a 100mg sample of fresh tissue (excluding midvein) was taken for analysis of defence-related secondary metabolites. Samples were weighed, immediately flash-frozen in liquid N_2, and stored at -80°C. Samples were then simultaneously homogenized and extracted on a FastPrep® tissue homogenizer (MP Biomedicals®, Solon, Ohio, U.S.A.) at 6 m/s for 90 s using 0.9g grinding beads (Biospec®, Zirconia/Silica 2.3mm) and 1mL 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, Torrance, CA, U.S.A.) 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, normalized 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 HPLC 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 exceeded a noise threshold that allowed quantification and/or if they possessed a clear UV spectrum. Counts were made blind to breeding status.

Finally, we grew a third set of plants from the greenhouse populations to conduct a bioassay to test for constitutive variation in resistance between mating systems using the model herbivore *Manduca sexta* L. (Lepidoptera: Sphingidae). From the youngest fully expanded leaf of each plant, we took 2.5 cm^2 leaf discs using a cork borer (average of 2 discs per plant). Using discs minimizes potential confounding effects that arise from the use of whole plants, which can be induced during the bioassay. Discs were mounted on a pin over moist filter paper (to facilitate larval preference for feeding on leaf undersides), and freshly hatched neonate *M. sexta* larvae were added and allowed to feed for 48-72 hours. Larvae did not run out of plant material during this period. Following removal from the discs, larvae were allowed to clear their gut contents for ca.12 hours and were then weighed; masses were normalized to the length of time spent feeding. Discs were scanned on a flat bed scanner and analysed for the amount of tissue consumption using ImageJ®.

Analyses

Field experiment data were analysed as a standard multi-factor linear model in a restricted estimate maximum likelihood (REML) framework using JMP® v9. Variables were checked for consistency with model assumptions where applicable (homoscedasticity among treatment variables and normality), and transformed as necessary when assumptions were not met. 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[population], breeding treatment, and herbivory treatment. 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, an herbivory×block term was specified as the error term for the test of the significance of herbivory (i.e., a split-plot design) (Littell *et al.* 2002). 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, while the contrast of sib-mated and outcrossed plants was used in the case of missing selfed plants. Confidence intervals (95%) were used to determine whether this difference (Inbred – Outcrossed) was significant based on whether the interval included zero (Sokal & Rohlf 1995). Metabolite data were analysed similarly to the fitness data, with maternal family, herbivory, breeding treatment and their interactions as model terms. While we analysed our trait and fitness data directly, we also derived estimates of the magnitude of the family-wise inbreeding load (Charlesworth & Charlesworth 1987), for one fitness trait (ramet production) to graphically illustrate genetic variation for inbreeding effects in the presence and absence of herbivores (Fox 2005). We focussed on asexual reproduction as it is probably the primary mode of reproduction for established populations of *S. carolinense* (Bassett & Munro 1986; Miyazaki 2008); SAC *pers*

obs). To avoid the asymmetry and instability inherent in conventional, population level ratio indices of δ (Johnston & Schoen 1994), we used the calculation of inbreeding load (Keller & Waller 2002; Escobar *et al.* 2007; Escobar *et al.* 2008). We calculated inbreeding load, or decline in log fitness as a function of the inbreeding level, *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 inbred progeny, respectively, and 0.1 corrects for zero fitness (Escobar *et al.* 2008). As in Escobar *et al.* (2007, 2008), we note that B is not equivalent to a simple logarithm of the conventional ratio estimate.

RESULTS

In the bioassay, herbivore growth and consumption (both in absolute and proportional terms) were greater on inbred plants (Fig. 2A,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 to outcrossed plants (Fig. 2C). There was considerable variation among maternal families in the magnitude of this damage, as indicated by the contrast between selfed and outcrossed progeny (Fig. 2D, Table 1); however there was significantly greater damage on inbred plants after controlling for genetic background (Fig. 2D).

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 and fitness traits when plants were exposed to herbivores (Fig. 3). 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. Horsenettle is naturally a slow-growing plant if grown from seed in the field, and only ca. 15% of plants had fruited in 2011. However, the proportion of fruiting plants was sevenfold higher in outcrossed relative to inbred plants under ambient herbivory, with only slight differences when herbivores were excluded (Fig 3). A similar pattern was found for fruit number, and though fruit production was variable, there were also significant overall reductions due to herbivory and inbreeding (Table 1). For most growth and fitness traits, ANOVA accordingly showed significant herbivory×breeding interaction terms for (Table 1), in addition to significant family effects.

The variation among families (Fig 2D, Table 1) prompted us to calculate inbreeding load for each family to examine genetic variation in the expression of inbreeding depression in the presence and absence of herbivores. Inbreeding load varied predictably among families, but was 0.93 ± 0.20 under herbivory, (mean ± 1 S.E.) and 0.07 ± 0.29 in the absence of herbivores; i.e., not different from zero (Fig. 4).

Analysis by HPLC revealed widespread changes to numerous defence-related metabolites as a function of simulated herbivory as well as inbreeding. Six phenolics (hydroxycinnamic acid derivatives) were consistently detectable in all family, breeding and herbivory treatments, and are the focus of our quantitative analysis (see Fig. 5 for representative chromatograms, and Table 2 for ANOVA results). Mechanical damage successfully induced plants: three of six phenolics were significantly upregulated under simulated herbivory (Fig 6). In two cases (Fig. 6A,B), this relationship appeared linear, with expression under 20% damage being 90% and 122% greater than controls for compounds A and B, respectively. In one case (Fig 6E),

induction was only apparent at 20% damage, and only in outbred plants. Simulated herbivory caused apparent down-regulation 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.C,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 greater expression of defence-related phenolics, either constitutively and/or after induction, for five of the six compounds we analysed (Fig 6). Outcrossed plants also produced a greater diversity of phenolics, with the number of hydroxycinnamic acid peaks being reduced under inbreeding by 35% (Fig 7).

DISCUSSION

Our study provides strong evidence that herbivory can be an agent of selection favouring outcrossing in this species. Our study is apparently the first to demonstrate significant herbivory×breeding interactions for fitness traits, and only the second for growth (Ivey *et al.* 2004), under natural environmental conditions. These interactive effects were most apparent for the likelihood of fruiting, 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. 3), 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. A recent phylogenetic analysis of the Solanaceae demonstrated that mating system transitions from outcrossing to selfing have influenced the evolution of defence strategies (Campbell & Kessler 2012); taken together, these studies suggest a coevolutionary relationship between 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 when plants were protected from herbivory (Fig. 3). In other words, field conditions other than herbivory (ambient nutrients, moisture, pH, light and competition with surrounding vegetation) did not appear to increase ID, and if anything, may ameliorate its expression relative to a greenhouse environment (Mena-Ali *et al.* 2008). While this result is inconsistent with some empirical studies (Cheptou & Donohue 2011; Fox & Reed 2011), other studies have also found non-significant ID under stress (Waller *et al.* 2008). The relationship between ID and stress may be more complex than usually thought, and specific to particular stressors. 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 4). 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 three years of this study. One implication of this ecologically-mediated ID is that purging of deleterious load for fitness related traits would be much more rapid under herbivory, suggesting a role for herbivores in the coevolution of inbreeding and ID.

While we found significant genetic variation for fitness, growth, and damage (Table 1, Fig 4), as well as population variation in the effects of herbivory on inbreeding depression (herbivory×inbreeding×population interactions), this variation did not appear to override the interactive effects of herbivory and inbreeding. This is in contrast to other studies that have found highly divergent, or even opposing effects of inbreeding among populations (Ouborg *et al.* 2000; Leimu & Fischer 2010). One reason for this difference may simply be the fact that our study species is predominantly outcrossing (though see (Travers *et al.* 2004), and may carry greater deleterious load on average than more inbreeding taxa, particularly given the opportunity for that load to be sheltered at the *S*-locus (Stone 2004). The strength of ecological ID may be greater in outcrossing taxa, 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 to inbred horsenettle individuals (Delphia *et al.* 2009a). Our analysis of foliar secondary metabolites suggests that this may be at least partly driven by substantial changes to secondary metabolite variation under inbreeding. In addition to reducing the number of compounds produced by 35%, overall investment in both constitutive and induced phenolic expression was quantitatively reduced 47% by inbreeding. Phenolics are ubiquitous defence-related secondary metabolites in many Solanaceae (Friedman 1997; Mithöfer & Boland 2011), and are significantly negatively correlated with flea beetle damage in the field in *S. carolinense* (e.g., chlorogenic acid, Campbell et al. *unpublished*), strongly implicating them as defensive metabolites. Our results 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 plant stress. Under a functional trait depression hypothesis, we would predict the effects of inbreeding on herbivory to be specific to the herbivore species identity, standing trait

variation, and the efficiency of load purging. Thus, this hypothesis may be consistent with the variable effects observed on resistance among families, populations and herbivores in prior studies (Carr & Eubanks 2002; Hull-Sanders & Eubanks 2005; Leimu *et al.* 2008). We propose that this hypothesis could be used as a framework for predicting inbreeding effects on defence within additional species. 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 insect communities on inbred and outbred plant populations. 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 equivalent *outbreeding* depression for both herbivore types (Hull-Sanders & Eubanks 2005). A non-specific stress hypothesis, under which ecological ID should covary purely with herbivore abundance in the field, could be used as an alternative hypothesis in this framework, since this hypothesis also has some support (Leimu *et al.* 2008).

Our findings of both quantitative (Fig. 6) and qualitative (Fig. 7) reductions in defence-related compounds in inbred plants also raise interesting questions on the location of genetic load in defence trait expression and the nature of the connection between homozygosity and phenotypic diversity. The expression of secondary metabolites is a complex process, but can be broken down into three stages: (1) Acquisition and processing of resources during primary metabolism; (2) allocation of precursors from primary metabolism (e.g., amino acids); (3) biosynthesis of secondary metabolites from these precursors. In this highly 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 given the sequential, hierarchical nature of metabolite biosynthesis. In

addition, pathways for the biosynthesis of defence molecules operate by feedback and signalling mechanisms that may themselves harbour genetic load, and it may be extremely 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 plant growth and defence. However, intrinsic differences in growth due to inbreeding (in the absence of herbivory) were minor in the field (Fig 3), and similarly, inbreeding has only minor effects on growth in greenhouse experiments (e.g., plant height was reduced only 11%; data not shown, and see also (Mena-Ali *et al.* 2008)), leading us to tentatively reject resource limitation. Mutations in allocation mechanisms 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 common products of the phenylpropanoid pathway, and derived from phenylalanine (Petersen *et al.* 2010), yet exhibited diverse responses to inbreeding, with some compounds apparently being lost. This suggests that the load associated with defence trait expression in this species may be predominantly localized 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 synthesis when the plant could still re-allocate precursors. Our finding that some compounds were lost (Fig. 7) while others are quantitatively reduced (Fig. 6) suggest that this load is variable, with reductions in biosynthetic efficiency for some compounds, but apparent loss-of-function mutations for others. The losses led to an apparent correlation between presumed allelic diversity (increased homozygosity under inbreeding) and chemical phenotypic diversity that may be of broader significance for other studies of ID and species interactions. Studies have suggested that phytochemical diversity may play an important role in

defence (Berenbaum *et al.* 1991). In support of this hypothesis for *S. carolinense*, a study of over 50 wild Solanaceae showed a weakly positive, but significant relationship between phenolic diversity and resistance to *Manduca sexta* larvae across species (Campbell and Kessler, *unpublished*). 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 the signalling and regulatory machinery involved in regulating 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 inducibility in inbred plants (Fig 6B, F). Moreover, inbred plants exhibited apparent down-regulation of two compounds in response to herbivory (Fig 6D,F). Together, these results suggest deleterious load at loci involved in the regulation of the defence response both in terms of ramping up defence-related metabolites, and maintaining production of others. These findings lead us to hypothesise that the production of plant hormones involved in the regulation of these traits (e.g., jasmonic acid, salicylic acid, gibberellic acid, among others) are likely to have been deleteriously affected by inbreeding.

In conclusion, our study shows strong, herbivore-mediated, ecological inbreeding depression in nature. The strength of ID is considered the 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. This conclusion is likely to be robust, since our study was based on replicate genetic families from multiple populations. 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, but also provide a new perspective on the evolutionary ecology of plant-animal interactions.

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FIGURE LEGENDS

Fig 1. Horsenettle, *Solanum carolinense* (Solanaceae) with evidence of flea beetle damage (shot-holes). Scale bar is approximate. Inset: *Epitrix fuscula* (Coleoptera: Chrysomelidae: Galerucinae) flea beetle, a dominant specialist herbivore of horsenettle in northeastern U.S.

Fig 2. Laboratory (A,B) and field (C,D) 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) performance growth of larval herbivores and (B) absolute and proportional tissue consumption. (C) Absolute damage on field grown plants, as estimated by the mean 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 the same maternal family, ± 95% confidence interval. Note that confidence intervals do not overlap/include zero, indicating significantly greater performance, consumption and damage on inbred relative to outcrossed plants.

Fig. 3. Growth (aboveground biomass), and absolute 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. Asterisks denote a significant interaction between herbivory and breeding treatments (Table 1).

Fig 4. 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 (red 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 δ=0.5) at which an outcrossing mating strategy is favoured over inbreeding. Families with very low values of *B* appear as almost absent.

Figure 5. 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.

Fig. 6. 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 5.

Fig. 7. Average diversity (±1 SE) of defence-related phenolics in undamaged inbred and outcrossed *Solanum carolinense (P* = 0.01).

