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ORIGINAL PAPER

Induced responses in *Ipomoea hederacea*: simulated mammalian herbivory induces resistance and susceptibility to insect herbivores

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Abstract Multispecies interactions between plants and natural enemies are ubiquitous, and often lead to diffuse interactions between plants and their herbivores. Non-specific induced responses, where responses induced by one species affect other species, are one potential mechanism generating diffuse interactions. Using 57 inbred lines of the Ivyleaf morning glory, *Ipomoea hederacea*, in a greenhouse experiment, we examined whether simulated mammalian herbivory induced responses that could affect plant resistance to the generalist insect herbivore, *Spodoptera exigua*. Inbred lines were highly variable for induced responses, ranging from induced resistance to induced susceptibility, with the rank-order for resistance in inbred lines changing between clipping and control treatments. We failed to detect significant genetic correlations between induced responses and trichome density, or that clipping modified the negative relationship between trichome density and *Spodoptera exigua* consumption and biomass. Our results suggest that non-specific induced responses can mediate the diffuse evolutionary relationship between *I. hederacea* and its herbivores, and that genetic variation in induced responses are an important component of this interaction.

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Introduction

Multi-species interactions are ubiquitous in natural communities, and their presence poses a significant challenge to understanding the ecological and evolutionary dynamics of subsets of the community (Wootton 1994; Abrams 1995; Inouye and Stinchcombe 2001; Strauss and Irwin 2004). Numerous factors influencing the interactions between species—for instance, their densities, the specific traits that affect the interaction and the strength of natural selection on those traits—can depend on the presence or absence of other species in the community. For example, nectar-robbing bees are typically thought to have negative effects on plants, but in some situations they may have indirect positive effects on plant fitness, if it leads to the recruitment of ant species that deter feeding by herbivores (Newman and Thomson 2005). Similarly, if the relationship between two species is mutualistic, the addition of a third species can reduce the reciprocal benefits of mutualism (Gange et al. 1999). The result is a complex pattern of direct and indirect interactions in the community, making it difficult to both understand and predict ecological and evolutionary dynamics (Inouye and Stinchcombe 2001; Wootton 2002).

The importance of multi-species interactions in affecting the evolutionary dynamics of plant-herbivore interactions has recently been demonstrated in several studies (Pilson 1996; Agrawal 2000; Stinchcombe and Rausher 2001, 2002; Hufbauer and Root 2002; Tiffin 2002; Lau 2006). A common result to these studies is that the pattern of natural selection imposed by herbivores is often dependent on the presence or absence of other species in the community. For example, Stinchcombe and Rausher (2001, 2002) found that the strength of directional selection on deer resistance and tolerance in *Ipomoea hederacea* changed depending on the presence or absence of insect herbivores. Similarly, Tiffin (2002) found that the presence of plant competitors changed selection patterns on insect resistance. These studies suggest that diffuse selection—that is selection that depends on community context (Iwao and Rausher 1997)—may be common in many plant-herbivore systems, and that any coevolution occurring between these plants and herbivores is likely to be diffuse (Janzen 1980; Rausher 1996).

Induced responses to herbivory are one potential mechanism mediating diffuse interactions between plants and multiple herbivore species (Van Zandt and Agrawal 2004a). If the effects of induced responses are species-specific (del Campo et al. 2001; Van Zandt and Agrawal 2004b; Viswanathan et al. 2005), and only serve to reduce the feeding and performance of the original herbivore that produced the response, induced responses should have no effect on other subsequently feeding herbivore species. Such species-specificity of induced responses would not lead plants to have diffuse evolutionary relationships with several herbivore species. In contrast, if induced responses are general or affect many species at once, there is the potential for induced responses to one species to have indirect effects on several species in the community (Agrawal 2000). There is mounting evidence suggesting that non-specific induced responses are common in nature (Bezemer and van Dam 2005), and as such, they may constitute an important mechanism creating diffuse evolutionary relationships between plants and multiple herbivore species.

