Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores

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ABSTRACT

Hypothesis: Genetic variation in plant defence structures a community of herbivores and ultimately mediates co-evolution.

Organisms: Common milkweed (*Asclepias syriaca*) and five natural insect herbivores (seed bug, leaf mining fly, monarch caterpillar and two beetles).

Methods: Quantitative genetic field experiment over 2 years, genetic selection analyses, and measurement of five defensive traits (cardenolides, latex, trichomes, leaf toughness and nitrogen content).

Results: All plant traits were genetically variable; directional selection favoured resistance to herbivory, latex production and the nitrogen content of leaves. Trichomes and latex were each negatively genetically correlated with abundances of herbivores, but not with herbivore damage; cardenolides and induced plant resistance were negatively genetically correlated with growth of monarchs.

Conclusion: Selection for plant defence was influenced by: (1) genetic correlations in plant susceptibility to multiple herbivores; (2) resistance traits affecting some, but not all, aspects of the insect community; and (3) early season herbivory inducing changes in milkweed influencing later season herbivores.

Keywords: cardenolides, genetic selection analyses, latex, monarch butterfly, plant–insect interactions, quantitative genetic field experiment, trichomes.

INTRODUCTION

Co-evolution is the microevolutionary process whereby reciprocal natural selection results in the evolution of adaptations in interacting species (Janzen, 1980). Such co-evolutionary interactions are thought to have shaped the defensive and offensive traits of many species, especially in plant–herbivore interactions (Ehrlich and Raven, 1964; Thompson, 1999; Karban and Agrawal, 2002; Zangerl and Berenbaum, 2003). It is increasingly recognized, however, that in systems where plants interact with multispecies assemblages of herbivores, co-evolution is more likely to be

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diffuse rather than the simple pair-wise manner typically envisioned between two species (Fox, 1981; Linhart, 1991; Poitrineau *et al.*, 2003). In this study, I estimate selection on plant defence by a community of insect herbivores and the effects of putative defensive traits on each of the species. Community-wide covariances among plant attackers suggest that herbivores may not be acting independently in their impact on plants (Maddox and Root, 1990; Roche and Fritz, 1997; Dungey *et al.*, 2000; Whitham *et al.*, 2003).

I conducted a quantitative genetic field experiment with common milkweed, *Asclepias syriaca*, measured five putative defensive traits, the abundance or performance of five herbivores, and plant fitness components after 2 years of growth. Few studies to date have examined the reciprocal effects between plants and herbivores mediated by defensive traits in a community context (Hare and Elle, 2002). Although I did not specifically estimate diffuse selection (Iwao and Rausher, 1997; Stinchcombe and Rausher, 2001), my goal was to conduct an ecological genetic exploration of the reciprocal interactions between a toxic host plant and its specialist herbivores. I tested some of the key assumptions of the diffuse co-evolution hypothesis: (1) that herbivore species covary in their abundances on particular plant genotypes; (2) that damage early in the season affects later season herbivores; and (3) that particular defensive traits simultaneously affect the community of attackers.

In addition, I employed genetic selection analyses (Rausher, 1992), because traditional estimates of phenotypic selection (Lande and Arnold, 1983) may be biased. The traditional approach is susceptible to environmental effects whereby trait values and fitness covary due to the environment (i.e. resource availability) (Rausher, 1992). Genetic selection analyses employ genetic family means as opposed to individual trait values in multiple regression analyses, thereby reducing this bias. Although this approach is highly limited by a reduced sample size (number of families not individuals), genetic selection analyses are superior to traditional estimates of phenotypic selection because they are more likely to reflect the response to selection (Mitchell-Olds and Shaw, 1987; Rausher, 1992; Stinchcombe *et al.*, 2002).

Thus, in this study I asked the following specific questions: (1) What is the strength of natural selection on plant resistance to a community of herbivores? (2) Which plant traits correlate genetically with the abundance and performance of the community of specialist herbivores naturally attacking milkweed?

MATERIALS AND METHODS

Natural history

This study was conducted at the University of Toronto's Koffler Scientific Reserve at Jokers Hill, in southern Ontario (44°03′N, 79°29′W; http://www.zoo.utoronto.ca/jokershill, hereafter Jokers Hill). At the site, common milkweed (*Asclepias syriaca*) is abundant in old-field habitats. *Asclepias syriaca* is a native perennial plant that reproduces by clonal production of underground stems and by sexual reproduction via hermaphroditic flowers (Woodson, 1954). Seeds from a single fruit of *A. syriaca* are typically full-siblings because of the pollinia pollination system of milkweeds (i.e. a single pollen sac or pollinium sires all the seeds in a follicle) (Gold and Shore, 1995).