Figure 1

Figure 2

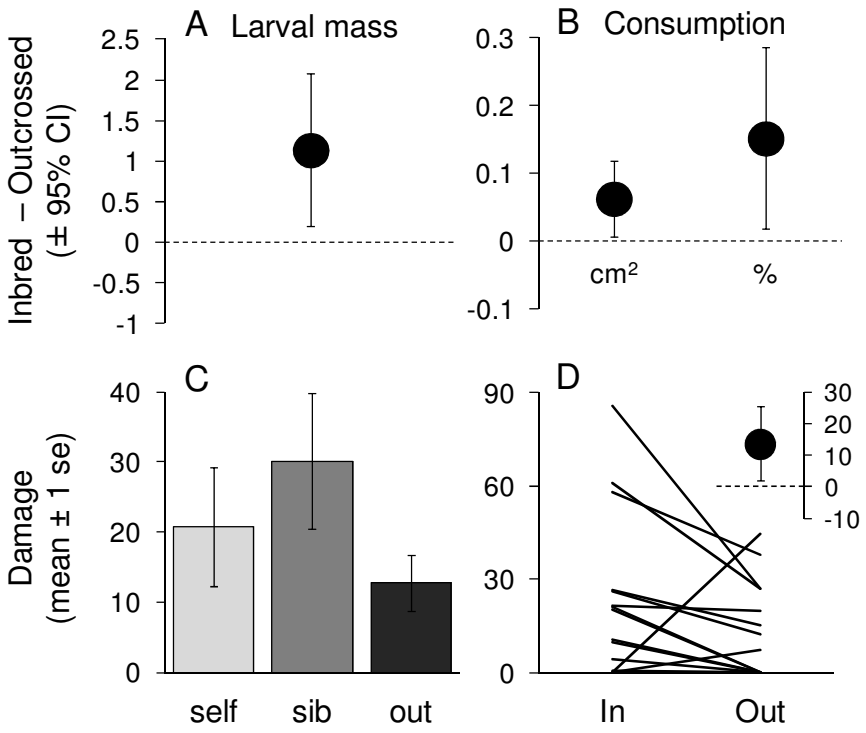
A Larval mass
B Consumption
Inbred – Outcrossed (± 95% CI)
2.5
2
1.5
1
0.5
0
-0.5
-1
0.3
0.2
0.1
0
-0.1
cm²
%
C
D
Damage (mean ± 1 se)
40
30
20
10
0
self
sib
out
90
60
30
0
In
Out
30
20
10
0
-10

Figure 3

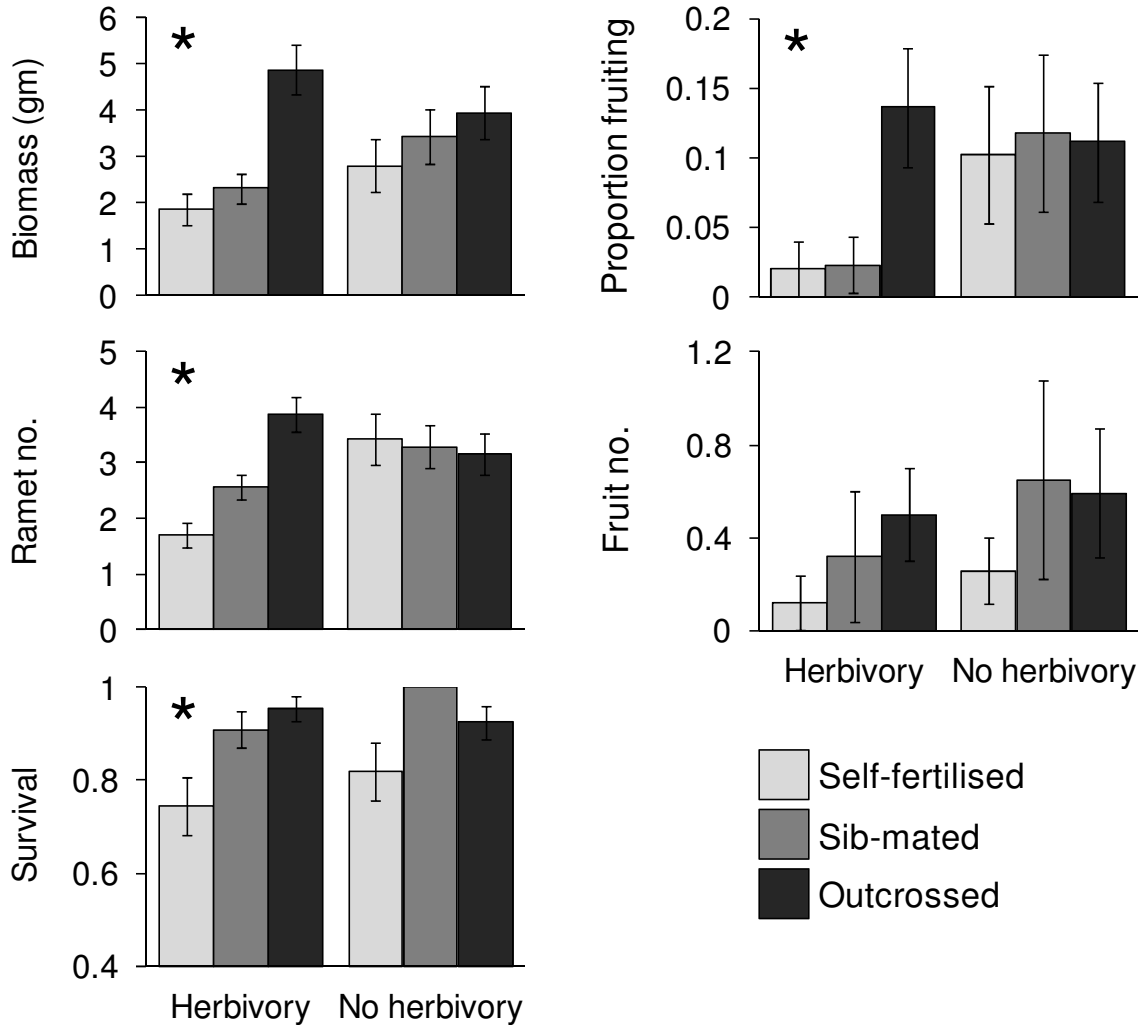
Biomass (gm)
Ramet no.
Survival
Proportion fruiting
Fruit no.
*
Herbivory
No herbivory
Self-fertilised
Sib-mated
Outcrossed

Figure 4

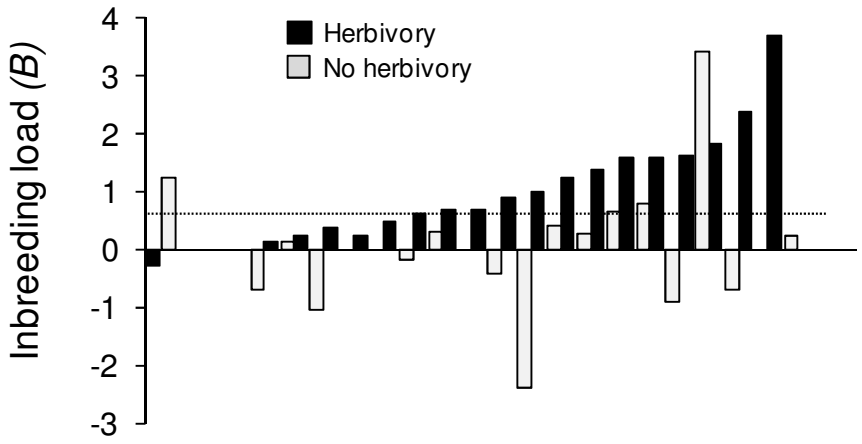

Figure 5

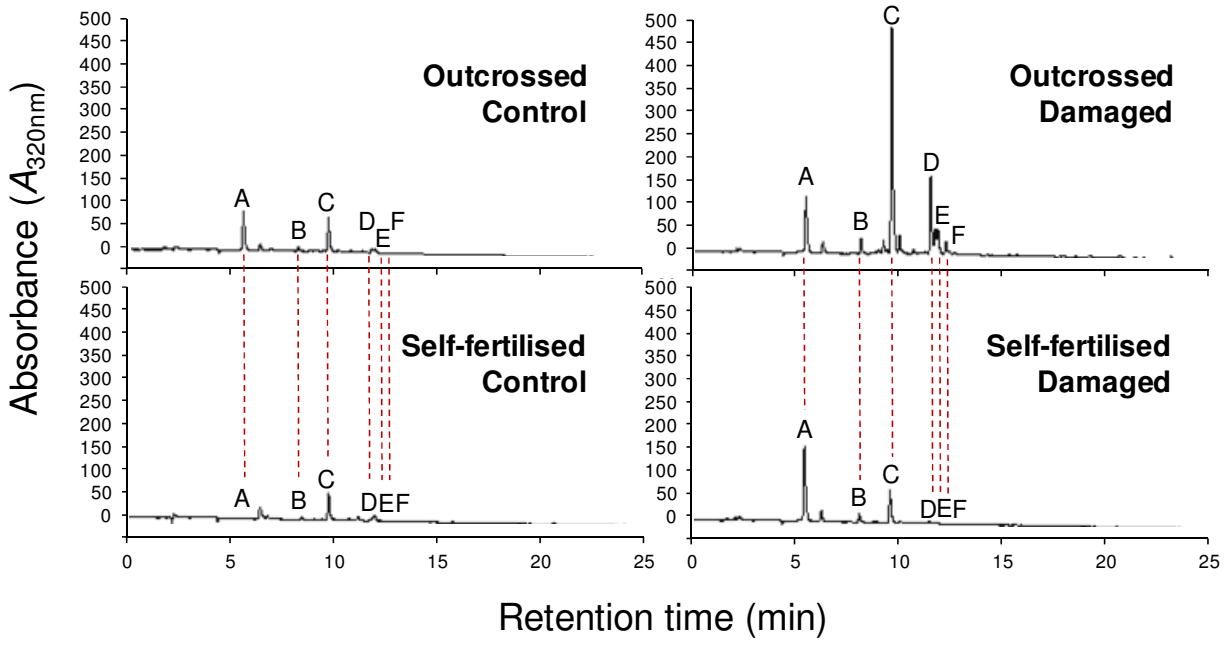
Absorbance (A_{320nm})
Outcrossed Control
Outcrossed Damaged
Self-fertilised Control
Self-fertilised Damaged
A
B
C
D
E
F
DEF
Retention time (min)

Figure 6

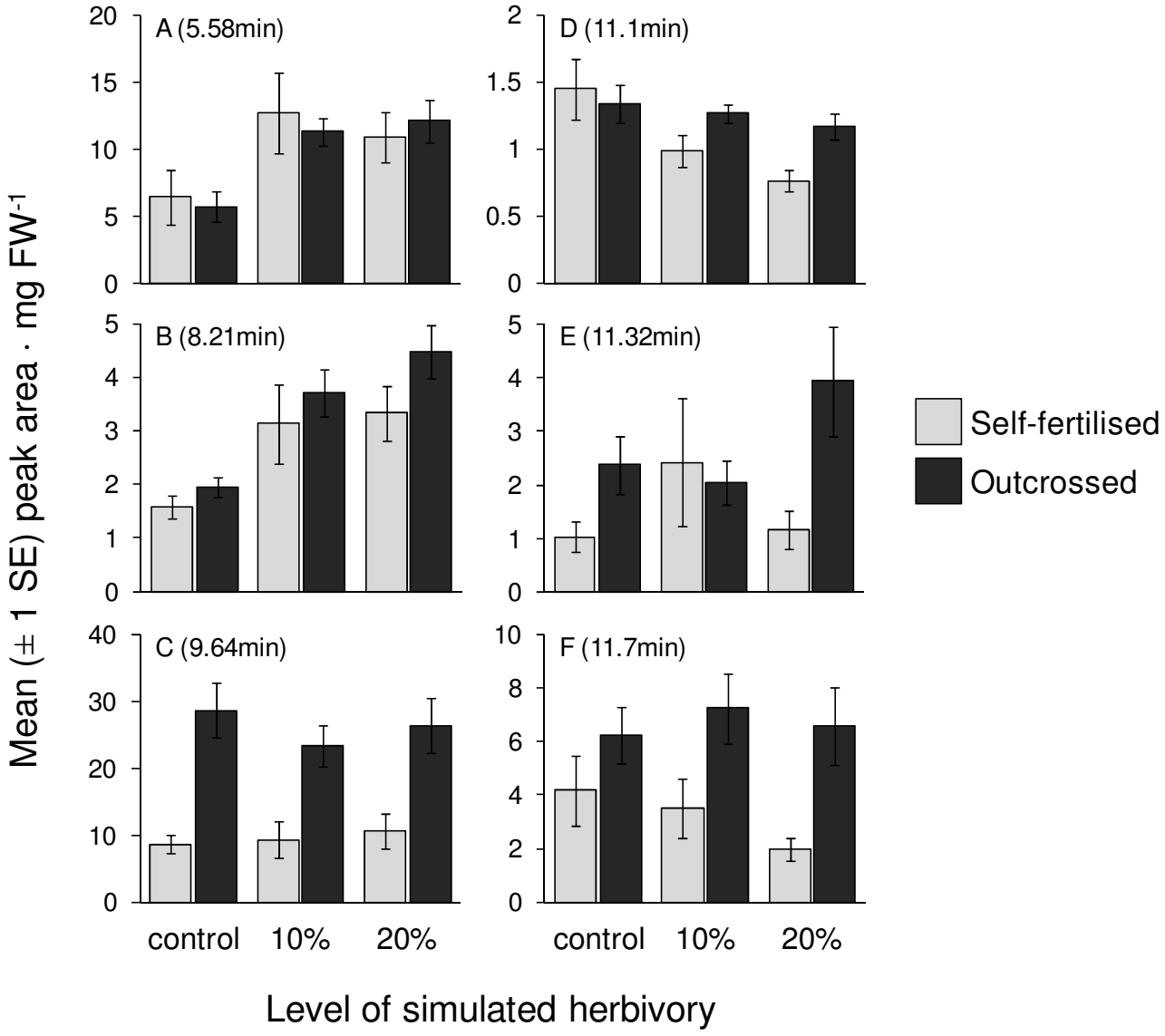
A (5.58min)
D (11.1min)
B (8.21min)
E (11.32min)
C (9.64min)
F (11.7min)
Mean (± 1 SE) peak area · mg FW-1
control
10%
20%
Level of simulated herbivory
Self-fertilised
Outcrossed

Figure 7

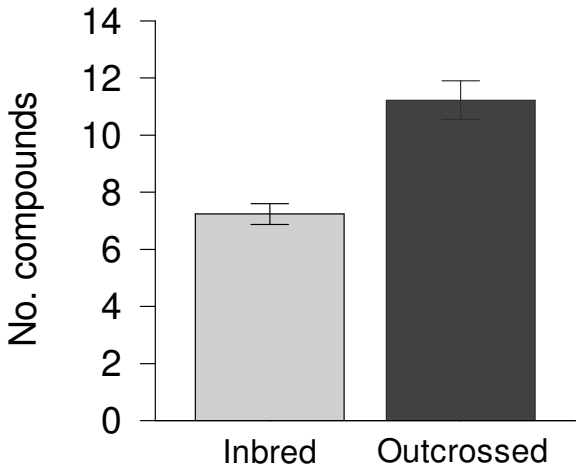

Table 1. Linear model results for growth and fitness traits from the field. Significant ($P \leq 0.05$) and marginally significant ($P < 0.1$) *P*-values denoted in bold and italics, respectively.