Non-specific induced responses can lead to diffuse evolutionary relationships between plants and herbivores through several mechanisms. In addition to the direct effects of induction on the performance and preference of subsequently feeding herbivores, indirect effects may alter the efficacy of other defense traits. For example, physical defenses such as trichomes, spines, or leaf toughness may be more effective at deterring herbivore feeding in induced plants rather than uninduced plants, simply because herbivores encounter two different plant defenses. Another indirect effect that can lead to diffuse evolution between plants and those herbivores is through genetic correlations between defense traits against multiple herbivores in the community, (Simms and Rausher 1993; Rausher 1996; Iwao and Rausher 1997; for a review, see Strauss et al. 2005). More specifically, the presence of genetic correlations between induced responses (species-specific or general) and constitutive defenses will lead to correlated responses to selection (Agrawal et al. 1999). Accordingly, the evolution of induced responses to one herbivore species can lead to a correlated evolutionary response in defenses against other herbivores species.

Here we evaluated whether induced responses to simulated mammalian herbivory could modify the interaction between the Ivyleaf morning glory (*I. hederacea*) and insect herbivores, and whether these interactions had the potential to alter the evolutionary dynamics of insect resistance traits. Previous work by Stinchcombe and Rausher (2001, 2002) has shown that the magnitude of selection on resistance and tolerance to deer herbivory increases in the presence of insect herbivores, indicating that these two taxa of herbivores have important ecological and evolutionary impacts on *I. hederacea*. However, it remains unclear whether the presence or absence of mammalian herbivory has the potential to affect the evolutionary dynamics of plant defense against insect herbivores. While the physiological mechanisms of induction are not well known in *Ipomoea* species, their ecological consequences can be studied by evaluating their effects on the growth and performance of other herbivores in the community (Rausher et al. 1993), regardless of the precise physiological mechanism. Specifically, we sought to answer three questions: (1) Does simulated mammalian herbivory induce responses that affect insect herbivores, and is there genetic variation for these induced responses that could lead to an evolutionary response to selection? (2) Do induced changes in plant traits by mammalian herbivory alter the efficacy of trichomes as a plant defense? (3) Are there genetic correlations between induced responses and physical defenses, such as trichome density?

Materials and methods

Study system and natural history

Ipomoea hederacea (L) Jacquin (Convolvulaceae), Ivyleaf morning glory, is an annual weedy vine distributed throughout the southeastern U.S.A., where it typically occurs in open fields, disturbed habitats and cultivated gardens. Ivyleaf morning glory is self compatible, but is capable of outcrossing, typically by bee pollinators (mainly *Bombus* sp.; Ennos 1981). Ivyleaf morning glory exhibits a genetic polymorphism for leaf shape, influenced by a single Mendelian locus.

A wide range of natural enemies in the southern United States, including fungal pathogens, and insect and mammalian herbivores, damage plants. Mammalian herbivory is typically due to white-tailed deer (*Odocoileus virginianus*), and invertebrate herbivores include generalist insects: beet and southern armyworm caterpillars, *Spodoptera* spp. (Lepidoptera: Noctuidea) (Hull-Sanders, pers. comm), as well as leaf beetles (Chrysomelidae), weevils (Curculionidae), cotton aphids (Aphididae), leaf hoppers (Cicadellidae), grasshoppers (Acrididae), and specialist tortoise beetles (Chrysomelidae, subfamily Cassidinae) (Bright 1998; Stinchcombe and Rausher 2001; Hull-Sanders and Eubanks 2005).

Experimental design

Our experimental approach was to manipulate the presence or absence of simulated mammalian herbivory on *I. hederacea*, and then to analyze whether the presence or absence of such herbivory modified plant defenses against subsequent insect herbivores. We evaluated this possibility in three ways. First, we determined whether simulated mammalian herbivory led to significant induced defense against insect herbivory. Second, we evaluated whether there were significant genetic correlations between induced and constitutive defenses. Third, we asked whether the responses induced by mammalian herbivory influenced the efficacy of a constitutive defense against insect herbivory.

We utilized 57 inbred lines of *I. hederacea* that have been self-fertilized by single-seed descent for three generations. Because many populations exhibit high selfing rates (Ennos 1981), the use of inbred lines is an appropriate mimic of the mating system typically found in natural populations. The inbred lines exhibited frequencies of entire and lobed leaf shaped morphs (11 and 89% respectively) similar to those observed in natural populations by Bright (1998).