Milkweed's well-known toxicity and arsenal of defences are thought to limit the herbivore community to about 10 species of mostly host-specific insects (Malcolm 1991; Agrawal and Malcolm, 2002). In this study, I focus on herbivory by five of the most abundant milkweed herbivores at my study site, each from a different feeding guild (Fig. 1). The first species is the monarch

Fig. 1. Images of the typically most abundant insect herbivores on common milkweed (*Ascelpias syriaca*) in southern Ontario. From left to right: first instar Monarch larva (*Danaus plexippus*), red milkweed beetle (*Tetraopes tetraophthalmus*), stem weevil (*Rhyssomatus lineaticollis*), small milkweed bug (*Lygaeus kalmii*) and the splotch mine of the milkweed leaf miner (*Liriomyza asclepiadis*). Aphids, which can be abundant in some years, were not seen in the present study.

butterfly (*Danaus plexippus*, Lepidoptera), whose caterpillars are foliage feeders of many milkweed species. The second species, *Rhyssomatus lineaticollis* (Coleoptera), are weevils that primarily feed on the stems of *A. syriaca*. Adults initially feed on the apical leaves and then, after feeding, female weevils walk to lower parts of the stem and chew several sequential holes in the stem, creating a continuous scar. Females lay a single egg per hole and larvae complete development inside the stem while feeding on pith tissue. Oviposition scar length is an accurate predictor of the number of eggs laid by the adult female ($n = 155$, $r = 0.88$, $P < 0.001$) (Agrawal and Van Zandt, 2003). The third species is the red long horn beetle (*Tetraopes tetraophthalmus*, Coleoptera), which can be very dense (upwards of 20 adults per square metre of milkweed plants). Adults feed on leaves and flowers, while larvae burrow down in the soil and feed on milkweed roots. The fourth species, the small milkweed

bug, *Lygaeus kalmii*, is a sap-feeding herbivore that feeds gregariously in the nymphal stage, and is frequently found feeding on milkweed pods. Finally, one of the least well-studied herbivores of milkweed is the leaf mining fly, *Liriomyza asclepiadis*, which can be very abundant (tens of miners per plant) and produces splotch mines up to 4 cm^2 on leaves.

Probably the two most potent aspects of plant resistance in milkweed are the production of cardenolides (cardiac glycosides) and latex. Cardenolides are bitter-tasting steroids that occur in all milkweed tissues, including the latex, act by disrupting the sodium and potassium flux in cells, and have toxic effects on most animals (Malcolm 1991; Fordyce and Malcolm, 2000). The sticky white latex is delivered via specialized canals (laticifers) to most plant parts, and is copiously exuded upon damage to the tissues. The latex of milkweed has been strongly implicated as a physical trait that impedes feeding by herbivores (Dussourd and Eisner, 1987; Dussourd, 1999). Other potentially defensive and nutritional constituents that may influence herbivory include leaf toughness (Coley, 1983; Kause *et al.*, 1999), trichome density (Hulley, 1988; Mauricio and Rausher, 1997) and nitrogen content (Mattson 1980; Lavoie and Oberhauser, 2004).

Common garden study

To study the genetic basis of milkweed resistance traits and their relationship with attack by herbivores, I established a common garden in 2001 employing 26 full-sibling families of *A. syriaca*. Families were established from seeds of single fruits collected from plants across a transect of the 350 ha Jokers Hill Reserve. No two families were established from the same parent plant.

The tips of approximately 40 seeds per family were nicked, germinated on moist filter paper in petri dishes, and grown in 500 ml plastic pots with Pro-Mix BX soil (Red Hill, PA) and about 0.6 g of slow-release Nutricote fertilizer (13:13:13 N:P:K) (Vicksburg Chemical, Vicksburg, MS). These plants were grown for 1 month in growth chambers (16 h/8 h light/dark cycle, 28°C/24°C temperature cycle) before planting them in the field. Seedlings were planted into a ploughed field at Jokers Hill in 4 litre plastic pots with field soil on 1 July 2001. Each pot was completely sunk into the ground and the plants were fully randomized within the common garden. Pots were used to restrain lateral spread of each clonal plant; in the second year of growth, milkweed plants typically emerge as multiple stems, sometimes over 1 m away from the original plant (personal observations). The ground around the pots was covered with a thin (water-permeable) sheet of landscaping fabric to reduce weeds.

A total of 530 plants from 23 families were included in the final analysis (where five or less plants survived, the family was not included in the analysis). On average, full-sib families were represented by 23 ± 2 (mean ± standard error, SE) individual plants per family.