Trait		Source of variation							
		Breeding	Herbivory	Breeding × Herbivory	Family	Pop	Breeding × Pop	Herbivory × Pop	Breeding × Herbivory × Pop
Ramet no.	F	34.24	17.72	5.85	3.87	112.15	15.65	12.31	4.96
	P	**<0.0001**	**<0.0001**	**0.0033**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0007**
Pr fruiting	χ^2	4.99	3.52	5.72	15.51	4.87	7.01	5.05	9.81
	P	*0.0824*	*0.0606*	**0.0573**	0.9472	*0.0875*	0.1354	*0.0799*	**0.0438**
Biomass	F	2.14	2.24	3.47	1.81	7.31	1.22	6.38	1.95
	P	0.1198	0.1359	**0.0326**	**0.0113**	**0.0008**	0.3015	**0.0020**	0.1024
Survival	χ^2	8.02	2.75	5.98	21.36	4.91	7.03	5.76	9.55
	P	**0.0181**	*0.0971*	**0.0504**	0.7234	*0.0858*	0.1344	**0.0560**	**0.0488**
Fruit no.	χ^2	13.95	4.09	9.42	151.70	4.84	34.63	5.47	26.70
	P	**0.0009**	**0.0432**	**0.0090**	**<0.0001**	*0.0891*	**<0.0001**	*0.0649*	**<0.0001**

Table 2. Linear model results for secondary metabolite traits from the greenhouse. Significant ($P \leq 0.05$) and marginally significant ($P < 0.1$) *P*-values denoted in bold and italics, respectively. Letters correspond to the compounds illustrated in Figure 5.

Phenolic		Source of variation			
		Breeding	Damage	Breeding × Damage	Family
A	F	0.634	19.044	1.461	7.199
	P	0.4270	**<0.0001**	0.2286	**<0.0001**
B	F	3.9894	35.160	0.182	7.283
	P	**0.0476**	**<0.0001**	0.6706	**<0.0001**
C	F	42.899	0.2143	0.0014	33.840
	P	**<0.0001**	0.6441	0.9706	**<0.0001**
D	F	5.3372	10.048	5.894	7.649
	P	**0.0222**	**0.0018**	**0.0164**	**<0.0001**
E	F	17.677	3.697	1.1528	5.278
	P	**<0.0001**	**0.0564**	0.2847	**0.0005**
F	F	14.9304	2.857	1.741	19.489
	P	**0.0002**	*0.0930*	0.1891	**<0.0001**

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3. Plant mating systems alter adaptive plasticity in response to herbivory

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ABSTRACT

Biologists have been studying the fitness consequences of mating system variation (e.g., inbreeding) for over 200 years, and yet the mechanisms and broader ecological consequences of this variation remain poorly understood. In particular, virtually nothing is known about the effects of inbreeding on behavioural and physiological responses to different environments (phenotypic plasticity). Most plant species are capable of inbreeding, and also exhibit a remarkable suite of adaptive phenotypic responses to environmental stresses such as herbivory, including upregulation of resistance and growth (tolerance) traits. We tested the consequences of experimental inbreeding for phenotypic plasticity in resistance and growth traits. Inbreeding reduced the ability of plants to upregulate resistance traits following damage. Inbreeding also disrupted growth trait responses to damage, indicating deleterious load at loci regulating growth under stress. Inbreeding reduced expression of the growth regulators absissic and indole acetic acid, and reduced upregulation of the defence signaling phytohormone jasmonic acid in response to wounding, indicating a phytohormonal basis to inbreeding effects on growth and defence trait regulation. We conclude that adaptive plasticity in plants is deleteriously affected by inbreeding, and suggest that this may be of concern in fragmented populations facing mate limitation under global environmental change.

KEYWORDS

Inbreeding depression, mating systems, phenotypic plasticity, inducible defence, tolerance, plant-insect interactions, phytohormones, Solanum carolinense, Solanaceae, jasmonic acid, abscisic acid, auxin/indole acetic acid

1. INTRODUCTION

Biologists have been studying the fitness consequences of inbreeding and outcrossing for at least 200 years (1-3). These consequences (e.g., inbreeding depression, heterosis) are predicted to influence the evolution of mating system variation (4, 5), which in turn can have widespread consequences for population genetics and dynamics (6-8). However, we still know relatively little about the effects of mating system variation on the interactions of species with their environment.

The broader effects of inbreeding and outcrossing are important from several perspectives. First, mating systems may influence fundamental aspects of a species' ecology, such as tolerance of abiotic stress (9, 10), and interactions with competitors (3, 11) or natural enemies (12). These effects presumably arise from deleterious genetic load at functional traits, though evidence for such mechanisms is sparse (but see (13). These interactions could in turn lead to coevolution of mating systems with functional traits (14). Second, environmental variation that mediates the magnitude of inbreeding depression (13) should influence mating system evolution (2, 15, 16). Finally, the effects of inbreeding on functional traits could alter species responses to anthropogenic environmental change, and such effects may be particularly severe in fragmented habitats with limited outcrossing opportunities (17, 18).

While evidence is accumulating that mating system variation can affect ecological interactions (and vice versa) almost nothing is known about the effects of mating systems on adaptive responses to different environments, i.e. phenotypic plasticity. From an ontogenetic perspective, inbreeding has been predicted to negatively affect developmental stability (19), and thereby contribute to greater phenotypic variation under stress (e.g., fluctuating asymmetry) (20). That is, inbreeding could amplify non-adaptive plasticity (21, 22). However, many species

exhibit adaptive phenotypic plasticity, that allows individuals and populations to tolerate environmental variation (23). Adaptive phenotypic plasticity requires the coordinated expression of genes involved in the perception of environmental cues and signal transduction, together with genes for biosynthesis, any of which could harbour deleterious genetic load. However, only a few studies have explicitly examined the effect of inbreeding on adaptive phenotypic plasticity. Population studies of the correlations among heterozygosity, population size and plasticity have not isolated the effect of inbreeding per se (24, 25). One manipulative breeding study has found evidence for reduced plasticity for an anti-predator trait under inbreeding (26), but several other studies have found little to no effect on plasticity (27-30). Thus, the relationships between mating systems, phenotypic plasticity and ecological interactions remain unclear.

The effects of inbreeding on phenotypic plasticity may be particularly important for plants, which are predominantly hermaphroditic and must cope with local fluctuations in both abiotic but also biotic stresses such as herbivory and disease. Accordingly, plants exhibit adaptive plasticity in a wide range of phenotypes in response to stress (31). For example, many plants upregulate defensive and immune responses only after initial damage or infection, via complex hormone signaling pathways (32, 33). A diverse suite of secondary metabolites are implicated as defence-related traits in many plants, and are induced following herbivory (34, 35). Both theoretical arguments and empirical data indicate that an inducible defence strategy primarily operates to limit the costs of trait expression in the absence of antagonists (36-38). Plant phenotypic responses to a wide range of abiotic stresses (e.g., drought, cold, salt) are similarly regulated through coordination of multiple hormone signaling pathways (39, 40). Plants can also alter the expression of growth or developmental traits in order to compensate for the effects of damage (i.e., tolerance) (41). A common set of plant hormones, including jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), auxin/indole acetic acids (IAA), gibberellic acid (GA) and ethylene, interacts to influence the expression of both chemical resistance traits, and tolerance-

related growth traits (32, 42). However, the molecular mechanisms and hormonal interactions that mediate plant tolerance to herbivory remain virtually unknown. Similarly, the physiological mechanisms for the effects of inbreeding on growth (e.g., phytohormones) are also poorly understood.

In this study, we used experimentally inbred (self-fertilised) and outcrossed plants of *Solanum carolinense* to test the effects of mating system variation on plant phenotypic plasticity. Previously, we showed that inbreeding reduced resistance to herbivores in the field and lab, and also deleteriously affected both the constitutive diversity and quantities of defence-related phenolic metabolites (13). Based on the observation that regulation of different phenolics differed between mating systems, we hypothesised that inbreeding deleteriously affects plant phenotypic plasticity in response to herbivory. Here, we test two fundamental predictions of this hypothesis, specifically that inbreeding will: (1) disrupt phenotypic plasticity (inducibility) of defence and growth traits; and (2) disrupt endogenous hormonal signalling involved in mediating phenotypic responses.

2. METHODS

(a) Study system and plant material

Horsenettle, *Solanum carolinense* L. (Solanaceae), is a short-lived perennial herb native to eastern North America (see (13) for a relevant description of the study system). *Solanum carolinense* possesses the gametophytic self-incompatibility system characteristic of over one third of the species in the nightshade family (Solanaceae), but exhibits plasticity in this system, with the potential for some inbreeding (43). This predominantly outcrossing mating system makes *S. carolinense* an ideal species for studies of the effects of inbreeding on ecological

interactions. We collected seed from three source populations near Ithaca, New York state, U.S.A., and grew maternal plants from these collections in a greenhouse. We experimentally self-fertilised and outcrossed 60 maternal plants to create full-sib families, and randomly sampled from these families for subsequent experiments. See Campbell et al. (13) for full details on the breeding protocol that produced these families. For the experiments described here, subsets of families were selected at random from the larger pool, and clonal replicates of 2-5 selfed and outcrossed offspring per family were created by subdividing main roots into 1.5g segments, and placing segments in a 1:1:1 mixture of vermiculite:perlite:potting soil (Metro-Mix 360 all-purpose potting soil, Scotts-Sierra Horticultural Products, Marysville, OH) in 27-well flats to sprout. Plants were grown in the greenhouse for two-weeks, transplanted to 355mL (four-inch) pots filled with soil, and grown for an additional three weeks under a 16hr light: 8hr dark schedule, *ad libitum* watering, and weekly fertilization (21-5-20 NPK, 150ppm), with trays randomized to minimise greenhouse position effects. Inbred and outcrossed plants grown in this way were then used in two experiments.

(b) Plant trait responses to herbivory and inbreeding

We conducted a greenhouse experiment to measure the effects of inbreeding on responses to damage, focussing on plasticity in defence and growth related traits. Plant induced responses can be highly sensitive to the rate of feeding, and because we had previously shown differential consumption rates on inbred and outcrossed *S. carolinense* (13), we chose to use controlled, simulated herbivory to test plant growth and metabolite responses. Inbred and outcrossed plants (n=20 families) were randomly assigned to receive 0%, 10%, or 20% manual tissue removal (n=520 plants). Damage levels (based on visual estimation) were imposed on every leaf using a standard paper hole-punch in order to approximate a stereotypical feeding pattern (see (13) for a full description of the experimental design). As reported in Campbell et al. (13), we quantified amounts of phenolic compounds, which correlate negatively with damage in field-

grown *S. carolinense* (Campbell et al. unpublished data), implicating them as defence-related secondary metabolites in this system. We used the phenolic data in (13) to explicitly compare inducibility in defence metabolites between mating systems. In brief, phenolic expression was quantified by excising a single leaf from each plant with a razor blade, and taking a100mg sample of fresh tissue (excluding midvein), which was weighed, flash-frozen in liquid N_2, and stored at -80°C. Samples were then extracted in an ice-cold 40% methanol, 0.5% acetic acid solvent, and analysed for secondary metabolites by high-performance liquid chromatography (HPLC) using a standard method targeted at phenolic compounds (13, 44). Plasticity in phenolic expression was calculated as the average of the proportional change in expression in damaged (20%) relative to control plants, across five of the six quantifiable phenolics. One phenolic (compound 'C'; a hydroxycinnamic acid derivative with retention time 9.58min), was removed because it showed no inducibility in either outcrossed or inbred plants. We compared plasticity in outcrossed vs. inbred plants for each family (to control for genetic variation among families, (13), in JMP® v9.0 (45) using an *F*-test; i.e., a matched-pairs design (46). At the time of tissue sampling (two weeks after damage), we also recorded plant height and leaf number. Plants were then moved outside to a rooftop, where they were watered daily and fertilized weekly (as above) until the first frost (two months). This allowed us to examine plant growth under semi-natural conditions following damage. No pests were observed on the plants. Plants were harvested, dried and weighed. Boxplots and distributions of growth traits were examined for outliers, normality and heteroscedasticity. Data were log-transformed (47), and analysed using JMP® v9.0 with linear mixed models in a restricted maximum likelihood (REML) framework. Genetic family, breeding status, treatment and breeding×treatment were specified as model terms. Family was set as a random effect, and treatment was modelled as a continuous variable. We also conducted pairwise multiple comparisons (Tukey's tests) among all breeding and damage treatment combinations, with experimentwise $\alpha = 0.05$.

