

Ecological play in the coevolutionary theatre: genetic and environmental determinants of attack by a specialist weevil on milkweed

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Summary

1 We studied the genetic and environmental determinants of attack by the specialist stem-attacking weevil, *Rhyssomatus lineaticollis* on *Asclepias syriaca*.

2 In natural populations, the extent of stem damage and oviposition were positively correlated with stem width, but not stem height. We hypothesized that both genotypic and environmental factors influencing stem morphology would affect attack by weevils.

3 In a common garden study with 21 full-sib families of milkweed, both phenotypic and genetic correlations indicated that weevils impose more damage and lay more eggs on thicker stemmed plants.

4 Of three other putative resistance traits, only latex production showed a negative genetic correlation with weevil attack.

5 When neighbouring grasses were clipped to reduce light competition, focal milkweed plants received up to 2.6 times the photosynthetically active radiation and 1.6 times the red to far red ratio of light compared with plants with intact grass neighbours. Focal milkweed plants were therefore released from the classic neighbour avoidance response and had 20% shorter internode lengths, were 30% shorter, and had 90% thicker stems compared with controls.

6 Clipping of grass neighbours resulted in nearly 2.7 times the damage and oviposition by stem weevils, thus supporting the hypothesis of an environmental or trait-mediated indirect influence on resistance.

7 Although attack of plants by weevils strongly increases the probability of stem mortality, thicker stems experience lower mortality, thus counteracting the selective impact of weevil-induced plant mortality.

8 The determinants of attack on milkweeds include both genetic variation for stem thickness and an indirect environmental influence of plant neighbours. If milkweeds and weevils are coevolving, the interaction is diffuse because the ecological neighbourhood is likely to modify the patterns of reciprocal natural selection.

Key-words: adaptive phenotypic plasticity, *Asclepias syriaca*, diffuse coevolution, herbivory, interaction modification, plant competition, plant–insect interactions, red : far red ratio of light, *Rhyssomatus lineaticollis*, trait-mediated indirect effect

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Introduction

Interactions between species may have evolutionary and ecological consequences. For example, coevolution, the reciprocal evolutionary adaptation between

interacting species, is thought to be important in both micro- and macroevolutionary processes, and has been implicated in driving the evolution of specialization within species as well as the adaptive radiation of taxa (Ehrlich & Raven 1964; Thompson 1994; Becerra 1997; Farrell & Mitter 1998; Rausher 2001). Two fundamental prerequisites for on-going coevolution to occur are: (i) genetic variation for the traits involved in mediating the interaction; and (ii) reciprocal fitness impacts imposed by the interacting species. Interactions between species

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may also have ecological consequences that influence communities (i.e. indirect effects). For example, trait-mediated indirect effects (Abrams 1995; Agrawal 2001) occur when a species modifies its phenotype in response to a second species, and this modification of a trait influences the interaction with a third species (cf. interaction modification, Wootton 1994). Recently it has been argued that (co)evolutionary interactions between species may be widely affected by such indirect ecological interactions (Miller & Travis 1996; Inouye & Stinchcombe 2001), suggesting a role for the biotic environment in modifying natural selection.

Although most studies of coevolution investigate pairwise interactions between two species, diverse interactions in natural communities abound and may strongly influence reciprocal evolutionary dynamics. Trait-mediated indirect effects allow community members to alter an interaction between two species, and may modify the pairwise reciprocal evolutionary interactions typically thought of as coevolution (Inouye & Stinchcombe 2001). In an attempt to reconcile the coevolutionary interactions that are likely to occur in nature with the diversity of interactions in natural communities, diffuse coevolution has been posed as an alternative model to pairwise coevolution (Janzen 1980; Fox 1981; Iwao & Rausher 1997; Stinchcombe & Rausher 2001). Diffuse coevolution occurs when the rate or direction of the reciprocal evolutionary interaction between two species is modified by the presence of an additional species. Although diffuse coevolution and trait-mediated indirect effects occur on different spatial and temporal scales, they are related and may influence each other (Inouye & Stinchcombe 2001).

The role of indirect interactions influencing coevolutionary interactions has rarely been tested. However, indirect effects should be particularly influential where organisms are faced with multiple selective agents in nature. For example, in natural communities of insects on plants, the plant neighbourhood may have a strong influence on plant–herbivore interactions. Considerable evidence indicates that plant competition may influence herbivory through several ecological paths, including changes in light availability, soil resources and the attractiveness of the neighbourhood (Atsatt & O'Dowd 1976; Louda *et al.* 1990; Karban 1997; Hamback *et al.* 2000). Competitors influence plant morphology (Morgan & Smith 1976; Ballare *et al.* 1994; Dudley & Schmitt 1996), defensive chemistry (Hjalten *et al.* 1994; Cipollini & Bergelson 2001), patterns of insect attack (Karbon 1993; Cipollini & Bergelson 2002; Tiffin 2002), and levels of predation on herbivores (Root 1973; Russell 1989). We thus propose that if the presence of plant competitors modifies the interaction between plants and herbivores via changes in the plant (i.e. a trait-mediated indirect effect), then the coevolutionary interaction between plants and herbivores will be diffuse. Most previous studies of how plant–herbivore coevolution is modified by a third species involved the influence of an additional herbivore (Rausher