Measures of plant resistance traits

I measured five plant traits that are potentially associated with resistance to insects: cardenolide concentration, latex production, leaf toughness, foliar trichome density, and nitrogen content of leaves. All measures were taken from a newly expanded leaf of plants in the common garden. Cardenolide concentrations were measured in 2002 as digitoxin equivalents (grams per gram dry tissue) extracted from 50 mg dry leaf tissue; I employed a spectrophotometric assay modified from Brower *et al.* (1972), following Nelson (1993). I adapted the assay for sampling using a microplate reader (PowerWave X, Bio-Tek

Instruments, Winooski, VT) and sampled 5–10 randomly selected replicates from each plant family ($n = 214$, mean $\pm$ SE $= 9.3 \pm 0.2$ replicates per family). Field-collected leaf tissue was kept on ice, then frozen, freeze-dried, ground with a mortar and pestle, and weighed in 2 ml boil-proof tubes (MCT 200-C, AXYGEN Scientific, Union City, CA). To each tube, I added 1.9 ml of 95% ethanol; the tubes were then vortexed, and floated in a sonicating water bath (65°C) for 10 min. I then centrifuged the tubes at 5000 rev$\cdot$min^{-1} for 5 min at room temperature. Two 45 μl aliquots of the supernatant from each tube were then pipetted into the wells of a 96 well-plate, one above the other in two rows (reacted sample and blank, respectively). Each plate also contained six samples of digitoxin for the standard curve used to determine concentrations of cardenolides (Sigma Chemical Co., 0.125–3.0 mg$\cdot$ml^{-1}). I then added 90 μl of ethanol to the blanks and 90 μl of 0.15% 2,2′4,4′-tetranitrodiphenyl (TNDP) in ethanol to the active samples. Finally, 70 μl of 0.1 mol$\cdot$l^{-1} aqueous NaOH was added to all wells to make the solutions basic and to catalyse the colorimetric reaction. After 15 and 20 min, all wells in the plate were read at 620 nm on the microplate reader.

I measured latex from all plants in both 2001 and 2002 ($n = 530$) by cutting the tip off (0.5 cm) of an intact leaf in the field and collecting the exuding latex onto a 1 cm disc of filter paper (#1 Whatman International, Maidstone, UK). Latex stopped flowing after $\approx$10 s, all latex was absorbed on the filter paper, and this disc was placed on top of another dry filter paper disc in a 24 well-plate. The discs were dried at 60°C and then weighed to the nearest microgram. This method is a repeatable method for determining latex exudation (see Results). In addition, this measure of latex production likely reflects what feeding insects must contend with, and has been shown to correlate negatively with the growth of milkweed herbivores (Van Zandt and Agrawal, 2004a; A.A. Agrawal, unpublished data).

Trichome density on the leaves was assessed on all plants ($n = 530$) in 2002 by counting the tops and bottoms of leaf discs (28 mm^2) under a dissection microscope. Leaf discs were taken from the tips of leaves. I measured leaf toughness on all plants in both 2001 and 2002 with a force gauge penetrometer (Type 516, Chatillon Corp., NY) that measures the grams of force needed to penetrate a surface. I sandwiched the leaf between two pieces of plexiglass, each with a 0.5 cm hole, pushed the probe of the penetrometer through the leaf, and recorded the maximum force required for penetration. For each leaf, I measured toughness on each side of the mid-rib; these two measures were averaged and used as a single data point per plant. Finally, total leaf nitrogen concentration was measured for 5–10 replicates from each plant family ($n = 219$, mean $\pm$ SE $= 9.5 \pm 0.3$) in 2002 by microcombustion, using 5 mg of dried ground leaf material in an Elemental Combustion System 4010, CHNS-O analyser (Costech Analytical Technologies, Valencia, CA).

Herbivory measures

In August 2001 and 2002, I measured the percentage of leaves on all plants ($n = 530$) that had foliar damage due to chewing herbivores. I employed this rapid assay for assessing leaf damage because plants were very large (mean $\pm$ SE $= 80 \pm 2$ leaves per plant in 2002). For each plant, the total number of leaves that had greater than 1 cm^2 removed by a chewing herbivore(s) was divided by the total number of leaves to obtain a herbivory index. In August 2002, I measured the length of stem scars imposed by *Rhyssomatus* as a measure of the number of eggs deposited (and larvae in stems).

In July 2002, all plants were censused for adult *Tetraopes* three times over a 2 week period. I report effects on the sum of *Tetraopes* observed on plants over those three dates. *Tetraopes* adults are very mobile and were dense in the study plot; thus, it is unlikely that individuals remained on plants over the censuses and were double counted. An analysis of the average number of *Tetraopes* is statistically identical. In late July, in a single census, I counted the number of adult *Lygaeus* and leaf miners per plant.

Danaus was rare in the summer of 2002 at my study site, probably due to the catastrophic die-off of over-wintering adults in Mexico (Brower *et al.*, 2004). To assess the effects of genetic variation in milkweed on *Danaus*, I conducted a bioassay by placing a freshly hatched caterpillar on all plants ($n = 530$) in the field on 12 August 2002. Caterpillars were from a laboratory colony established from local individuals collected in summer 2001 and maintained on frozen milkweed foliage. Each caterpillar was caged in a spun polyester bag (Rockingham Opportunities Corporation, Reidsville, NC) on the apical meristem with four fully expanded leaves. After 5 days, I collected each caterpillar and weighed the fresh mass of all living individuals.