We used five replicates per inbred line; each replicate was placed in an individual pot, randomly distributed in a single greenhouse room. We randomly assigned three of the five replicates for each inbred line to a simulated mammalian herbivory treatment. After 70 days of plant growth, we imposed simulated mammalian herbivory by removing 50% of the leaves from each plant with scissors. We clipped leaves on the leaf petiole, roughly 3 cm from the stem. Because saliva from browsing mammals can significantly affect regrowth characteristics of damaged plants (e.g., Bergman 2002), one of us (AKS) licked the scissors between every cut. We initially attempted to obtain Cervid saliva from a nearby reindeer farm, although this proved to be impossible without causing undue distress to the animals. We elected not to include a treatment of clipping without saliva for three reasons: (1) Our goal was not to distinguish the proximate mechanism of whether it was physical damage or damage and saliva that induced responses, but rather to study their potential ecological effects on other species (2) Purely mechanical damage that removes whole leaves, without saliva, does not occur naturally in *I. hederacea*, unlike other forms of herbivore damage (e.g., apical meristem damage) that can be mimicked by fire, frost, flooding and other abiotic factors, and (3) Past studies, albeit in a field setting and with different methods, failed to detect an effect of purely mechanical damage on either plant fitness or subsequent herbivore damage (Stinchcombe 2002). Although human and deer saliva clearly differ, we believe our approach is a reasonable compromise between the inclusion of saliva, which is likely to be ecologically important in this system, and the demands of a randomized design in which plants are individually exposed to herbivory treatments. Our experimental protocol mimics not only how deer typically feed on morning glory (removing whole leaves at a time), but also its intensity: damage levels by deer in experimental populations of *I. hederacea* commonly approach 50% (Stinchcombe and Rausher 2001).

After 95 days of growth (25 days after the simulated herbivory treatment), we conducted a single bioassay on each plant using beet armyworm, *Spodoptera exigua* to estimate resistance to leaf damage by the caterpillar, and induced responses due to prior herbivory. We used first instar larvae, following standard methods (e.g., Barrett and Agrawal 2004; McGuire and Johnson 2006), to avoid potentially confounding effects from rearing insects on artificial diet and switching them to morning glory leaves at later instars. To conduct the bioassay, a newly hatched caterpillar was given two leaf cuttings using a manufactured stamp (McGill Craftivity; part # 95901) with a fixed area of 5.6 cm^2, taken from two leaves of the same plant. *Spodoptera exigua* were obtained from Benzon Research (Carlisle, Pennsylvania, USA). We chose leaves of approximately the same age, because ontogenetic stage may cause differential effects on herbivore preference (Ikonen 2002). Caterpillars were allowed to feed on the tissue for 4 days and were then removed. For simplicity, initial caterpillar weights were assumed to be constant and insect performance was estimated from the dry biomass, after drying caterpillars at 65°C for 5 days. To estimate plant resistance to insect damage, we measured the amount of leaf area damaged by using an acetate grid sheet with a resolution of 1 mm that was overlaid on the leaf.

Our estimate of a putatively constitutive resistance trait was trichome density (we also tested to see if trichomes were induced, see below). We estimated trichome density by taking three standard hole punches (1.13 cm^2 each), and counting the number of trichomes per punch. We took hole punches from the two leaves sampled for the bioassay, and an additionally sampled leaf. We used the mean from these three punches to estimate trichome density (trichomes/cm^2) for each experimental plant.

Statistical analysis

We first evaluated whether there were significant effects of simulated mammalian herbivory on trichome density, leaf damage, and insect weight gain, and whether there was genetic variation among our inbred lines in these same traits. We analyzed each of these response variables using mixed-model ANOVA (Proc Mixed; SAS Institute v9.1) The full model included treatment as a fixed effect and inbred line and the inbred line* treatment interaction as random effects. The significance of random effects was estimated with log likelihood ratio tests by comparing the—2 log—likelihoods of models with and without random effects. Specifically, differences in log likelihoods were tested with a 1-tailed χ^2 test with 1 degree of freedom (1-tailed because variances cannot be less than zero; Littell et al. 1996). To improve the normality of residuals, leaf damage, trichome density, and insect weight gain were ln(y+1) transformed prior to these analyses. In these models, significant treatment effects indicate that simulated mammalian herbivory led to induced responses in the plant that affected insect resistance traits. Similarly, significant inbred line or the inbred line*treatment terms indicate significant genetic variation for the trait, or that the inbred lines differed significantly in their response to the treatment, respectively.