Fig. 1 An adult female weevil, *Rhysomatus lineaticollis*, preparing to oviposit on a milkweed (*Asclepias syriaca*) stem.

et al. 1993; Agrawal 1998, 2000; Hufbauer & Root 2002; Stinchcombe & Rausher 2002; but see Tiffin 2002). Nonetheless, any ‘third’ organism that modifies an interaction may cause selection to be diffuse, and the third organism need not itself be coevolving with either of the other two species.

We have been studying the genetic and environmental determinants of resistance in milkweed plants (*Asclepias syriaca* L.) to their fauna of specialized insect herbivores in order to understand the potential for coevolution in a community context. In this study we focus on a stem inhabiting weevil, *Rhysomatus lineaticollis* (Say), an abundant specialist herbivore of common milkweed (Fig. 1). We asked the following specific questions: (i) What stem traits of milkweed correlate with attack by weevils in natural populations? (ii) Is there a genetic basis to these traits, and do they correlate with resistance in a common garden? (iii) How does the plant neighbourhood influence these traits, and do environmentally induced phenotypes modulate differences in resistance? (iv) Does weevil herbivory cause plant mortality? Through these questions we address the potential for coevolution between weevils and milkweed and assess how competing plants can alter this interaction.

Materials and Methods

NATURAL HISTORY

Our studies were conducted at the Koffler Scientific Reserve at Jokers Hill (henceforth Jokers Hill) in southern Ontario, Canada (44°03' N, 79°29' W, <http://www.zoo.utoronto.ca/jokershill>). Common milkweed (*Asclepias syriaca*) is a long-lived native herbaceous perennial and is abundant in old-field sites in our study area. The above ground tissues die back each winter, but extensive networks of modified stems underground allow for rapid vegetative growth and clonal reproduction. Adult weevils attack milkweed stems very early in the season each spring (mid-May to July), and the appearance of weevils is often synchronized with emergence of milkweed plants. Adults initially feed on the apical leaves, and after feeding, female weevils walk to lower parts of the stem and chew several sequential holes in the stem (Fig. 1). Females generally lay a single egg per hole, larvae hatch in 7–10 days, and larvae complete development inside the stem feeding on pith tissue (Fordyce & Malcolm 2000). The holes expand with the milkweed stem as it develops and this damage forms long prominent scars. Total oviposition scar length not only describes the physical damage imposed on the stem, but is also an accurate predictor of the number of eggs laid by the adult female ($n = 155$, $r = 0.88$, $P < 0.001$).

OBSERVATIONAL STUDY

During the summer of 2000, we surveyed 10 patches of milkweed along transects through Jokers Hill to identify patterns of attack by weevils. Weevils were only present in eight of the patches. In each of these eight patches we measured 10 milkweed stems for (i) total scar length, (ii) diameter of the stem at the base, and (iii) height of the plant. We hypothesized that stem morphology might be correlated with the pattern of attack, as stem morphology may be representative of the resources available to the pith-feeding larvae. We employed Pearson correlations to assess the association between stem diameter and height with weevil attack ($n = 80$).

GENETIC EFFECTS: COMMON GARDEN STUDY

To study the genetic basis of stem characteristics and their relationship with weevil attack, we established a common garden in 2001 employing 26 milkweed families. Plants were established from seeds of a single fruit per family and no two families were established from the same maternal plant. Seeds from a single fruit of *A. syriaca* were assumed to be full-siblings because of the pollinia pollination system of milkweeds (Gold & Shore 1995). An examination of isozyme polymorphisms from a nearby site in southern Ontario found no evidence for multiple paternity within fruits (Gold & Shore 1995) supporting this assumption. Fruits were

collected from the 10 patches of plants used in the original survey. Approximately 20 individuals from each full-sib family were grown in 500-mL plastic pots in growth chambers for 1 month before planting into a ploughed fallow field at Jokers Hill. In the second year of growth, milkweed plants typically emerge as multiple stems, sometimes over a metre away from the original plant. Therefore, each plant was grown in a 4-L plastic pot with field soil to limit lateral spread of clones and to allow us to distinguish individual plants for more than one season. Each pot was completely sunk into the ground and the plants were fully randomized within the common garden.