Plant fitness measures

When plants began to senesce in late August 2002, I harvested all fruits and above-ground vegetative parts. These materials were dried in a large forced air oven at 60°C and were measured for: (1) above-ground vegetative biomass, (2) number of fruits and (3) dry mass of fruits as components of plant fitness.

Statistical analyses

I used one-way analysis of variance to test for genetic variation in the defensive, herbivory and fitness-related traits. I calculated a full-sib heritability for each of the traits by dividing two times the variance component of family by total variance [estimated in SAS Proc Mixed (Littell *et al.*, 1996)]. Full-sib heritabilities equal narrow-sense heritabilities when all genetic variance is additive, and are typically lower than broad-sense heritabilities (Roff, 1997). Family was the only factor included in the model (random effect). These analyses examine multiple response variables (i.e. 15 heritabilities); I use the binomial expansion test (Zar, 1996) to examine how many heritabilities would be expected to be significant by chance alone. To assess the year-to-year stability of family-level variation, which is indicative of a genetic basis, I examined the family mean correlations between traits that were measured over 2 years (latex, toughness, percent of leaves damaged). Genetic correlations between all traits were also estimated by family mean correlations. Genetic correlations could not be estimated using the less biased jackknife procedure (Roff and Preziosi, 1994) because not all plant trait measures were taken from the same replicate plants.

Because my measures of the herbivore community and plant fitness traits were numerous, I used principal components analysis (PCA) to reduce the data in each of these categories separately. I employed PCA with a correlation matrix extraction method, a minimum eigenvalue of 1 for extraction, and the Varimax rotation procedure in Systat (Wilkinson, 1997). This method produces uncorrelated principal components (PCs) that maximize the loadings of variables to one axis. Variation in the abundances of the four common herbivores was largely explained by one PC factor (all loadings were positive and >0.5, in total explaining 49% of the variation). Plant fitness traits were correlated and also collapsed to one PC

factor (all loadings were positive and >0.5, explaining 73% of the variation). Separate selection analyses on components of asexual versus sexual reproduction were qualitatively similar.

Selection gradients measure the strength of direct, adaptive selection, after correcting for correlations among measured traits (Conner and Hartle, 2004). I employed Rausher's (1992) modification of Lande and Arnold's (1983) selection gradient method by using the family mean estimates for plant fitness and the plant and herbivore traits measured. Selection differentials estimate the strength of total selection, including both direct selection and indirect selection caused by correlations. Genetic correlations among traits, and between each trait and the fitness PC (analogous to selection differentials), were estimated as Pearson product–moment correlations among the family mean values. Relative values of the estimates of fitness were regressed on standardized estimates for the traits [(value – mean)/standard deviation]. The fitness and herbivore community PC values were relativized by adding the smallest possible number to all of the values to make all of these values positive, and then dividing by the new mean; this transformation does not affect the P-values of the regressions and is necessary because PC factors are standardized to a mean of zero. Directional selection gradients were estimated in models containing the linear terms only; estimates of quadratic selection gradients, which estimate curvature of the fitness function, were generally not significant and are not reported because of limited statistical power ($n = 23$ genetic families).

To test for genetic selection, the plant fitness PC was regressed on the five defensive traits, herbivore community PC and plant damage. To estimate the effects of plant resistance traits on the herbivores, each of the five resistance traits was regressed on the herbivore community PC and plant damage. A separate multiple regression was employed to examine the effects of plant resistance traits on monarch growth, because these data were collected from an independent bioassay at the end of the season. I included leaf damage as an additional factor in this analysis because monarch larvae are sensitive to induced plant resistance (Van Zandt and Agrawal, 2004a) and because the assay was conducted at the end of the growing season when plants were considerably damaged.

RESULTS

Heritabilities of defensive traits and fitness components

Nearly all the plant traits measured exhibited high levels of variation and heritability across the 23 full-sib families examined (Table 1); the observed frequency of significant heritabilities (13 out of 15) is unlikely to have occurred by chance [binomial expansion test: $P < 0.001$ (Zar 1996)]. Leaf toughness was the least variable plant trait, varying only 30% between the families with the lowest and highest values, while latex production varied well over five-fold across families (Table 1). Similarly, the abundances of the herbivore species varied over four-fold across plant families (Table 1). The abundance of the *Tetraopes* was correlated with the other three species of herbivores, although not with total leaf damage (Table 2). Although vegetative biomass varied nearly two-fold across families, more surprising was the range of fruits produced, varying from a mean of 0.5 per plant to 17 times this amount. My measures of asexual reproduction (vegetative biomass of all stems) and sexual reproduction (fruit production) were weakly positively correlated ($R^2 = 0.03$, $F_{1,21} = 4.024$, $P = 0.058$).