We also tested whether the presence or absence of simulated herbivory treatment modified or altered the effectiveness of trichome defenses. Using a general linear model (GLM), we tested for significant effects of trichome density, the herbivory treatment, and the trichome density*treatment interaction on log(y+1) transformed leaf consumption and insect biomass. These analyses were performed on inbred line means, to reduce the effects of micro-environmental variation in the greenhouse (Rausher 1992; Stinchcombe et al. 2002).

To determine whether plant responses induced by simulated mammalian herbivory were correlated with constitutive defenses, we examined the correlation between trichome density and two estimates of induced responses. The first estimate of induced responses was the difference in leaf damage between clipped and control plants for each inbred line. The second estimate was the difference in insect weight gain between insects reared on leaf discs gathered from clipped and control plants. For both of these measures, we used the difference in inbred line means (i.e., the mean leaf damage and insect weight gain for control plants was subtracted from the means for clipped plants for each inbred line). We then examined the Pearson correlation between these two measures of induced responses and inbred line mean estimates of trichome density.

Results

Induced responses and genetic variation in plant defenses

The simulated herbivory treatment, by itself, did not have significant main effects on trichome density, leaf damage by caterpillars, or insect weight gain ($F_{1,56} < 0.66$, $P > 0.42$ for all three traits), suggesting that there was no consistent overall effect of simulated mammalian herbivory on these insect resistance traits.

The putatively constitutive resistance trait we examined, trichome density, showed no evidence of being induced by simulated mammalian herbivory (Treatment effect: $F_{1,56} = 0.04$, $P = 0.84$; Inbred Line * Treatment: $\chi^2 = 0$, $P > 0.99$). Based on these results, we refer to trichome density as a constitutive trait for the remainder of the paper.

Trichome density, leaf damage by caterpillars, and insect weight gain all exhibited significant genetic variation among the inbred lines ($\chi^2 > 14$, $P < 0.001$ for all three traits). The consequences of simulated mammalian herbivory for leaf damage by caterpillars and insect weight gain also differed among the inbred lines used in the experiment, as indicated by significant Treatment * Inbred line interactions (leaf damage; $\chi^2 = 3.7$, $P = 0.0272$ and insect weight gain $\chi^2 = 4$, $P = 0.0228$; Fig. 1A and B). These data suggest the inbred lines are variable in their induced responses to simulated mammalian herbivory. Plots of the reaction norms for these traits (Fig. 1A and B) suggest that some inbred lines exhibit induced resistance (i.e., increased resistance to leaf damage and decreased insect weight gain) while others exhibit induced susceptibility to damage.

Effects of trichome density on insect consumption and performance

Generally, trichomes had a strong negative effect on insect consumption and performance—insects feeding on leaf cuttings from inbred lines with higher densities of trichomes consumed less leaf area and gained less dry biomass (log-transformed leaf damage: $F_{1,110} = 11.56$, $P = 0.0009$; log-transformed insect dry biomass; $F_{1,110} = 12.28$, $P = 0.007$; Fig. 2A and B). Simulated mammalian herbivory did not significantly alter effects of trichomes on insect consumption or performance, as indicated by the absence of a significant trichome density*treatment interactions ($F_{1,110} < 0.62$, $P > 0.433$ for both; Fig. 2A and B).

Correlations between induced and constitutive responses

The two estimates of induced responses—changes in leaf damage and changes in insect dry biomass between control and clipped plants of the same inbred line—were highly correlated ($r = 0.88$, $P < 0.0001$). However, neither estimate was correlated with trichome density (induced leaf damage responses: $r = 0.14$, $P = 0.31$; induced insect biomass responses: $r = 0.08$, $P = 0.55$).