(c) Phytohormone analysis

We conducted a controlled artificial wounding experiment to compare inbred and outcrossed plants for their constitutive and induced expression of four key plant hormones that regulate plant growth, defensive metabolite expression, and responses to environmental stresses such as herbivory. This wounding technique specifically allows us to conservatively examine endogenous hormone production independent of mating system differences in the effects of physical damage (tissue removal), herbivore physiology (e.g., saliva) and behavior (e.g., feeding rate). Leaves on inbred and outcrossed plants (n=8 maternal families, 2-5 replicates per family) were wounded by applying rows of punctures parallel to the midrib at 5 mm spacing using a fabric pattern wheel, thereby providing a proportionally standardized wound to all plants. Damaged leaves were sampled exactly 60 min after wounding based on a conservative estimate of the plateau in hormone expression (48). Jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and auxin/indole-3-acetic acid (IAA) were extracted from the damaged leaves using the protocol of Pan et al. (49) with the modifications described in Thaler et al. (50). In brief, 1 mL of an iso-propanol:H_2O:$HCl_{conc.}$ (2:1:0.005) extraction buffer was added to ca. 300mg of frozen tissue, and 0.8 ng each of d_4-SA, d_5-JA, d_6-ABA and d_5-IAA (C/D/N Isotopes Inc., Pointe-Claire, QC, Canada) were added as internal standards. Samples were homogenized on a FastPrep® homogenizer (MP Biomedicals®, Solon, Ohio, U.S.A.) at 6 m/s for 45 s using 0.9g grinding beads (Biospec®, Zirconia/Silica 2.3mm), re-extracted with dichloromethane, dried and dissolved in 200µL methanol. A 10µL aliquot of each sample was analyzed on a triple-quadrupole LC-MS/MS (Thermo Scientific®, Waltham, Massachusetts, U.S.A.) equipped with a C_{18} reverse-phase HPLC column (Gemini-NX, 3 µm, 150 × 2.00 mm; Phenomenex, Torrance, California, U.S.A.), using the method described in Thaler et al. (2010). Auxin/IAA was analysed by positive electrospray ionisation, and JA, SA and ABA were analyzed by negative electrospray ionization (spray voltage: 3.5 kV; sheath gas: 15; auxiliary gas: 15; capillary temperature: 350°C), collision-induced dissociation (argon CID gas pressure 1.3 mTorr

[1.3 micron Hg], CID energies 16V [JA,SA], 13V [ABA] and 18V [IAA]) and selected reaction monitoring (SRM) of compound-specific [parent→product ion] transitions: SA [137→93]; d_4-SA [141→97]; JA [209→59]; d_5-JA [214→62]; ABA [263→153]; d_6-ABA [269→159]; IAA [176→129]; d_5-IAA [181→134]. Analyte quantities were normalised to the mass of fresh sample tissue, log-transformed to improve residual normality and heteroscedasticity, and analysed identically to the growth traits. In addition, we conducted a test of whether inbreeding altered coordinated hormone expression in both wounded and control plants. Plant hormones are known to interact with one another in their effects on gene expression (42), and since the treatments could alter both the magnitude of the traits, but also the relationships among them, we compared the phenotypic variance-covariance matrices of the four combinations of breeding and wounding treatment. Covariance matrices were estimated using REML in JMP® v9.0 (45), and compared using the random skewers method of Cheverud (51). The random skewers method compares the overall structure of matrices by calculating the average vector correlation of the products of random vectors and the two covariance matrices being compared. It is therefore an holistic method of matrix comparison (52). The method produces vector correlation coefficients that correspond to a test of whether the structures of two covariance matrices are correlated with one another. We used the *skewers* software, courtesy of L. Revell (http://faculty.umb.edu/liam.revell/programs/index.html), with 10^6 skewers. We conducted the analysis with and without JA, since this hormone is often detectable only after wounding, and its inclusion was predicted to bias the analysis in favour of finding correlations based on wounding alone, rather than breeding. For each treatment/breeding combination, we also estimated product-moment correlations for all pairs of hormones.

3. RESULTS

Outcrossed plants were significantly more inducible than inbred plants in the expression of defence-related phenolics. We had previously shown that six different phenolics varied in the effects of inbreeding on expression (13). Analysis of the plasticity of the five compounds that showed significant induction reveals that plasticity in total phenolic expression was significantly greater in outcrossed plants ($F_{1,13} = 34.76$; $P = 0.0041$) (figure 1).

Growth trait responses to artificial herbivory differed between inbred and outcrossed plants (figure 2, table 1a). Two weeks after damage, leaf number did not differ due to either inbreeding or damage level (table 1). However by the end of the growing season, average investment in leaf biomass declined in inbred plants as a function of increased herbivory, but increased in outcrossed plants (figure 2a), leading to a significant breeding×damage treatment interaction (table 1). Conversely, damage induced an increase in stem biomass in inbred, but not outcrossed plants: compared to outcrossed plants, stem biomass of inbreds was significantly lower in the control treatment, but increased to the level of outcrossed plants as a result of damage (figure 2b). A similar pattern was also found for plant height (figure 2c), measured only two weeks after damage, although there were no statistically significant differences between inbred and outcrossed plants in any treatment.

Three of the four phytohormones showed significantly reduced expression as a result of inbreeding, and divergent responses to the wounding treatment under each breeding condition (figure 3, table 1b). Salicylic acid expression was not affected by inbreeding or wounding. Jasmonic acid levels were almost undetectable in control plants, and were differentially upregulated 116-fold and 51-fold in outcrossed and inbred plants, respectively. Thus, there was a significant breeding×wounding interaction, but also significant main effects of inbreeding and

wounding (table 1b). Abscisic acid was reduced 55% in inbred, relative to outcrossed plants, and was upregulated 32% in response to wounding, with similar inducibility in outcrossed and inbred plants (figure 3, table2b). Indole acetic acid was reduced significantly by 27% due to inbreeding, but was not affected by wounding. As a result of these effects, the relative ratios among hormones showed striking differences among breeding and wounding treatments (figure 3). The significance of correlations between specific pairs of phytohormones also differed strikingly as a result of inbreeding in both control and wounded plants (table 2). As predicted, the overall structure of the hormone variance-covariance matrix was similar in both breeding treatments, but differed between control and wounded plants, when JA was included (table 3a). When JA was excluded, there was a significant correlation between the control and wounded outcrossed matrices, but not control and wounded inbred matrices; inbred and outcrossed hormone matrices were not significantly correlated in either treatment (table 3b).

4. DISCUSSION

Our results strongly support the hypothesis that inbreeding reduces adaptive plasticity in plant defence and growth traits. Despite genetic and compound-specific variation (13), inbreeding reduced the ability of *Solanum carolinense* to induce its suite of defence-related phenolics, which may contribute to significant increases in damage experienced by inbred *S. carolinense* in the field, and significant herbivore-mediated inbreeding depression for fitness traits (13). As an independent, additional test of inbreeding depression for plasticity, we conducted a bioassay of inducibility in resistance to *Manduca sexta* (Supplementary Materials). Consistent with the phenolic analysis, inbred *S. carolinense* were significantly less inducible compared to outcrossed plants ($p = 0.015$, figure S1), providing independent corroboration of our hypothesis. Our results add to a growing appreciation that plant mating system variation can have significant

effects on antagonistic species interactions such as parasitism (53), disease (54, 55), and herbivory (13, 56-58). In an earlier study (13) we demonstrated that inbreeding has deleterious effects on the production of defence-related secondary metabolites, resulting in reduced diversity and quantities of metabolites. The results of the current study indicate that fundamental regulatory mechanisms of plant defence also shelter deleterious load. Given the effect of inbreeding depression on inducibility, we hypothesise that mating systems could alter natural selection for an inducible vs. constitutive strategy of plant defence. Specifically, we hypothesize that selection for constitutive defences should be greater in populations and species which harbour significant inbreeding depression for plasticity, as in *S. carolinense*. To the extent that constitutive and inducible defence are relative alternative strategies within a population, this should be true regardless of the magnitude of inbreeding depression for overall defence trait expression. Inbreeding depression is significantly higher in predominantly outcrossing taxa (2, 59), and thus our hypothesis would predict the coevolution of an outcrossing mating strategy with higher constitutive resistance, a pattern which was found in a phylogenetically-controlled comparison of defence strategies in outcrossing and inbreeding wild Solanaceae (14).

Inbreeding also altered plasticity in plant growth traits in response to damage, suggesting that mechanisms of tolerance to herbivory (60) are also deleteriously affected by inbreeding. Only one other study has examined the effect of inbreeding on putative tolerance traits, and showed that plant growth was reduced to a greater extent in inbred plants (61). In the present study, inbred and outcrossed plants exhibited dramatically different growth responses to standardized damage. Damage induced a significant increase in leaf biomass in outcrossed plants (figure 2), a response that has been considered an adaptive mechanism for compensating for lost source (photosynthetic) tissue (62). In contrast, herbivory induced a significant decrease in leaf investment in inbred plants, suggesting that inbred plants suffered greater resource

(photosynthetic) limitation and reduced ability to compensate for herbivory. Outcrossed plants were also able to maintain constant stem growth in response to damage. Interestingly, inbreds appeared to increase investment in stem growth, measured as both biomass and plant height, in response to damage (figure 2). Stem biomass was significantly lower in inbred compared to outcrossed control plants, but increased 31% in inbred plants following damage. A similar pattern was found for height (measured only two weeks after damage), with significant main effects of breeding and damage that appear to have been driven by the response of inbred plants (figure 2c): when the effect of damage on plant height was analysed separately by breeding status, the relationship was significant for inbred ($p = 0.038$) but not outcrossed plants ($p = 0.3725$). In contrast to leaf investment, a putatively adaptive (or maladaptive) consequence of increased plasticity in inbred stem growth is less clear. Reduced resource acquisition due to inbreeding might have been predicted to cause a trade-off in investment in leaf vs. stem growth in inbred plants. However, at the individual plant level there was no evidence for such a trade-off, with significant, positive correlations among growth traits (all $p \leq 0.01$) in all combinations of breeding and damage treatment. Thus, we conclude that inbreeding effects on these growth traits were due to independent disruption of growth regulation (see below). Finally, we note that a few studies have examined the combined effects of herbivory and inbreeding on fitness ((13) and citations therein), and have occasionally concluded effects of inbreeding on tolerance in the field, where there may be simultaneous inbreeding depression for resistance traits and differential damage between inbred and outcrossed plants (63). While tolerance has conventionally been defined as the absence of a fitness cost of herbivory (41), we suggest that progress in this area may benefit from considering the effects of inbreeding on specific herbivore-induced re-growth traits (or other so-called ‘mechanisms’ of tolerance) that, together with specific resistance traits, can underlie variation in fitness.

Our results strongly implicate differential regulation of several key plant hormones as

mechanisms for altered plasticity under inbreeding. All four measured phytohormones play important roles in regulating the expression of plant growth and defence traits (64-67), and the significant reductions in foliar concentrations of three of these provides compelling evidence for deleterious effects of inbreeding on the regulation of gene expression. Jasmonic acid (JA) is critical in regulating induced resistance and upregulation of defensive metabolites, particularly the phenolics produced by the phenylpropanoid pathway (32). Thus, the 60% reduction in JA upregulation under inbreeding is consistent with the significant reduction in inducible resistance and plasticity in phenolic production. This finding is also consistent with inbreeding depression for herbivory (damage) and fitness in 29 field-grown families of *S. carolinense* (13); it is also consistent with a study with three inbred clones suggesting reduced upregulation of volatiles under inbreeding (68), although neither study tested for differences in plasticity per se. Overall, we predict that a wide variety of JA-mediated phenotypes and interactions will be affected by inbreeding. Consistent with other studies (69, 70), salicylic acid (SA) was not upregulated under this wounding treatment, but was also unaffected by inbreeding. This result suggests that interactions mediated by this hormone (71) may be less affected by inbreeding, although it is possible that pathogen cues (rather than wounding) would have revealed inbreeding effects on SA upregulation. SA-mediated pathogen resistance might also be positively affected by reduced JA-SA and IAA-SA cross-talk (42) in inbred plants with reduced JA and IAA expression.

Abscisic acid (ABA) and indole acetic acid (IAA) were reduced 55% and 27%, respectively, under inbreeding. While ABA is primarily considered a growth and abiotic stress response hormone (64, 72), ABA-deficient mutant tomato plants show reduced resistance to caterpillar herbivores (73), and ABA modulates JA expression (74, 75), implicating this hormone in inbreeding depression for plant resistance. ABA-deficient mutants show variable resistance to different pathogens (76), again suggesting that the effects of inbreeding on disease may be weaker or more variable (55). ABA has also been shown to inhibit stem elongation (77),

providing a possible mechanism for the stem growth observed in inbred plants, particularly with the correlated reduction in IAA (66). Both ABA and IAA have diverse and fundamental roles in leaf cell division and growth (67), and are implicated in defence trait expression (78-80), suggesting their reduced expression in inbred plants may be linked to reduced leaf investment following damage. While our results indicate severe inbreeding depression for phytohormone production, they do not indicate whether deleterious load is localized at particular stages of damage perception and signal transduction (e.g., systemin production), within phytohormone biosynthetic pathways (e.g., the octadecanoid pathway), or both. Moreover, our hormone experiment did not examine features of real herbivory, such as feeding rate (81) or salivary/chemical elicitors (82). If additional deleterious load exists at loci governing plant responses to these factors, our experiment may be a conservative indication of the magnitude of inbreeding depression for endogenous signalling. These remain open questions for future studies of the effects of mating system variation on plant physiological responses to stress.

Plant hormone signalling pathways are assumed to act in a coordinated fashion (42, 83), and the effect of inbreeding on hormone cross-talk and the interaction of induction pathways could be as important as the effect on specific phytohormone quantities. Accordingly, ratios among hormones clearly change as a function of inbreeding (figure 3). We also tested the hypothesis that inbreeding disrupted the correlated expression of hormones, by comparing the overall pattern of trait covariances among phytohormones. A disruption of hormone cross-talk would have significant consequences since these correlations represent functional constraints on the ability of the plant to regulate downstream gene expression. In support of this hypothesis, the strength and significance of specific correlations differed widely as a result of inbreeding (table 2). Consistent with this finding, the overall pattern of relationships changed under inbreeding, as indicated by a comparison of covariance matrix structure (51). As predicted, when JA was included in the analysis, its strong induction appeared to drive correlations between inbred and

outcrossed control matrices, and inbred and outcrossed wounded matrices. When removed, there was a significant correlation between control and wounded trait matrices in outcrossed, but not inbred plants (table 3b), indicating that the correlated pattern of expression of ABA, IAA and SA was conserved under wounding in outcrossed, but not inbred plants. In addition, outcrossed and inbred matrices were not significantly correlated in either the control or wounded treatment confirming that inbreeding fundamentally altered the coordinated hormonal response to wounding.

These results have important implications for the study of inbreeding depression in plants. In particular, our study provides a mechanistic hypothesis for the common observation that inbreeding reduces plant growth and vigour (2). Very few studies have examined the mechanisms of inbreeding depression for growth and fitness at the level of plant physiology and metabolism (84). Research on maize cultivars has suggested a role for gibberellic acid in hybrid vigour (85); however, our study is apparently the first to show severe inbreeding depression for key growth hormones such as ABA and auxin/IAA. Inbreeding depression appears to be greater under environmental stress in many species (15, 86), including *S. carolinense* (13), and our data indicate that a mechanism for this ecologically-mediated inbreeding depression is deleterious load in hormonal stress responses. Inbreeding depression in hormone expression and hormone cross-talk could mediate responses to wide range of abiotic stressors (e.g., drought) and a wide range of interactions in addition to herbivory (e.g., pollination).