Table 1. Family-mean range, one-way ANOVA (F-ratio), variance components (VC) and heritability (H^2) for defensive, herbivory and fitness-related traits of common milkweed at Jokers Hill, Ontario

	N	Range	F	$VC_{full\text{-}sib}$	VC_{error}	H^2
Resistance trait						
Latex 2001	530	1.3–2.2	**2.937*****	0.051	0.580	**0.161***
Latex 2002	528	0.8–4.5	**5.596*****	0.582	2.576	**0.368****
Toughness 2001	524	97–128	**2.504*****	44	661	**0.124***
Toughness 2002	525	135–185	**2.303*****	76	1389	**0.109***
Trichomes 2002	524	162–305	**5.162*****	1033	5719	**0.306****
Cardenolides 2002	214	0.07–0.17	**1.485***	<0.001	0.008	**0.155***
Nitrogen 2002	230	2.87–3.89	**2.464*****	0.293	2.088	**0.246***
Herbivory						
Damage 2001	529	0.20–0.33	0.559	0	0.032	–
Damage 2002	522	0.18–0.41	**4.988*****	0.004	0.022	**0.284****
Lygaeus	524	0.00–0.09	0.797	0	0.029	–
Rhyssomatus 2002	524	15–74	**1.584***	40	2149	0.037
Liriomyza 2002	524	1.0–4.4	**2.056****	0.304	6.470	**0.090***
Tetraopes 2002	522	0.7–4.7	**2.266*****	0.297	5.476	**0.103***
Danaus mass 2002	362	1.9–3.0	1.274	24218	948529	0.050
Fitness						
Vegetative biomass	527	42–72	**2.429*****	46.246	726.000	**0.120***
Fruit number	527	0.5–8.5	**2.244*****	2.166	38.424	**0.107***
Fruit mass	527	1.0–14.8	**2.243*****	9.227	165.800	**0.105***

Note: Significance levels for heritabilities are from z-tests of full-sib family variance components in SAS Proc Mixed. Sample sizes (N) vary because of variations in how the data were collected (see methods). **Bold** values are significant at $P < 0.05$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2. Genetic (full-sib) correlation coefficients between (a) herbivore abundances and (b) milkweed resistance traits ($n = 23$ families)

(a)	*Lygaeus*	*Liriomyza*	*Rhyssomatus*	*Tetraopes*
Liriomyza	0.054			
Rhyssomatus	0.177	0.292		
Tetraopes	**0.481***	0.357†	**0.462***	
Damage	−0.149	0.041	0.058	−0.024

(b)	Cardenolides	Latex	Toughness	Trichomes
Latex	0.077			
Toughness	0.062	−0.272		
Trichomes	−0.168	−0.072	0.051	
Nitrogen	−0.392†	−0.169	0	0.161

Note: Family means were typically calculated from ~23 replicate individuals, except for cardenolide and nitrogen analysis, which had ~9 individuals. **Bold** values are significant at $P < 0.05$. †$P < 0.10$, *$P < 0.05$.

Full-sib family level differences had a genetic component, as family mean trait values for latex and leaf toughness were each correlated across years (latex: $R^2 = 0.52$, $F_{1,21} = 23.13$, $P < 0.001$; toughness: $R^2 = 0.23$, $F_{1,21} = 6.23$, $P = 0.021$). However, the two years did vary in mean levels of latex and toughness, with plants having 41% higher latex and 47% higher toughness in 2002 than in 2001. The entire above-ground parts of the plants die back each winter, thus the observed genetic correlations reported here are not estimated from the same tissues. The proportion of leaves damaged across years, however, was not correlated ($R^2 < 0.01$, $F_{1,21} = 0.02$, $P = 0.882$), and likely represent yearly variation in herbivore assemblages (Van Zandt and Agrawal, 2004b).

Natural selection on plant resistance to a community of specialist herbivores

The genetic selection analyses revealed a reasonable level of consistency between the selection gradients (slopes from the multiple regression) and selection differentials (slopes from the pairwise correlations between traits and plant fitness PC). From this I infer that indirect selection was not driving the net selective impact (i.e. differentials) because selection gradients estimated by multiple regression account for correlations among predictor variables (Tables 3 and 4) (Conner and Hartle, 2004). Plant and herbivore traits explained 68% of the variance in the plant fitness PC (full model, $F_{7,17} = 4.556$, $P = 0.007$) and revealed two intuitive findings: selection against herbivore damage (or selection for resistance) and marginally significant selection favouring the production of latex (Fig. 2, Table 3). In addition, there was weak evidence of selection favouring higher nitrogen concentrations in leaves (Table 3). Somewhat counterintuitively, selection strongly favoured the herbivore community PC, apparently indicating selection for high abundances of insects on plants (Table 3). This effect is likely due to a positive association between plant size and herbivore abundances (see Discussion below).