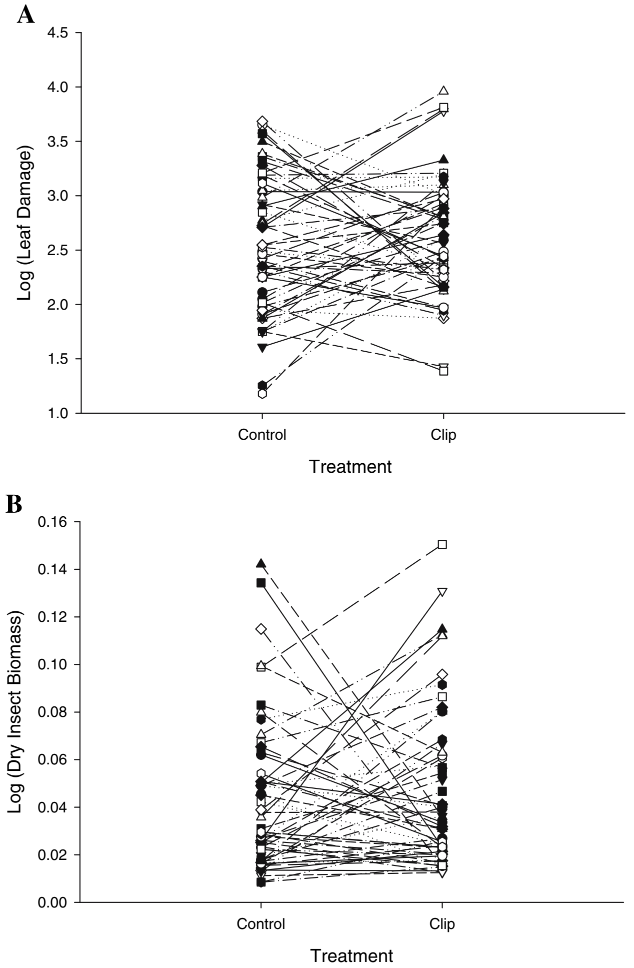

Fig. 1 Reaction norms illustrating genetic variation in induced responses to mammalian herbivory, using total leaf area damaged (mm^2) (**A**) and performance measured by weight gain (mg) (**B**) of *Spodoptera exigua*, a generalist insect herbivore. Plotted lines connect the same inbred line in each treatment

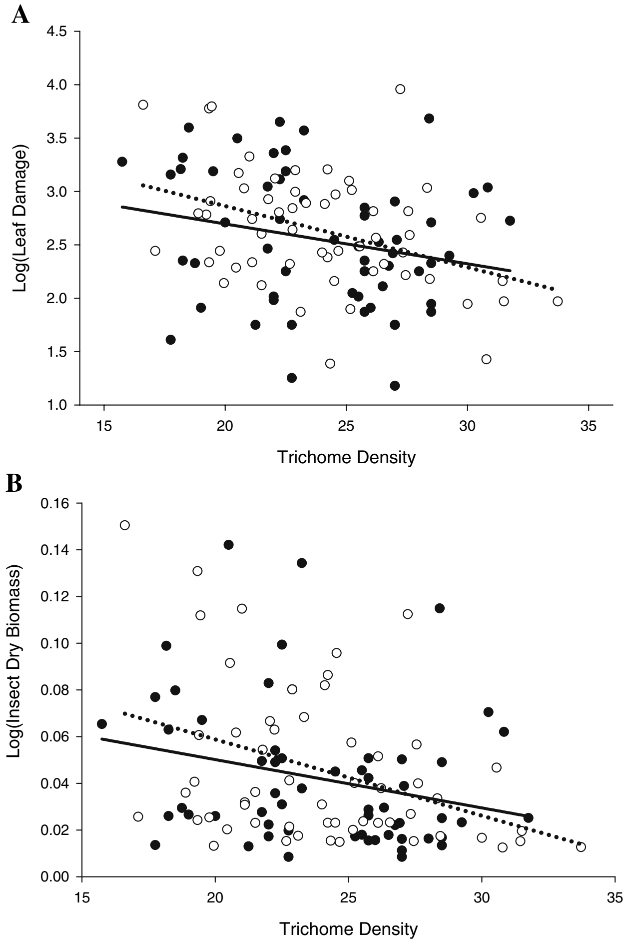

Fig. 2 The relationship between trichome density ($trichomes/cm^2$) and insect consumption (mm^2) (**A**) and performance, measured by weight gain (mg) (**B**), of *Spodoptera exigua* on plants that underwent simulated mammalian herbivory (clipped treatment, open circles) and undamaged plants (control, solid circles). Plotted points are inbred line means, estimated in each treatment