In conclusion, our study represents the first explicit demonstration that inbreeding significantly alters plasticity in functional plant phenotypes. Other studies have shown the potential for inbreeding effects on responses to stresses such as herbivory (13, 61, 68), but have not tested for plasticity and in some cases had limited genetic replication (68). Phenotypic plasticity can be critical to plant fitness, particularly in response to herbivore attack and other environmental

stresses. Plasticity is also considered critical for colonisation and population persistence at range edges (87), and for plant responses to anthropogenic environmental change (88). Our study indicates that mating systems may play a role in these interactions. For example, habitat fragmentation and population isolation are more pronounced at range edges, which already represent the limits of environmental tolerances where plasticity should be most adaptive (89, 90). Inbreeding in these habitats, coupled with inbreeding depression for plasticity, could thus contribute to limits on range expansion (91). Finally, increasing anthropogenic habitat fragmentation is expected to increase mate limitation and inbreeding depression, particularly for outcrossing taxa (17, 92). These detrimental effects on population growth could be exacerbated by reduced phenotypic tolerance of environmental variation, and make isolated populations more susceptible to the detrimental effects of climate change. Thus, population genetic and dynamics studies, as well as demographic studies of vulnerable species, may benefit from understanding the interactions among population size/isolation, phenotypic responses to environmental factors, and mating systems.

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FIGURE LEGENDS

Figure 1. Plasticity in defence-related phenolics in inbred (light grey point) and outcrossed (dark point) progeny of *Solanum carolinense*. Data points represent the proportional change in phenotype in previously damaged relative to control plants ± 95% confidence limits.

Figure 2. Growth trait responses (mean ± se) of inbred (light points, dashed line) and outcrossed (dark points, solid line) *Solanum carolinense* exposed to 0, 10 and 20% simulated herbivory. For clarity, inbred and outcrossed data points are offset within each treatment. Asterisks denote significant pairwise differences between inbred and outcrossed plants within each treatment (Tukey's tests).

Figure 3. Constitutive and wounding induced expression of the phytohormones salicylic (SA), jasmonic (JA), abscisic (ABA) and indole-3-acetic acids (IAA) (structures at top) in leaves of inbred and outcrossed *Solanum carolinense*. Data are means ± 1 SE. Note that IAA is plotted on the right-hand axis for clarity. Pie charts show relative hormone ratios within each combination of breeding and wounding treatment. *,† and ‡ denote significant effects of breeding, wounding treatment and their interaction, respectively (see table 1); *ns* indicates no significant effects.

TABLE LEGENDS

Table 1. Fixed-effects linear model results for effects of breeding (inbreeding vs. outcrossing) and damage/wounding treatment on (a) plant growth trait responses, and (b) expression of plant phytohormones. Significant *p*-values are in bold.

Table 2. Pairwise phenotypic correlation matrices for phytohormone traits in (a) control and (b) wounded plants. Values below the diagonals are for outcrossed plants, while values above the diagonals are for inbred plants. Bold denotes $p \leq 0.05$; * denotes $p \leq 0.01$; † denotes $p \leq 0.001$.

Table 3. Random skewers analysis comparing the covariance matrices for hormone expression in control and wounded plants in each breeding condition. Out = outcrossed; In = inbred. Pairwise vector correlation coefficients between matrices given below the diagonal; *p*-values given above the diagonal, indicating whether two covariance matrices are significantly correlated. (a) Analysis of all four phytohormones. (b) Analysis excluding jasmonic acid (JA).

Table 1

(a) Growth trait		Source of variation: Breeding	Treatment	Breeding× Treatment	R^2
Leaf mass	*F*	9.411	0.574	4.071	0.20
	p	**0.0023**	0.4493	**0.0445**	
Stem mass	*F*	25.562	0.852	0.103	0.37
	p	**<0.0001**	0.3567	0.7482	
Height	*F*	20.807	4.771	1.800	0.25
	p	**<0.0001**	**0.0294**	0.1804	
Leaf no.	*F*	1.153	0.6795	0.9149	0.25
	p	0.2828	0.4102	0.3393	
(b) Hormone[1]					
SA	*F*	0.045	1.305	0.499	0.18
	p	0.8317	0.2579	0.4826	
JA	*F*	4.889	77.101	5.342	0.63
	p	**0.0311**	**<0.0001**	**0.0245**	
ABA	*F*	45.585	5.159	0.0209	0.54
	p	**<0.0001**	**0.0268**	0.8856	
IAA	*F*	4.030	0.028	0.027	0.61
	p	**0.0493**	0.8689	0.8703	

[1]SA, salicylic acid; JA, jasmonic acid; ABA, abscisic acid; IAA, indole-3-acetic acid

Table 2

(a) Control	SA	JA	ABA	IAA
SA	–	0.018	0.229	-0.026
JA	0.278	–	**0.535**	**0.779**†
ABA	**0.470**	-0.173	–	**0.671***
IAA	**0.558**	**0.541**	**0.549**	–

(b) Wounded	SA	JA	ABA	IAA
SA	–	0.316	0.263	0.187
JA	**0.466**	–	0.219	0.381
ABA	0.328	0.132	–	**0.682***
IAA	0.330	**0.612***	0.216	–

Table 3

(a) All hormones		*Out*		*In*	
		Control	Wound	Control	Wound
Out	Control	–	0.21	**0.03**	0.28
	Wound	0.470	–	0.21	**0.02**
In	Control	**0.871**	0.467	–	0.29
	Wound	0.347	**0.905**	0.335	–

(b) Without JA		*Out*		*In*	
		Control	Wound	Control	Wound
Out	Control	–	**0.03**	0.06	**0.03**
	Wound	**0.932**	–	**0.01**	0.05
In	Control	0.878	**0.972**	–	0.06
	Wound	**0.943**	0.901	0.884	–

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Figure 1

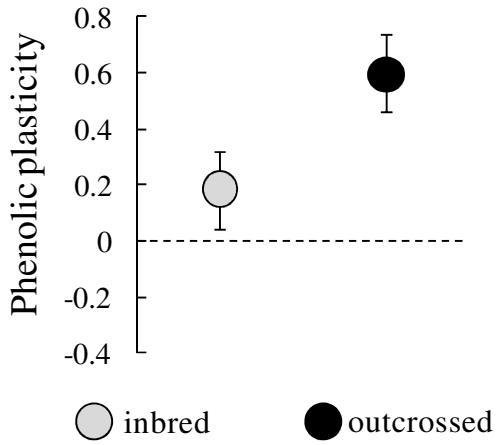


Figure 2

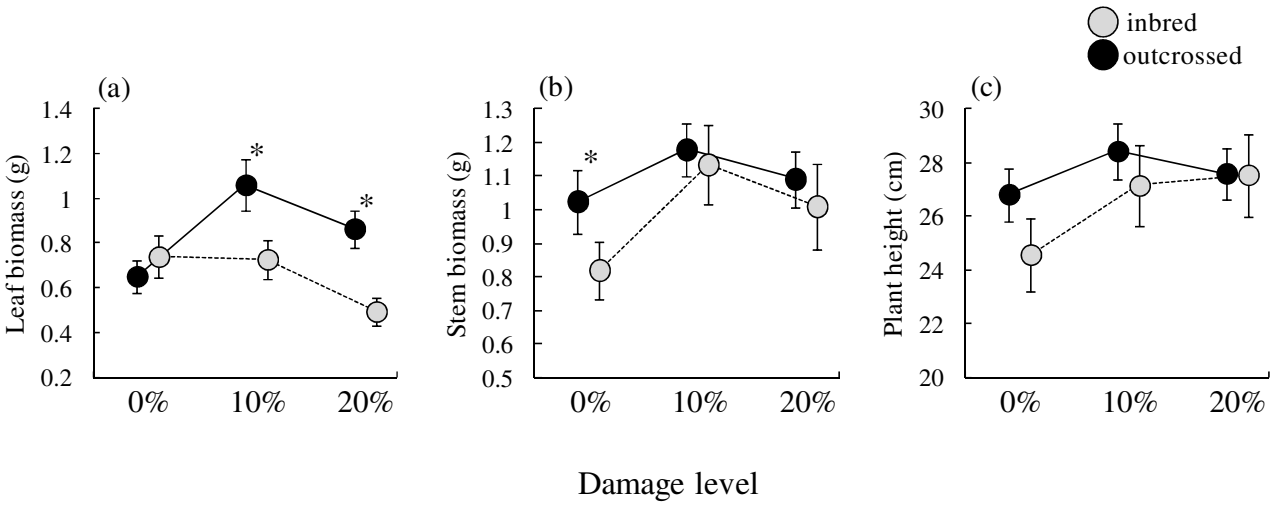
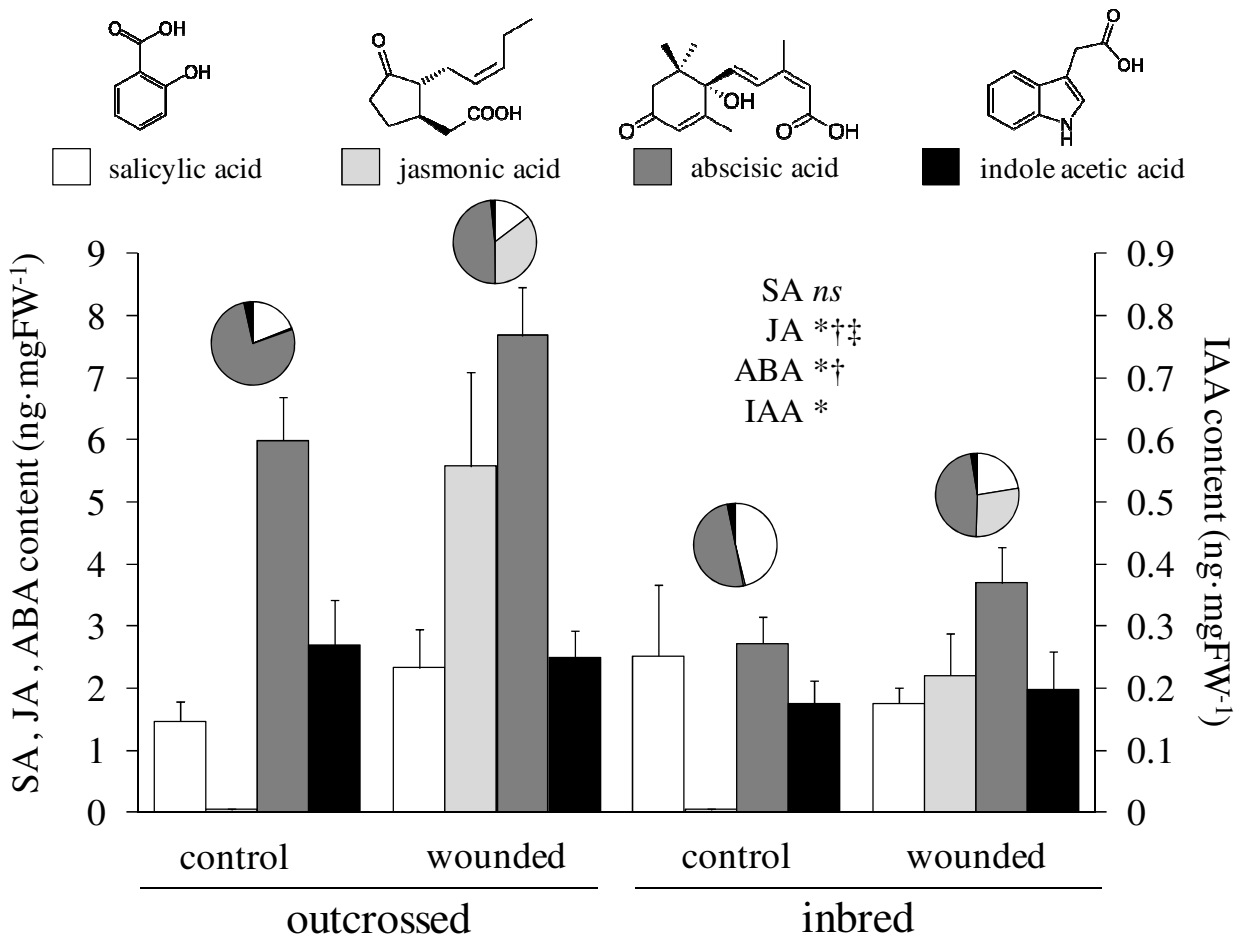

inbred
outcrossed
(a)
Leaf biomass (g)
1.4
1.2
1
0.8
0.6
0.4
0.2
*
*
0%
10%
20%
(b)
Stem biomass (g)
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
*
0%
10%
20%
(c)
Plant height (cm)
30
28
26
24
22
20
0%
10%
20%
Damage level

Figure 3


salicylic acid
jasmonic acid
abscisic acid
indole acetic acid
SA, JA, ABA content (ng·mgFW-1)
IAA content (ng·mgFW-1)
SA ns
JA *†‡
ABA *†
IAA *
control
wounded
control
wounded
outcrossed
inbred

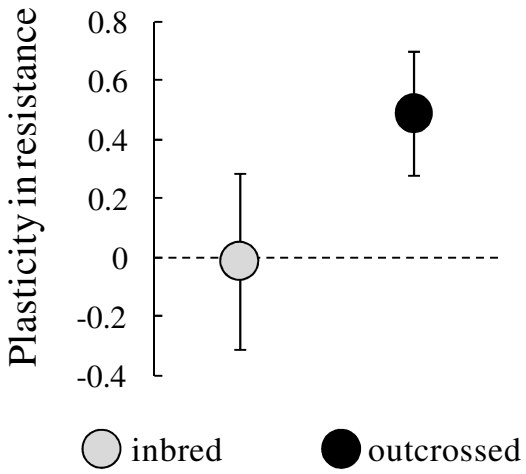
Electronic Supplementary Material

Induced resistance experiment

Solanum carolinense is host to numerous insect herbivores, including the tobacco hornworm, *Manduca sexta* L., (Lepidoptera: Sphingidae), a solanaceous specialist [93, 94] and the generalist four-lined plant bug, *Poecilocapsus lineatus* (Hemiptera: Miridae) [95]. As an additional independent test of the hypothesis that inbreeding reduced plasticity in defence traits, we conducted a bioassay that manipulated damage by the generalist bug and measured resistance to *M. sexta.* Inbred and outcrossed plants (n=14 families) of approximately equal size (4-6 true leaves) were exposed to damage by freshly caught *Poecilocapsus lineatus*. Single bugs were allowed to feed on individual bagged plants for ca. 48 hours, until bugs had damaged ca. 20-30% of the leaf area of each plant based on visual inspection of feeding lesions [95]. Control plants were also bagged. Following damage, bags were removed, and a bioassay was conducted to test for differences in constitutive and induced resistance. We used the model herbivore *Manduca sexta*, since it is sensitive to variation between inbred and outcrossed *S. carolinense* [13]. From the youngest fully expanded leaf of each plant, we took 2.5 cm^2 leaf discs using a cork borer. Using discs minimizes potential confounding effects that arise from the use of whole plants, which can be induced during the bioassay. Discs were mounted on a pin over moist filter paper (to facilitate larval preference for feeding on leaf undersides), and freshly hatched neonate *M. sexta* larvae were added and allowed to feed for 48-72 hours. Following removal from the discs, larvae were allowed to clear their gut contents for ca.12 hours and were then weighed; masses were normalized to the length of time spent feeding. Larval growth was converted to estimates of resistance as (1 – [relativised average growth for each maternal family]). We analysed the effect of inbreeding on resistance using the proportional change in resistance on outcrossed vs. inbred plants for each family (to control for genetic variation among families, [13], and compared these contrasts in JMP® v9.0 [45] using an *F*-test;

i.e., a matched-pairs design [46]. We removed one outlier (a single outcrossed genotype that showed striking induced susceptibility, in contrast to every other outcrossed family); inclusion of this datum did not qualitatively affect the results. The results of this experiment demonstrate that outcrossed plants exhibit significantly greater inducibility of resistance, relative to inbred plants ($F_{1,13} = 7.89$; $P = 0.015$) (figure S1), consistent with plant chemistry results (figure 1).