Table 3. Genetic selection analysis (Rausher, 1992) for milkweed plants based on full-sib family means and regressions between the standardized traits and relative plant fitness

Effect	β	P	r_g	P
Plant damage	−0.324	**0.007**	−0.233	0.080
Herbivore community PC	0.687	**0.001**	0.411	**0.001**
Cardenolides	0.074	0.493	−0.009	0.948
Latex	0.285	0.067	−0.183	0.176
Trichomes	0.184	0.199	−0.211	0.114
Leaf toughness	−0.024	0.809	0.019	0.893
Nitrogen	0.207	0.083	0.002	0.989

Note: Only the linear selection gradients (β) are presented from a multiple regression analysis because no quadratic terms were significant. The r_g column represents the pair-wise genetic correlations between the trait and fitness (analogous to selection differentials). $N = 23$. The herbivore community PC was determined by the abundances of four species of herbivorous insects. **Bold** values are significant at $P < 0.05$.

Table 4. Genetic selection analysis (Rausher, 1992) for effects of five standardized plant resistance traits on the relativized herbivore community (PC), plant damage and monarch larval growth

	Effect	β	P	r_g	P
Herbivore community PC	Cardenolides	−0.011	0.893	0.056	0.629
	Latex	−0.303	**0.001**	−0.272	**0.012**
	Trichomes	−0.315	**0.001**	−0.305	**0.004**
	Toughness	0.023	0.765	0.089	0.441
	Nitrogen	−0.091	0.271	−0.086	0.455
Plant damage	Cardenolides	0.067	0.251	0.046	0.365
	Latex	0.067	0.230	0.055	0.277
	Trichomes	0.002	0.976	−0.002	0.965
	Toughness	0.021	0.695	0.007	0.888
	Nitrogen	0.069	0.237	0.032	0.530
Monarch growth	Cardenolides	−0.067	**0.003**	−0.071	**0.001**
	Latex	−0.017	0.393	−0.023	0.353
	Toughness	−0.021	0.250	−0.023	0.353
	Trichomes	−0.014	0.427	−0.004	0.862
	Nitrogen	−0.012	0.558	0.009	0.723
	Previous damage	−0.043	**0.034**	−0.063	**0.006**

Note: Only the linear selection gradients (β) are presented from a multiple regression analysis because no quadratic terms were significant. The r_g column represents selection differentials as the pair-wise genetic correlations between the traits and the herbivore variable. $N = 23$. **Bold** values are significant at $P < 0.05$.

Traits responsible for the abundance and performance of specialist herbivores

The abundance of insects in the herbivore community (PC) was strongly negatively influenced by two physical resistance traits, plant latex and trichomes, and the full model explained 68% of the variance ($F_{5,17} = 7.141$, $P < 0.001$; Fig. 3, Table 4). Because each of the herbivores loaded positively on the PC axis, this result represents a common response of the herbivores to latex and trichomes. In contrast, I found absolutely no evidence that leaf damage on the whole could be predicted by any of the factors measured ($R^2 = 0.170$, $F_{5,17} = 0.696$, $P = 0.633$; Table 4).

Because *Danaus* larvae were essentially absent from my study site, I conducted a bioassay of caterpillar growth on the plants towards the end of the season. In this selection analysis based on full-sib variation in plant traits, plant cardenolide concentrations and the extent of previous plant damage explained 64% of the variation in monarch larval growth (full model, $F_{6,16} = 4.738$, $P < 0.006$; Fig. 4, Table 4). Plant damage and cardenolides were not correlated ($R^2 = 0.039$, $F_{1,21} = 0.858$, $P = 0.365$).

DISCUSSION

In the population of common milkweed that I studied, five putative defensive traits were highly variable among full-sib families. The genetic selection analysis revealed only weak (marginally significant) selection favouring one of the resistance traits (latex) and one
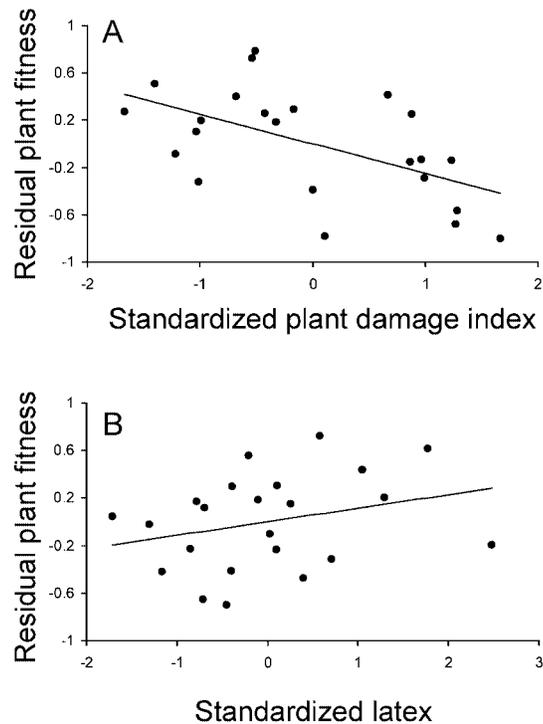
Fig. 2. Genetic selection analysis indicating (A) selection against herbivore damage (i.e. directional selection for resistance) and (B) directional selection favouring latex exudation. Residual fitness represents the residuals of the relativized fitness PC values from the multiple regression with all other traits included in the model except that on the x axis.