Discussion

Our study suggests that mammalian herbivory has the potential to modify the evolutionary interaction between *I. hederacea* and generalist insects but that a constitutive resistance trait, trichome density, is uncoupled from this modification. Three main results support this interpretation. First, we found that simulated mammalian herbivory did not consistently decrease or increase generalist insect performance or subsequent herbivore damage, but that there was significant genetic variation for induced responses to herbivory that included induced resistance and induced susceptibility. To our knowledge, this is the first report of genetic variation for responses induced by simulated mammalian herbivory that affect later-feeding herbivores. Second, damage by simulated mammalian herbivory does not affect the ability for a constitutive defense, trichomes, to reduce insect damage, nor to reduce insect performance. Third, the induced plant response is not correlated with one constitutive defense, trichome density. Below, we consider the implications of these results for understanding the potential for indirect effects between mammalian herbivores and insects, and the effects of mammalian herbivory on the efficacy of plant defense against insect herbivores.

The effects of mammalian herbivory induction on insects

Mammalian herbivory can lead to both positive and negative effects on subsequently feeding insect herbivores. For instance, there are several reports of increased insect densities, feeding, or survival on previously browsed plants (Danell and Huss-Danell 1985; Haukioja et al. 1990; Hjalten and Price 1996; Roininen et al. 1997; Martinsen et al. 1998; Olofsson and Strengbom 2000; Bailey and Whitham 2006) or plants that suffered shoot damage from abiotic disturbances (e.g., Nakamura et al. 2005). In contrast, there are also several reports of decreased insect damage or density on previously browsed plants (Shimazaki and Miyashita 2002; Bailey and Whitham 2003; den Herder et al. 2004). Still other studies report a mixture of positive and negative effects of mammalian browsing on subsequently feeding insect herbivores (Roininen et al. 1994; Riipi et al. 2005). A recent meta-analysis of 68 studies suggested that, across all studies, prior browsing or clipping led to positive effects on insect pupal mass, but has no overall effect on insect consumption or survival (Nykanen and Koricheva 2004).

The results of our study indicate that simulated mammalian herbivory did not produce a general, ecological effect of either increasing or decreasing insect herbivore consumption or biomass. Although we utilized simulated mammalian herbivory in our experiment, our findings follow the general trend found in many other studies (see above). However, our results clearly reveal significant genetic variation among inbred lines in their induced responses: some inbred lines showed induced resistance, while other lines showed induced susceptibility. The effects of genetic variation in our experiment were large, in that clipping produces nearly 4-fold variation in the performance of insect herbivores. Our results thus raise the possibility that previous findings of weak or non-significant overall effects of induced responses on subsequent herbivores species may simply reflect the inability or failure of previous studies to account for genetic variation in induced responses. In statistical terms, past studies may have failed to detect a main effect of herbivory or herbivore treatment on the performance of subsequent herbivores because of a failure to account for Genotype * Herbivore treatment interactions. Thus while the exact nature of induced responses may differ between natural and artificial herbivory, or due to differences between human and deer saliva, we suggest that our findings of the importance of genetic variation and G * E interactions demonstrate, *in principle*, the need to incorporate these factors into studies of induced responses and the ecology of plant-mammal-insect interactions.

The genetic variation in induced responses we detected has several important implications. First, the heterogeneity of effects of browsing on subsequently feeding insect herbivores (described above) could be, in part, due to differences in genetic structure of populations that have been sampled by investigators. Thus, in addition to traditional ecological and behavioural influences on the effects of browsing—e.g., timing and intensity of damage, frequency of browsing (Danell et al. 1985, 1994)—our data strongly suggest the need to consider explicitly the effects of genetic variation in studies of indirect interactions between herbivores. Although genetic variation in induced responses has previously been detected in a variety of systems in response to insect herbivory (Siemens and Mitchell-Olds 1998; Agrawal et al. 2002; Cipollini et al. 2003; Agrawal 2004; Barrett and Agrawal 2004; Agrawal 2005; McGuire and Johnson 2006), all of these studies examine the effects of induction by insects on other species of insects. To our knowledge, genetic variation in responses induced by simulated or natural mammalian herbivory on insect herbivore feeding and performance has not previously been reported.