Figure S1. Plasticity in resistance to *Manduca sexta* herbivores in inbred (light grey point) and outcrossed (dark point) progeny of *Solanum carolinense*. Data points represent the proportional change in phenotype in previously damaged relative to control plants ± 95% confidence limits



4. Plant mating system transitions drive the macroevolution of defence strategies

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ONE SENTENCE SUMMARY:

The transition from an obligately outcrossing to an inbreeding mating system in plants leads to the evolution of a more plastic and specialised strategy of resistance to herbivores, indicating coevolution between a plant's sex life and antagonistic species interactions.

ABSTRACT: **Alternative strategies of sexual reproduction (inbreeding vs. outcrossing) have divergent effects on population genetic structure, and could influence the evolution of species interactions. Across a phylogeny of 56 wild species of Solanaceae (nightshades), we show that the unidirectional transition from self-incompatibility (outcrossing) to self-compatibility (inbreeding) leads to the evolution of an inducible (vs. constitutive) strategy of plant defence. We demonstrate that inducible and constitutive defence strategies represent evolutionary alternatives, and that the magnitude of this macroevolutionary constraint is dependent on the mating system. Loss of self-incompatibility has also promoted the evolution of increased specificity in induced plant responses. The macroevolution of sexual reproductive variation has profound effects on the evolution of plant-herbivore interactions, providing the basis for a new hypothesis of plant defence evolution.**

MAIN TEXT:

Both plant and animal taxa exhibit remarkable variation in sexual reproduction, and this variation can have important consequences for population genetic structure and evolution. In general, outcrossing taxa exhibit larger, more genetically variable populations, while inbreeding taxa tend to exhibit smaller population sizes with lower genetic variability *(1,2)*. Mating systems can influence many aspects of a species' evolutionary ecology, including gene flow, extinction risk *(3),* local adaptation *(4)* and interactions with other species *(5-8)*. At a macroevolutionary scale, however, the study of how mating strategies influence species interactions has focussed almost exclusively on the interactions that are directly relevant to the mating system itself (*9*). For example, the evolution of self-fertilisation in plants is correlated with the loss or reduction of floral traits that attract pollinators and with reductions in floral visitation (*10*). Evolution of inbreeding strategies may occur more often at species range edges, allowing the persistence of small, isolated populations in which pollinators and/or mates are limiting (*11*). Such populations must overcome the costs of inbreeding (e.g., inbreeding depression), but can thereby fill new niches and potentially speciate (*11*). Ecological studies have shown that such isolated marginal populations, while lacking mutualistic pollinators, may also escape antagonists such as herbivores and parasites (*12*,*13*), suggesting a role for mating system transitions in antagonistic species interactions. However, to our knowledge, there have been no evolutionary studies on the broader effects of mating system transitions on the evolution of functional traits that are not directly related to the mating system itself; thus our understanding of how mating systems shape phenotypic evolution remains limited.

We tested the role of mating system evolution in the macroevolution of defence. Plant defence against natural enemies (herbivores, pathogens, parasites) comprises a complex range of phenotypes, including physical traits such as trichomes and leaf toughness, and chemical traits such as toxins and anti-nutritive compounds (*14*). There are numerous hypotheses for the

evolution and maintenance of this variation, each emphasizing to varying degrees the relative importance of herbivore frequency, physiological constraints, resource limitation and the costs of trait expression (*15*). Fundamentally however, plants may adopt two different primary strategies in how they deploy those traits: they may express defence traits at all times (i.e., constitutive defence strategy), or they may save the cost of producing defences in the absence of attackers by only inducing defences after initial attack (inducible strategy) . One hypothesis predicts that plants that grow in environments in which the probability of attack is variable should exhibit the latter strategy and show a greater phenotypically plastic response to herbivory (inducibility) (*16*). However, decades of theorizing and research on plant defence have yielded little consensus (*15*), indicating that important predictive factors may be missing from models of defence evolution, particularly in relation to macroevolutionary patterns (*17*).

Plants also exhibit a remarkable diversity of traits that have ostensibly evolved to promote outcrossing and limit the costs of inbreeding depression, including aspects of floral morphology, the timing and location of gender expression and gamete recognition (*18*,*19*). Some of the most potent mechanisms preventing inbreeding include the self-incompatibility systems found in over 19 orders and 70 families of angiosperms (*20*). Gametophytic self-incompatibility (SI), in which pollen from relatives is prevented from reaching the ovule, creates a predominantly outcrossing mating system, although some species exhibit plasticity in this system (*21*). Self-incompatibility is the ancestral condition in the nightshades (Solanaceae), however, the loss of SI has independently occurred over 60 times in this plant family (*22*). The transition from SI to self-compatibility (SC), i.e., from an outcrossing to more inbreeding reproductive strategy, appears to be irreversible (*22*). We used the repeated, unidirectional losses of SI in the Solanaceae to investigate the consequences of mating system transitions for the evolution of strategies of resistance to herbivores. In a phylogenetic framework, we conducted manipulative experiments with 56 wild species (over 900 plants) from 13 genera, including SI and SC petunia, tobacco,

groundcherry, pepper, tomato and potato taxa. We selected taxa *a priori*, based on mating system status, to ensure that we assessed independent, replicate mating system transitions. For each species, we induced (damaged) plants with larvae of *Manduca sexta* (Lepidoptera: Sphingidae), an herbivore that naturally feeds on most Solanceae, and with a mechanical wounding treatment (*23*). Biological and mechanical damage were standardised (20% leaf area) to control for covariation between damage level and induction. Performance bioassays with fresh *M. sexta* larvae on treatment and control plants were used to generate estimates of induced and constitutive resistance, respectively (*23*). Such estimates integrate all the individual traits (e.g., specific metabolites) that are relevant to the plant's defence, and thereby address the issue of different taxa using different suites of secondary metabolites.

Using Bayesian analyses of trait evolution on a molecular phylogeny of our experimental taxa (*23*), we find a moderate decrease in constitutive resistance in SC compared to SI taxa, as indicated by a Bayes Factor test and a marginally significant likelihood ratio test (Fig 1). However, there were no consistent differences between mating systems in the absolute induced resistance. Thus, neither obligate outcrossing (SI), nor its loss, confers a consistent evolutionary advantage in terms of the magnitude of resistance traits. Prior damage by *Manduca* did induce a 21% increase in average resistance across all species (Fig 1).

We next examined evolutionary patterns in how species deploy those traits (defence strategies). Estimates of inducibility (*23*) reveal that the transition from outcrossing to inbreeding has been accompanied by a shift to a more inducible strategy of plant defence: Despite considerable variation among species, self-compatible taxa are on average 63% more inducible, indicating that phenotypic plasticity in response to herbivore attack has coevolved with plant mating strategies (Fig 2). Since there is no direct ecological interaction between mating and defence strategy, we propose that this coevolutionary relationship is an indirect result of the selective

environment that accompanies the shift to increased self-fertilisation. Self-compatible taxa often persist in marginal habitats and/or at range edges with a paucity of mates and pollen vectors (*11*). Theoretical models predict that these habitats should in turn favour the evolution of phenotypic plasticity (*24*), particularly if under variable herbivory (*16*), and our data support this hypothesis. Alternatively, there may exist indirect interactions among herbivores, plants and pollinators that may collectively favour the evolution of greater inducibility in inbreeding taxa. Herbivory can alter floral chemical phenotypes and influence pollinators (*25-27*), and the induction of deterrent or toxic metabolites in reward tissues such as pollen and nectar could be disproportionately costly to obligately outcrossing, as compared to inbreeding, taxa. Overall, mating system evolution could have widespread impacts on a range of species interactions, including parasitism, competition, predation as well as mutualism, provided that mating system transitions were accompanied by changes in genetic and/or ecological factors important to the interaction. Understanding the strength of these impacts becomes an important issue in the study of these interactions.

The observed increase in plasticity under the loss of SI could also be interpreted as the evolution of developmental instability under an inbreeding mating system (*28*), rather than adaptive coevolution of mating and defence strategies. Inbreeding-generated developmental instability has been shown to increase plasticity to variable environments, and is considered maladaptive (*29*). Under this hypothesis, we would predict that SC taxa would show equally strong induced responses to herbivore attack and mechanical wounding; that is, SC taxa would show a lower degree of specificity to herbivore attack than SI taxa. Many plant species are capable of inducing responses that are specific to herbivore consumption (vs. mechanical wounding). A strategy of specificity allows plants with multiple attackers, and plants at risk of wounding from stochastic incidents of e.g., trampling or wind damage, to fine-tune their defences in a putatively adaptive manner (*30*). A developmental instability hypothesis would not

be supported if SC taxa exhibited such adaptive specificity.

We analysed variation in plant responses for evidence of the evolution of specificity. Our null hypothesis was that if there had been no evolution of specificity, the behavioural response to *Manduca* damage and the response to mechanical wounding would be strongly and positively correlated across the phylogeny, indicating that plants were poorly able to differentiate the two types of damage. In contrast, no relationship would be found if the two traits were evolving independently, indicating the evolution of specificity. We find that SI taxa show a pattern consistent with low specificity (a strong and significant correlation between responses), while SC taxa showed a pattern consistent with a high degree of specificity (Fig 3). Such differential specificity is inconsistent with an instability hypothesis for SC taxa. In ruling out this alternative hypothesis, we have shown that the loss of SI in this diverse plant family has led to the evolution of increased specificity to herbivory (vs. wounding). No other study to our knowledge has examined the macroevolution of specificity in plant responses, and additional studies are now needed to examine the role of mating systems for specificity to different herbivores.

The contrasting effects of mating systems on constitutive resistance and inducibility prompted us to investigate how these traits covaried across the phylogeny. A long standing tenet of so-called plant defence theories is that a trade-off should exist between these two strategies, primarily because plants should benefit from being inducible when defences are costly and herbivory uncertain, since this saves the cost of defence expression in the absence of herbivores (*31*). This hypothesis has been supported by comparisons within species (*32*); conversely, prior phylogenetic studies have demonstrated positive correlations in the expression of individual defence-related secondary compounds (*33*), but have not examined actual plant resistance among wild species when the amount of damage was controlled (cf. [*34*]). Plant resistance (i.e., based on the performance of an herbivore) is necessary for fully understanding

the evolution of defence strategies, since it integrates all the individual physical and metabolic traits that are relevant to the herbivore, but that may be highly divergent even between closely related species. Across the Solanaceae, we find a highly significant, negative relationship between direct, constitutive resistance and inducibility of resistance (Fig 4). To our knowledge, this is the first robust comparative evidence that these represent macroevolutionary alternative strategies in plant defence. This is significant because it demonstrates a potential correspondence between microevolutionary processes (defence trade-offs) and constraints on the evolution of species-level variation in strategy, regardless of the particular defensive tactics (e.g., chemicals) employed by each species. Mating systems differ in the magnitude of the constraint (Fig 4), indicating that this fundamental relationship in plant defence theory is shaped in part by coevolution of mating systems and inducibility.

Our study indicates that variation in plant sexual reproduction has broadly shaped the macroevolution of defence strategies across the Solanaceae. Our findings should be particularly robust, first, because our analyses are based on repeated losses of a key reproductive trait (self-incompatibility). Second, our conclusions are based on measures of plant resistance, rather than secondary metabolite variation, and thus are robust to taxon-specific differences in which traits comprise the defence phenotype. Finally, our comparative study is among the few to consider plant defence as a set of behavioural strategies, rather than simply using 'fixed', constitutive phenotypes (*33*). Accordingly, we propose incorporating mating system variation into models of defence trait evolution, and herbivory into models of mating system evolution. While sex *per se* (vs. asexuality) is long thought to have been favoured in part by coevolution of antagonistic interactions (*35-39*), we have shown that such interactions may also coevolve with variation in the mode and expression of sexual reproduction (see also [*8*]). We conclude that host strategies of sexual reproduction may represent a previously unappreciated predictive factor that could be used to better understand defence trait variation in

nature, and that ecological and comparative studies of plant trait evolution would benefit from integrating information on the mating system.