physiological trait (leaf nitrogen content). Though other defensive traits may have been beneficial at some level, costs of resistance may have constrained selection. Although I did not manipulate the putative selection agent (herbivory) as some other researchers have (Mauricio and Rausher, 1997; Shonle and Bergelson, 2000), I did detect directional selection against herbivory, suggesting that herbivores were likely agents of selection. In other studies from the same site, we have shown that natural and experimental damage by *Rhyssomatus* is associated with increased mortality of ramets compared with undamaged plants (Agrawal and Van Zandt, 2003). Similarly, both adult and larval feeding by *Tetraopes* is associated with reduced milkweed fitness (Matter, 2001; Agrawal, 2004b). Conversely, at natural levels of attack, it appears that milkweed is quite tolerant of attack by *Danaus, Lygaeus* and *Liriomyza* at our site (unpublished data).

Somewhat paradoxically, I found directional selection favouring high abundances of the herbivores (herbivore community PC). I interpret this pattern as large plants being favoured by natural selection, which results in the attraction of more insects (Price, 1991). For example, plant traits not included in the selection analyses, such as maximum height and probability of flowering, were significantly correlated with insect abundances (data not shown). Although some analyses of insect abundances standardize abundance data by plant biomass (Root, 1996), this was not appropriate in my analyses because vegetative biomass
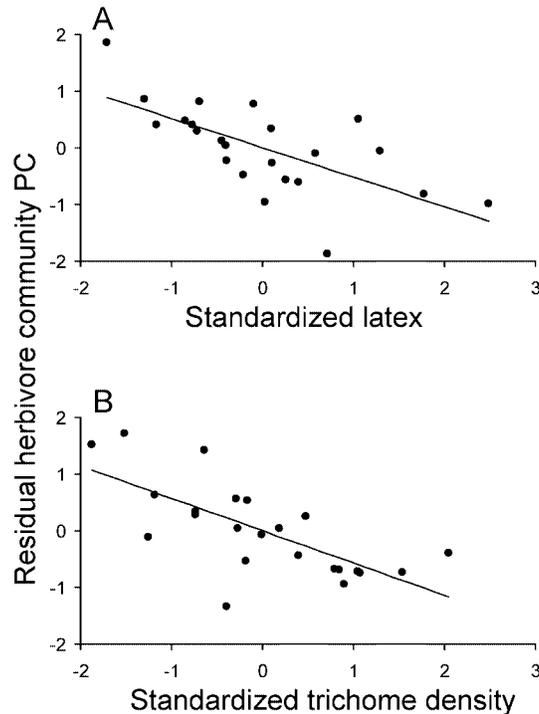
Fig. 3. Selection analyses indicating the influence of milkweed latex and trichome density on the suite of herbivore abundances (community PC). Residual PC represents values from the multiple regression with all other traits included in the model except that on the x axis.

was included as a component of the fitness PC. Thus, given that the index of herbivory did not correlate with any of the herbivore abundance data (Table 2), it is safe to conclude that the opposing selection gradients for the herbivore community PC and plant damage (Table 3) are not driven by selection favouring herbivory by any insect.

Plant latex and trichomes were quite generally effective at reducing the abundances of milkweed herbivores. Indeed, the abundances of *Tetraopes, Rhyssomatus* and *Liriomyza* were negatively genetically correlated with these traits in individual analyses (Agrawal and Van Zandt, 2003; Agrawal, 2004b), as well as in this study in the herbivore community PC. Both latex and trichomes are physical traits (latex volume is not correlated with cardenolide concentration). Although each of the milkweed herbivores has a behavioural mechanism to deactivate the latex exudation, this remains a dangerous activity, frequently taking tens of minutes and often resulting in mortality (Dussourd and Eisner, 1987; Malcolm, 1991; Dussourd, 1999; Zalucki *et al.*, 2001; Agrawal and Malcolm, 2002). Trichomes may prevent feeding, especially by small herbivores. First-instar *Danaus* larvae, for example, must graze the layer of trichomes before feeding on the leaf tissue (Fig. 1) (Malcolm, 1995). Latex and trichomes may thus primarily be targeted at the pre-ingestive phase, especially against the host-seeking adult stage of herbivorous insects, where mobile herbivores can assess the level of physical resistance traits.
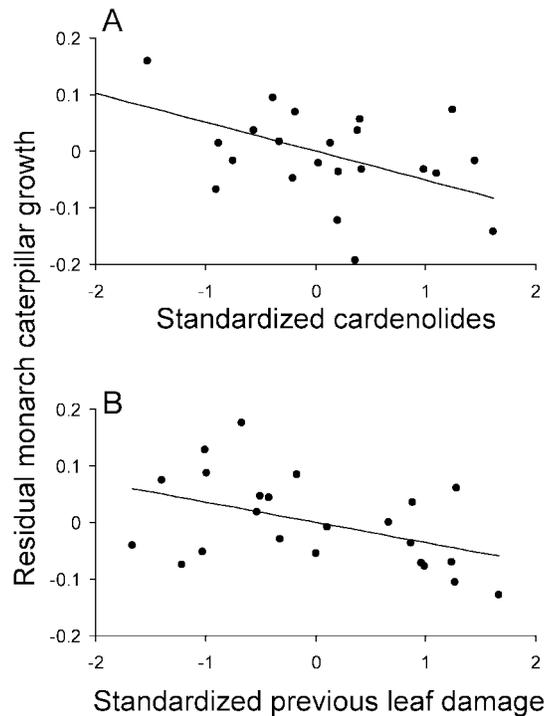
Fig. 4. Selection analysis on monarch larval performance as influenced by milkweed cardenolide concentration and previous leaf damage by other herbivores. Residual monarch growth represents values from the multiple regression model with all other traits included in the model except that on the x axis.