The presence of genetic variation in induced responses in *I. hederacea* has two important implications for how mammalian herbivory can potentially affect the evolution of plant defense against insect herbivores. First, mammalian herbivory induced increased or decreased insect resistance in many inbred lines, and as such, the subsequent pattern of selection on insect resistance may change depending on the presence or absence of mammalian herbivory. (Fig. 1A and B). Second, this data suggest that selection on induced responses to mammalian herbivory has the strong potential to affect insect herbivore performance. Thus, the evolution of induced responses to mammalian herbivores will have cascading effects on the performance of insect herbivores, due to the non-species specific nature of the induced response. These data, when coupled with the results of Stinchcombe and Rausher (2001, 2002), suggest that *I. hederacea* has a diffuse (co)-evolutionary relationship with its mammalian and insect herbivores. Interestingly, the presence or absence of fungal and oomycete pathogens does not affect the evolutionary dynamics of plant defense against deer (Stinchcombe and Rausher 2001, 2002).

Much of the past work on indirect effects of mammalian herbivory has focused on woody plants (e.g., *Salix*: Hjalten and Price 1997; Roininen et al. 1997; Olofsson and Strengbom 2000; Bergman 2002; Bailey and Whitham 2003; den Herder et al. 2004; Hochwender et al. 2005; Nakamura et al. 2005; *Populus*: Martinsen et al. 1998; Bailey and Whitham 2006; *Betula*: Danell and Huss-Danell 1985; Riipi et al. 2005; *Viburnum*: Shimazaki and Miyashita 2002). Despite the importance of mammalian herbivores, especially white-tailed deer (*Odocoileus virginianus*) in North America, for the population dynamics of herbaceous plants (e.g., Miller et al. 1992; Vellend et al. 2003; McGraw and Furedi 2005), very little is known about the indirect ecological effects produced by browsing on herbaceous plants (Rooney and Waller 2003). Our study, albeit utilizing simulated herbivory, suggests strong potential for indirect effects on insect populations of browsing on herbaceous plants.

The effects of mammalian herbivory on the function and evolution of trichome defense

Ipomoea hederacea was found to have genetic variation for trichome density, and increased trichome densities decreased both the amount of leaf area consumed and the amount of mass gained by *S. exigua*. These data suggest that selection on trichome number, for instance, to reduce the amount of insect herbivore damage (e.g., Mauricio and Rausher 1997), has the potential to lead to reciprocal selection on caterpillar traits.

Our study showed that the efficacy of plant defense against *S. exigua* herbivory provided by trichomes does not change when *I. hederacea* is damaged by mammalian herbivory (see Fig 2). In other words, trichomes are no more or less effective in deterring herbivory or decreasing herbivore performance depending on whether or not plants have been previously damaged. In addition, the absence of a genetic correlation between induced responses to mammalian herbivory and trichome (a physical defense) suggests that these two traits can evolve independently from each other. Our results are similar to findings on black mustard, *Brassica nigra*, which found no genetic correlation between constitutive trichome defense and induced physical and chemical defense traits (Traw 2002). Our data strongly suggest that the indirect effects of mammalian herbivory on insect performance are not mediated by trichomes.

Conclusions

Our study suggests that *I. hederacea* is likely to have a diffuse interaction with its herbivore natural enemies, and thus is consistent with previous studies documenting diffuse selection and in plant-herbivore systems (Pilson 1996; Juenger and Bergelson 1998; Stinchcombe and Rausher 2001; Stinchcombe and Rausher 2002; see Strauss and Irwin 2004; Strauss et al. 2005 for reviews). Our results also suggest that induced responses have the potential to be one of the mechanisms underlying these diffuse relationships. However, we note that we did not specifically measure natural selection on resistance to insect herbivores (i.e., the relationship between plant fitness and resistance) in the presence and absence of mammalian herbivory, or natural selection on insect herbivore traits. As such, our results only indicate the strong potential for diffuse patterns of selection mediated by induced responses in *I. hederacea*, and future work will have to examine these possibilities explicitly in more ecologically realistic conditions.

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