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Fig 1

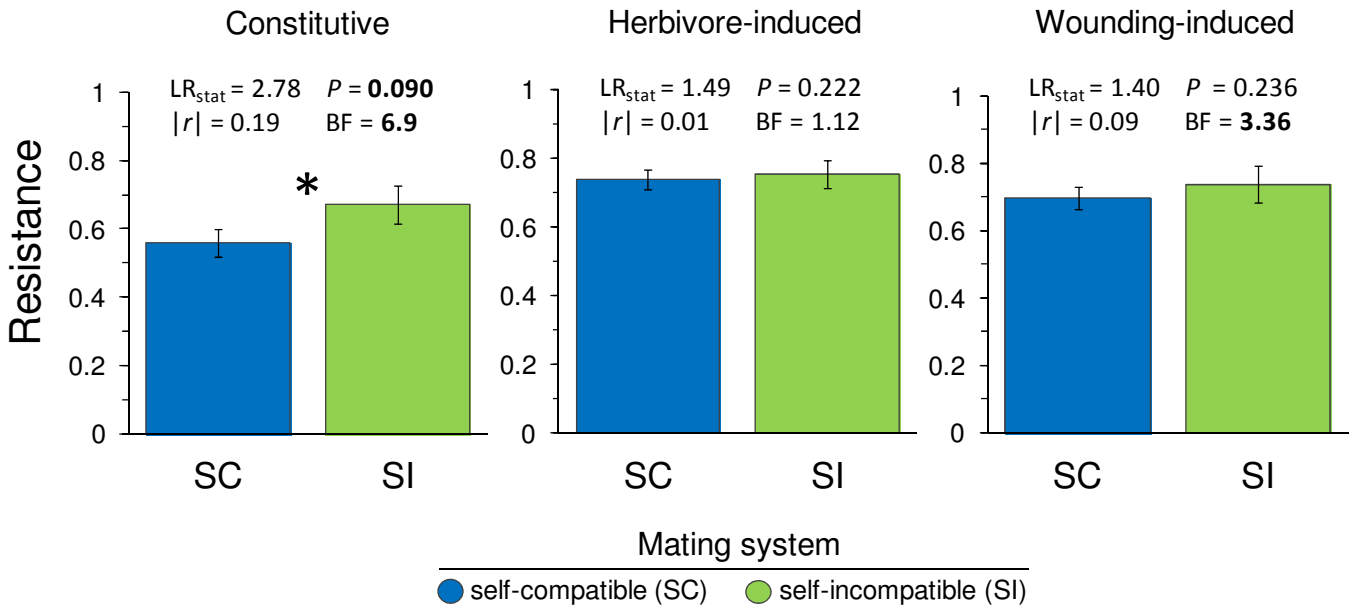
Constitutive
Herbivore-induced
Wounding-induced
LRstat = 2.78 P = 0.090
|r| = 0.19 BF = 6.9
LRstat = 1.49 P = 0.222
|r| = 0.01 BF = 1.12
LRstat = 1.40 P = 0.236
|r| = 0.09 BF = 3.36
Resistance
1
0.8
0.6
0.4
0.2
0
*
SC
SI
Mating system
self-compatible (SC)
self-incompatible (SI)

Fig 2

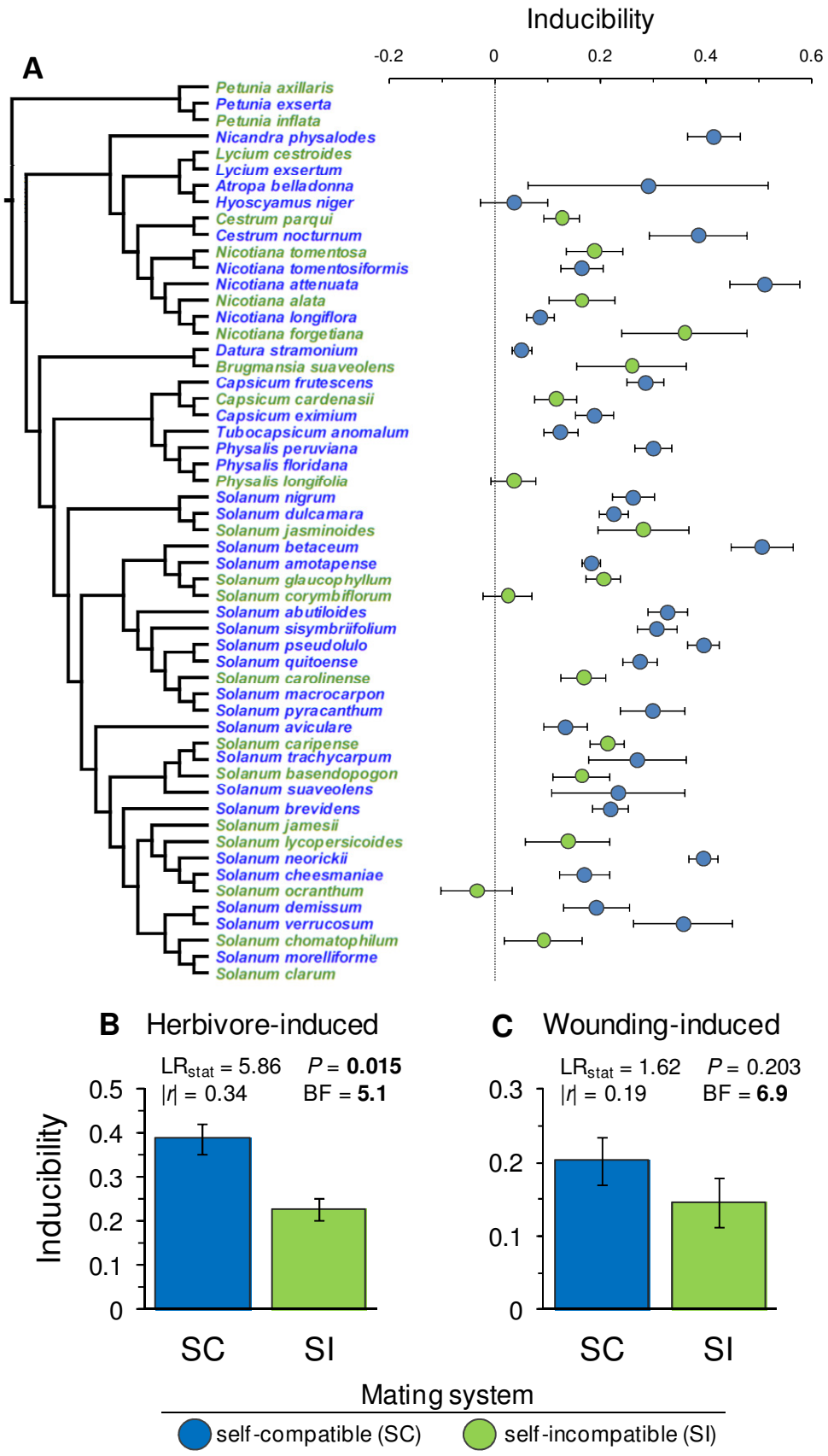
A
Inducibility
-0.2
0
0.2
0.4
0.6
Petunia axillaris
Petunia exserta
Petunia inflata
Nicandra physalodes
Lycium cestroides
Lycium exsertum
Atropa belladonna
Hyoscyamus niger
Cestrum parqui
Cestrum nocturnum
Nicotiana tomentosa
Nicotiana tomentosiformis
Nicotiana attenuata
Nicotiana alata
Nicotiana longiflora
Nicotiana forgetiana
Datura stramonium
Brugmansia suaveolens
Capsicum frutescens
Capsicum cardenasii
Capsicum eximium
Tubocapsicum anomalum
Physalis peruviana
Physalis floridana
Physalis longifolia
Solanum nigrum
Solanum dulcamara
Solanum jasminoides
Solanum betaceum
Solanum amotapense
Solanum glaucophyllum
Solanum corymbiflorum
Solanum abutiloides
Solanum sisymbriifolium
Solanum pseudolulo
Solanum quitoense
Solanum carolinense
Solanum macrocarpon
Solanum pyracanthum
Solanum aviculare
Solanum caripense
Solanum trachycarpum
Solanum basendopogon
Solanum suaveolens
Solanum brevidens
Solanum jamesii
Solanum lycopersicoides
Solanum neorickii
Solanum cheesmaniae
Solanum ocranthum
Solanum demissum
Solanum verrucosum
Solanum chomatophilum
Solanum morelliforme
Solanum clarum
B Herbivore-induced
LRstat = 5.86 P = 0.015
|r| = 0.34 BF = 5.1
Inducibility
0.5
0.4
0.3
0.2
0.1
0
SC
SI
C Wounding-induced
LRstat = 1.62 P = 0.203
|r| = 0.19 BF = 6.9
0.3
0.2
0.1
0
SC
SI
Mating system
self-compatible (SC)
self-incompatible (SI)

Fig 3

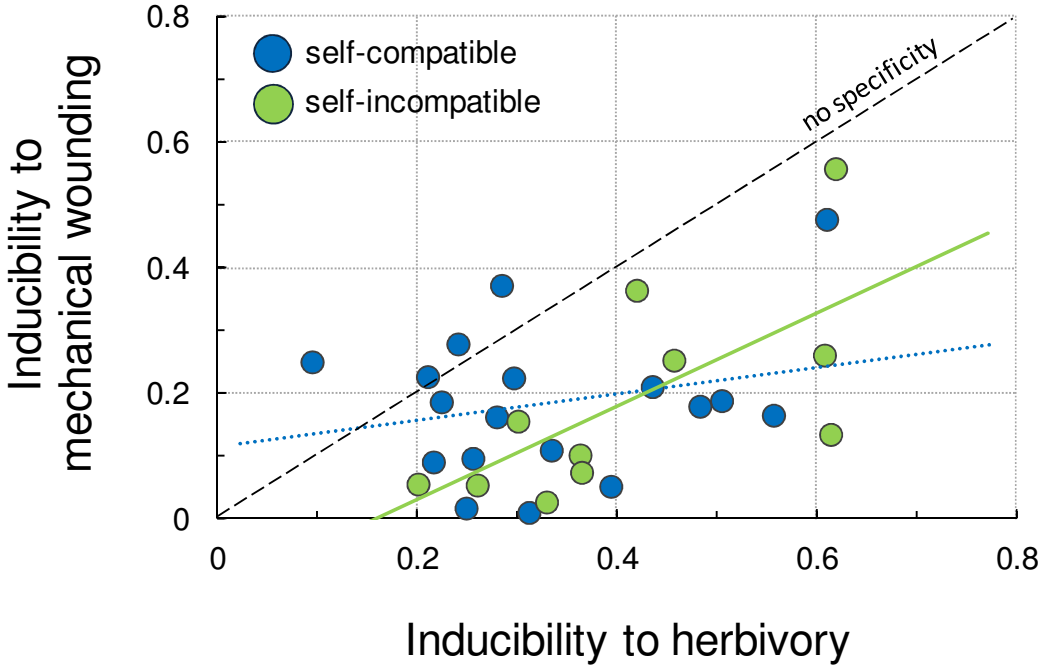
self-compatible
self-incompatible
no specificity
Inducibility to mechanical wounding
Inducibility to herbivory
0
0.2
0.4
0.6
0.8

Fig 4

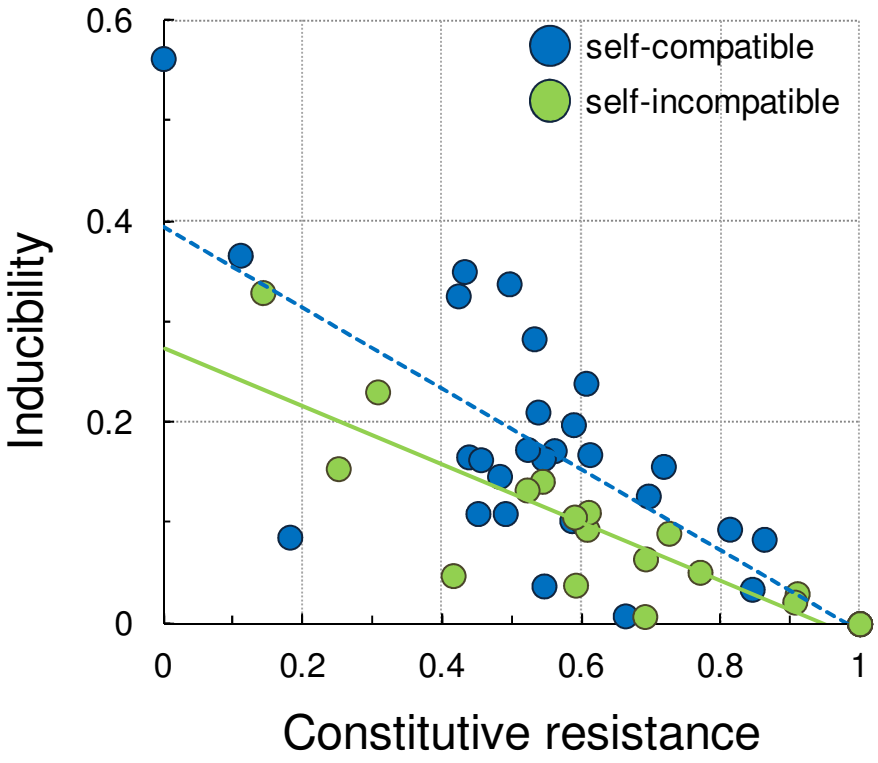
0.6
0.4
0.2
0
Inducibility
self-compatible
self-incompatible
0
0.2
0.4
0.6
0.8
1
Constitutive resistance

Figure 1. Absolute levels of constitutive and induced resistance (1 – [relativised performance of bioassay larvae]) across self-incompatible (green, SI) and self-compatible (blue, SC) Solanaceae. Data are raw phenotypic means (±1 se), while statistics account for phylogenetic relationships among taxa. Above each panel are the results of likelihood ratio (LR) tests of the hypothesis that resistance has been shaped by mating system (LR_{stat} and associated *P*-value), and corresponding Bayes factor (BF) tests: BF ≥ 2 indicates positive support, BF ≥ 5 indicates strong support, and BF ≥ 10 indicates very strong support in favour of a model of correlated evolution. $|r|$ is the phylogenetically-corrected correlation coefficient. Asterisk denotes a marginally significant difference in constitutive resistance between mating systems ($P = 0.080$) prior to correcting for phylogeny.

Figure 2. (A) Bayesian maximum clade credibility tree of experimental taxa, with outgroups removed. Taxa and data are colour-coded by mating system, as either self-compatible (blue) or self-incompatible (green). Inducibility to *Manduca sexta* herbivore damage (mean ± SE) is mapped on the phylogeny. Summary bar graphs show the average difference between mating systems in inducibility to *Manduca* herbivore damage (B) and inducibility to mechanical wounding (C). Above each panel are the results of likelihood ratio (LR) tests of the hypothesis that resistance evolution is a function of mating system (LR_{stat} and associated *P*-value), and corresponding Bayes factor (BF) tests: BF ≥ 2 indicates positive support, BF ≥ 5 indicates strong support, and BF ≥ 10 indicates very strong support in favour of a model of correlated evolution. $|r|$ is the phylogenetically-corrected correlation coefficient.

Figure 3. Evolution of specificity differs between mating systems. Correlation of inducibility to mechanical wounding (ordinate) and inducibility to *Manduca sexta* herbivory (abscissa) for self-incompatible (solid green line, $\beta_{SI} = 0.74$; $P = 0.02$) and self-compatible (dashed blue line, $\beta_{SC} = 0.21$; $P = 0.32$) taxa. Data are raw phenotypic mean values, while statistics are corrected for

phylogenetic relationships among taxa. Null expectation for an hypothesis of no specificity denoted by black, dashed line ($\beta_{null} = 1.0$).