I measured larval performance of *Danaus* caterpillars near the end of the season, when this species occurs naturally on plants. The selection analysis revealed that cardenolides and previous damage strongly reduced *Danaus* growth (cardenolides and previous damage were not correlated). These data are consistent with previous phenotypic analyses (Zalucki *et al.*, 1990, 2001) and experiments showing induced plant resistance to monarch growth (Van Zandt and Agrawal, 2004a). Although aphids were also essentially absent from the study plot, my previous experiments have shown that cardenolides and nitrogen correlate (negatively and positively, respectively) with the population growth of *Aphis nerii* (Agrawal, 2004a). I thus hypothesize that the plant chemical constituents (i.e. cardenolides, nitrogen) may be more effective in reducing the performance of feeding herbivores (post-ingestion), while physical resistance traits may be most effective at reducing the preference of herbivores pre-ingestion. The physical traits (trichomes and latex) were effective at reducing the abundance of the community of common attackers. I attribute the effects on these herbivores primarily to preference because abundance was typically measured on mobile adults or immobile larvae that were deposited by mobile adults. The extent to which this hypothesis can be generalized is unclear, although assessing the chemical constituents of a host-plant may be more challenging than assessing the physical impediments to feeding.

Beyond pair-wise versus diffuse co-evolution

The diffuse co-evolution hypothesis simply posits that when a third species modifies the strength or direction of interaction in a pair of species, then the resulting responses to natural selection between the pair are also likely to be modified. The two 'diffusing' conditions considered in this study are the correlations between attackers and the influence of past herbivory on subsequent performance of other herbivores. Indeed, all of the abundant attackers were (weakly) positively correlated with each other and were generally affected by a similar set of plant defensive traits. In addition, this and our previous work (Van Zandt and Agrawal, 2004a,b) suggests that early season herbivory can influence the assemblage of subsequently feeding herbivores on the plant. Induced plant responses to herbivory are particularly interesting because they may cause diffuse selection on both herbivores and plants. On the one hand, induced responses to one herbivore can modify the selective impact that a plant trait has on other herbivores (Stout *et al.*, 1998; Agrawal, 2000), while on the other hand, induced responses to one herbivore can modify the fitness impact that another herbivore has on a plant (e.g. Agrawal, 1998, 1999).

The fitness reduction in plants imposed by a potent herbivore may drive natural selection, and this may be intensified or suppressed depending on the associated responses (and impact) of the community. In this scenario, each herbivore influences plant fitness and, depending on the strength of selection imposed by each herbivore, the responses to selection may be altered. Not all herbivores in a community, however, may be imposing selection on the plant. For milkweed and its insect community, there may be 'drivers' and 'followers' in the (co)evolutionary interaction. A driver imposes selection and, in the simplest case, other organisms in the community do not impose selection (followers), yet are still affected by resistance traits. For milkweeds and the community of about 10 herbivore species at my study site, the two beetles (*Tetraopes* and *Rhyssomatus*) appear to be the drivers (Agrawal and Van Zandt, 2003; Van Zandt and Agrawal, 2004a,b). Still, followers may be strongly affected by the defensive traits selected for by drivers or the pleiotropic consequences of these traits. The effect of the driver may be positive or negative on the followers. For example, resistance favoured by selection imposed by several herbivores of *Datura wrightii* cause plant susceptibility to another herbivore, but the latter does not have a selective impact on the plant (Hare and Elle, 2002). Thus, although the evolution of plant resistance may be targeted at a particular herbivore(s), the entire community of organisms attacking the plant may, in part, be structured by such resistance traits (Maddox and Root, 1990; Whitham *et al.*, 2003).

Ultimately, the challenge is to develop predictions of the resultant evolution depending on the independent effects and ecological associations of herbivores. For milkweed and its herbivore community, trichomes and latex were effective at reducing the abundance of herbivores. Given the similar response of the insects to these traits, it is likely that diffuse selection has amplified the responses for these traits. However, damage was selected against, and no single resistance-related trait explained variation in damage. Thus, diffuse interactions make it much more difficult for the plant to evolutionarily respond to reduce plant damage as a composite trait.

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