Figure 4. Inducible and constitutive resistance strategies represent evolutionary alternatives. Shown is the relationship between inducible and constitutive strategies of plant resistance for self-incompatible (solid green line, $R^2 = 0.822$; $\beta_{SI} = -0.275 \pm 0.030$) and self-compatible (dashed blue line,$R^2 = 0.499$; $\beta_{SC} = -0.390 \pm 0.075$) taxa. Data are raw phenotypic mean values, while statistics are corrected for phylogenetic relationships among taxa. Likelihood ratio (LR) tests for both SI ($LR_{stat} = 25.22$; $P < 0.0001$) and SC taxa ($LR_{stat} = 21.56$; $P < 0.0001$) strongly support a model of a negative phylogenetic relationship between resistance strategies, as do Bayes factor (BF) tests ($BF_{SI} = 25.84$; $BF_{SC} = 23.78$, where BF > 10 indicates extremely strong support for a model of correlated evolution). Mating systems diverge in the steepness of the relationship based on a LR test that $\beta_{SI} < \beta_{SC}$ ($LR_{stat} = 3.72$; $P = 0.05$).

Supporting Online Material

Materials and Methods

Tables S1, S2

SUPPLEMENTARY MATERIALS:

Materials and Methods:

Taxon sampling

We focussed our taxon sampling on as many independent evolutionary losses of self-incompatibility as possible, based on the simultaneous availability of botanical seed, published information on mating system status (*22*,*40*), and molecular sequence data. We selected self-incompatible (SI) species with available seed, and for each of these we selected closely related self-compatible (SC) species for comparison. We generally avoided sampling the same independent loss of SI. We attempted to select diploid species in both mating systems to avoid confounding any effects of mating system transitions with any effects of polyploidisation, since polyploidisation is a common route to SC (*41*). Nevertheless, the correlation between ploidy and mating system is almost perfect in some clades, and we sampled known polyploids in the case of *Lycium exsertum*, *Physalis peruviana,* and *Solanum demissum*. Exclusion of these three taxa from our analyses did not affect our results.

The dataset for this study is based on 58 species of Solanaceae from 13 genera (Table S1), including *Petunia, Nicotiana, Datura, Brugmansia*, *Cestrum*, *Capsicum*, *Physalis* and *Solanum*. Within *Solanum*, we sampled many of the major clades (*42* - *44*), including representatives of the Archaeosolanum, Dulcamaroid, Cyphomandra, Geminata, Brevantherum, Leptostemonum, Petota and Lycopersicon (wild true tomato) clades. The ratio of SI to SC taxa was ca. 2:3, which is very close to published estimates for the family (*40*). Some clades were not sampled if

seeds for both SI and SC taxa were unavailable. Our dataset is among the largest to date with respect to comparative studies of plant defence, and as the largest such study within the Solanaceae, it is moderately representative of the family as a whole. Nevertheless, we emphasize that our taxon sampling was targeted at mating system variation across the family, and thus was neither random nor proportional to clade species richness. However, the *a priori* selection of taxa based on SI status (and specifically, independent losses of SI) allows us to rigourously test the hypothesis that mating system transitions have independently caused changes in the evolution of defence.

We report details of phylogeny reconstruction elsewhere. In brief, we used only previously published sequence data from three chloroplast regions: ndhF, maturaseK (matK), and an intergenic spacer region (trnL-trnF); and two nuclear regions: granule-bound starch synthase I (*waxy*) and internal transcribed spacer 1 (ITS1). Sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/), concatenated and aligned in the *Geneious* v5.5 software package (*45*) using first-pass multiple alignment in MAFFT, and a gene-partitioned data matrix was analysed in Mr Bayes v.3.1.2 (*46*) for over 180 million post burn-in generations using a GTR + I + G model of evolution. Bayesian maximum clade credibility trees (N=1000) were selected at random from the posterior distribution and manually curated, adding four taxa lacking sequence data (*Solanum basendopogon, S. conocarpum, S. suaveolens* and *S. trachycarpum*) based on published phylogenies, all of which were highly congruent with our analyses (*42,44,47-49*).

Experimental protocol

Seeds were obtained from gene-banks and seed repositories, primarily the Radboud University Botanical Garden (Nijmegen, NL), the C.M. Rick Tomato Genetics Resource Centre (Davis, CA,

USA), the Botanical Garden Berlin-Dahlen, and the USDA-ARS National Genetic Resources Program. We selected 2-3 replicate wild accessions wherever possible. All species were germinated from seed on moist vermiculite, and transferred as seedlings to four-inch (355 mL) pots filled with moist Metro-Mix 360 all-purpose potting soil (Scotts-Sierra Horticultural Products, Marysville, OH) and grown in a greenhouse under a 16hr light : 8hr dark schedule, watered *ad libitum* and fertilized weekly (21-5-20 N:P:K, 150 ppm). To control for ontogenetic variation in resistance, all plants were grown for a minimum of one month until they had 4-8 true leaves, and were used prior to bolting and/or reproduction in all cases expect *Petunia* spp., which bolted extremely rapidly.

We imposed three treatments on replicate plants (N = 6-20, total N ≈ 930) of each species: Damage by *Manduca sexta* larvae; mechanical wounding; and controls (no damage). *Manduca sexta* is a Solanaceous specialist herbivore that naturally feeds on most Solanceae (*50*). *Manduca*-damaged plants received single neonate (1st instar) larvae of *M. sexta* (obtained from an in-house colony) to each of three leaves. A few species (e.g., *S. morelliforme, S. clarum*) only produced sufficient seedlings for the control treatment. Since different damage levels can induce dramatically different responses, we standardised the amount of proportional damage across species. Larvae were left to feed until each leaf was damaged ca. 20% based on visual estimation, at which point larvae were removed. For the mechanical wounding treatment, a hole-punch was used to create equivalent damage, and a standard fabric pattern wheel was used to apply puncture wounds to the distal third of the damaged leaves, at 5mm spacing perpendicular to the longitudinal leaf axis. While mechanical wounding alone does not provide the same signals as real herbivore attack, it allowed us to ask whether plants differed in their specific responses to herbivory, as compared to simple wounding and tissue removal. Within 24 hours of cessation of damage (biological and mechanical), damaged leaves from all plants were harvested. Three leaves of undamaged control plants were sampled from similar positions

as the damaged plants. From each damaged leaf, and similarly positioned leaves from control plants, we took leaf discs using a cork borer (diam = 3.1 cm) for bioassays (average of 2 discs per plant). Using discs minimizes potential confounding effects that arise from the use of whole plants, which can be induced during the bioassay. In taxa with compound and/or finely divided leaves (e.g., tomatoes), we excised the terminal leaflet as our bioassay unit. Discs and leaflets were mounted on a pin over moist filter paper (to facilitate larval preference for feeding on leaf undersides), and freshly hatched neonate *M. sexta* larvae were added and allowed to feed for 48-72 hours. Following removal from the discs, larvae were allowed to clear their gut contents for ca.12 hours and were then weighed. Only larvae that had initiated feeding (as indicated by frass and/or feeding marks) were included in the analysis. Larvae initiated feeding on all species in our study, but those that had not fed due to falling off the leaf shortly after placement were excluded.

Results from multiple discs (larval growth rate) were averaged for each plant. Following protocols developed for the analysis of relative fitness (*51*), treatment averages for each species were relativised to the most resistant species to allow comparison among species and mating systems on a common scale. Performance measures were converted to measures of resistance (1-[relativised treatment average]) to facilitate interpretation. In addition to absolute values of resistance using treatment means, we estimated species level inducibility (phenotypic plasticity) in two ways. First, we generated estimates of inducibility using a relative distance plasticity index (RDPI), which uses the average pairwise difference in performance between damaged and control plants relative to control values (*52*). This approach is advantageous in that the plasticity measure obtained is a random, normally distributed variable, allowing the estimation of standard errors and confidence limits for the purposes of statistically comparing species pairs (something which is not possible using single inducibility values for each species) (*52*). However, proportional estimates of inducibility lead to biases in the comparison of inducible and

constitutive resistance strategies (*53*), and for such comparisons, we also estimated inducibility as the bias-corrected contrast between induced and constitutive resistance (as in [*53*]). Some taxa (e.g., *Petunia* spp.), were excluded from measurements of inducibility when they were extremely constitutively resistant to *Manduca*, since it would have been inappropriate to conclude low inducibility *per se* when feeding damage was so low. The wild status of *Solanum macrocarpon* may be questionable, leading to us to conduct analyses with and without this species. Although these exclusions represent a conservative approach, inclusion or exclusion of these taxa did not qualitatively change any result. We estimated inducibility to mechanical wounding using the same method. In analysing the relationship between inducibility to *Manduca* damage and inducibility to mechanical wounding, we used the proportional change in resistance relative to controls in order to avoid confounding interspecific differences in constitutive resistance with the test for specificity.

Analyses

We analysed the relationship between mating strategy (SI vs. SC) and measures of constitutive resistance, induced resistance (to both *Manduca* damage and mechanical wounding), and inducibility, in the software package *Bayestraits* v.2.0 (*54*) (available online at www.evolution.rdg.ac.uk) using our 1000 Bayesian phylogenetic trees, allowing us to incorporate phylogenetic uncertainty in our analyses. However, using a single Bayesian consensus tree gave equivalent results. All analyses utilised standard Bayesian, Markov chain Monte Carlo (MCMC) methods, with at least 5 million post-burn-in generations, uniform priors, a rate deviation parameter adjusted such that acceptance rates were between 20% and 40%, and phylogenetic scaling parameters δ, κ and λ allowed to take their maximum likelihood values (*55*),(*56*). Hypothesis tests were conducted by comparing the nested null and alternative models using standard likelihood-ratio tests and Bayes factor tests (*54, 57*). For each trait, we

first estimated λ (phylogenetic signal) under both random walk and directional models of trait evolution, using the *Continuous* routine, and used log-likelihood tests to compare the maximum likelihood values for these models with those when we constrained λ to take a value of 1.0 (strong phylogenetic signal), and 0.0 (no phylogenetic signal). Maximum likelihood estimates of λ (Table S2) confirmed the need for phylogenetically-controlled analyses (*58*). The relationships between mating system and resistance traits were analysed as the phylogenetic correlation between resistance and mating system, since the binary mating system character can be considered a discrete version of an underlying continuously distributed trait (i.e., realised outcrossing rate) (*59*).

Relationships between continuous traits (resistance) were analysed as phylogenetic correlations under a directional model of trait evolution. No other study to our knowledge has examined the macroevolution of specificity, and we therefore employed a novel approach for analysing the macroevolution of herbivore-specific plant responses. Specificity was analysed by assessing the strength of the among-species correlation in inducibility to *Manduca* damage vs. inducibility to mechanical wounding (as described above). Our (null) expectation was that if there had been no evolution of specificity (i.e., if plants were incapable of differentiating real from simulated herbivory), then inducibilities would be strongly and positively correlated across the phylogeny. Conversely, under an evolution of specificity model, inducibilities to real and simulated herbivory would be expected to be evolving independently, leading to a non-significant correlation. Our primary motivation for conducting this analysis was to ascertain specifically whether SC taxa exhibited low specificity relative to SI taxa. Since using different herbivores introduces could introduce potential confounding effects (e.g., due to herbivore host range or feeding type), we used a mechanical wounding treatment. Thus, we note that this approach tests the evolution of specificity to real vs. simulated herbivory, and not specificity to different herbivores (a separate question). To analyse the relationship between constitutive and inducible resistance strategies,

we tested the significance of the phylogenetic relationship within each mating system using likelihood ratio tests, and compared their phylogenetically-corrected slope estimates using a dummy-variable approach (*60*) and a likelihood ratio test. This analysis was confirmed by a one-tailed *Z*-test that $\beta_{SI} < \beta_{SC}$ ($P = 0.06$).

Table S1. List of taxa used in experiments, with breeding system information (SC = self-compatible; SI = self-incompatible).

Species	Mating system
Atropa belladonna	SC
Brugmansia suaveolens	SI
Capsicum cardenasii	SI
Capsicum eximium	SC
Capsicum frutescens	SC
Cestrum nocturnum	SC
Cestrum parqui	SI
Datura stramonium	SC
Hyoscyamus niger	SC
Lycium cestroides	SI
Lycium exsertum	SC
Nicandra physalodes	SC
Nicotiana alata	SI
Nicotiana attenuata	SC
Nicotiana forgetiana	SI
Nicotiana longiflora	SC
Nicotiana tomentosa	SI
Nicotiana tomentosiformis	SC
Petunia axillaris	SI
Petunia exserta	SC
Petunia inflate	SI
Physalis floridana	SC
Physalis longifolia	SI
Physalis peruviana	SC
Solanum abutiloides	SC
Solanum amotapense	SC
Solanum aviculare	SC
Solanum basendopogon	SI
Solanum betaceum	SC
Solanum brevidens	SC
Solanum bulbocastanum	SI
Solanum caripense	SI
Solanum carolinense	SI
Solanum cheesmaniae	SC
Solanum clarum	SI
Solanum conocarpum	SI
Solanum corymbiflorum	SI
Solanum demissum	SC

Solanum dulcamara	SC
Solanum glaucophyllum	SI
Solanum havanense	SC
Solanum jamesii	SI
Solanum jasminoides	SI
Solanum lycopersicoides	SI
Solanum macrocarpon	SC
Solanum morrellifome	SC
Solanum neorickii	SC
Solanum nigrum	SC
Solanum ocranthum	SI
Solanum penellii	SI
Solanum pseudolulo	SC
Solanum pyracanthos	SC
Solanum quitoense	SC
Solanum sisymbriifolium	SC
Solanum suaveolens	SC
Solanum trachycarpum	SC
Solanum verrucosum	SC
Solanum chomatophilum	SI
Tubocapsicum anomalum	SC

Table S2. Maximum likelihood estimates of λ (phylogenetic signal) for plant resistance traits, *P*-values of log-likelihood tests comparing a model with λ_{ML} to models in which λ was constrained to be either 0 or 1, and a tentative summary interpretation of the results.

Trait	λ_{ML}	$P_{\lambda > 0}$	$P_{\lambda < 1}$	Interpretation
Constitutive resistance	0.53	0.031	0.078	Moderate phylogenetic signal
Herbivore-induced resistance	0.49	0.023	0.238	Moderate phylogenetic signal
Wounding-induced resistance	0.70	0.014	0.211	Moderate phylogenetic signal
Inducibility (herbivore)	0.38	0.233	0.036	Weak phylogenetic signal

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