In May 2002, a subset of the plants from each family was inoculated with two adult weevils collected from nearby natural populations. Most weevils were removed by hand after 1 week. Natural infestation by other herbivores was not controlled. One month later, when all adult weevils had disappeared from the landscape, we measured stem width, maximum height and scar length on all plants. The sum of all stem widths and scars on stems from a single pot (i.e. one genet) were employed as single data points. We used a simple one-way ANOVA to show that there was genetic variation for stem thickness across all plant families and then proceeded to conduct correlations between family means for plant traits and weevil attack. The use of full-sib family means in such correlations overestimates the genetic component of heritable traits (i.e. includes non-additive genetic variance), but nonetheless reflects an underlying genetic basis for the correlation (Falconer & Mackay 1996).

We measured three traits putatively associated with insect resistance: production of latex, foliar trichome density, and leaf toughness. All measures were taken on a single stem from the youngest fully expanded leaf. We measured latex by cutting the tip (0.5 cm) off the leaf and collecting the latex onto a 1-cm disc of filter paper. Latex stopped exuding after *c.* 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 microgram. This is a repeatable method for determining latex exudation; we have found positive phenotypic and genetic correlations of latex exudation across 2 years when plants were measured in a common garden (A.A. Agrawal, unpublished data). In addition, this method of assessing latex production is likely to reflect what feeding insects must contend with, and has been shown to be negatively associated with the growth of other milkweed herbivores (Van Zandt & Agrawal 2004). Trichome density on the leaves was assessed by counting trichomes on the tops and bottoms of leaf discs (28 mm², punched from near the tip of the leaf) under a dissecting microscope. We measured leaf toughness with a force gauge penetrometer (Type 516, Chatillon Corp., New York, USA) that measures the grams of force needed to penetrate a surface. We 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. Two measures were taken for each leaf, one on each side of the mid-rib, and these data were averaged and used as a single data point per plant.

A total of 330 plants from 21 families received weevil damage and were measured (where five or less plants were treated in a full-sib family, the family was not included in the analysis). One-way ANOVA was used to test for genetic variation in latex, toughness and trichomes. Both phenotypic and family level (genetic) correlations were conducted to assess the effect of traits on weevil damage and oviposition. A Bonferroni correction was applied to the correlations between weevil scars and latex, trichomes and toughness because we did not have a strong a priori expectation for the direction of the effect for each of the traits.

Although weevils do not frequently move from plant to plant, in the above experiment weevils were occasionally found on non-treated plants. Therefore, in order to assess the oviposition of weevils in a no-choice arena, 41 single stemmed plants in a separate experiment were enclosed with spun-polyester sleeves. Two wild-collected weevils were placed in each bag and were allowed to feed and oviposit for 3 days. We then used a Pearson correlation to assess the relationship between stem diameter and oviposition scars.

COMPETITIVE EFFECTS: NEIGHBOUR REMOVALS IN NATURAL POPULATIONS

To test for effects of neighbouring vegetation on plant morphology and weevil attack, we studied plants in four natural populations of milkweed at Jokers Hill, all within 1 km from each other. We selected individual stems of milkweed in a dense old-field and randomly assigned them to either neighbour removals or left them as controls ($n = 46$ for each treatment). Experiments were initiated in late May 2001, when milkweeds were still emerging and expanding. Neighbour removals consisted of clipping all vegetation surrounding the focal milkweed stem within a 20-cm radius. We used scissors and cut the vegetation as low as we could without disturbing the soil.

On clear days, we measured the quantity and quality of light reaching the centre of clipped or intact plots. At three time points throughout the day (8, 12, 16 hours), we measured photosynthetically active radiation (PAR, 400–700 nm, in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ using a Licor 250 metre with quantum cosine-corrected sensor, LiCor, Lincoln, NE, USA) and the ratio of red to far red light (R : FR, using a Skye SKR-1102-channel sensor with narrow band filters centred at 660 nm and 730 nm, Skye Instruments, Llandrindod Wells, UK) ($n = 12$ –18 for each treatment-time combination). We measured PAR in intact and neighbour-removal treatments to assess the potential for light competition; R : FR was measured because this ratio is the cue used

by plants to detect light competition from neighbouring plants (Ballare 1999). Light values for plants with and without neighbours were compared using separate variance t -tests and subsequent Bonferroni correction for multiple tests at the different daytime measurements. Separate variance t -tests do not require equal variances among the two treatment groups (Wilkinson 1997).

Ten days following clipping, we re-visited the plots and assessed the stem width, height, scar length and percentage leaf damage by herbivores. Foliar herbivory was estimated by visually dividing each leaf into four quadrants to calculate the percentage leaf area missing on each plant. Effects of neighbour removals on plant morphology and herbivory were analysed using a multivariate analysis of variance (MANOVA) model. We employed a MANOVA approach because (i) our multiple response variables were potentially correlated, and (ii) the multivariate approach is more powerful than univariate analyses because it incorporates several possible effects of our treatments (Scheiner 1993). Based on the results of the observational studies, our a priori hypothesis was that plant neighbours would cause changes in the stem architecture of milkweed (increased height and decreased width) and reduce weevil attack. We proceeded with univariate analyses to decompose our analysis only when the MANOVA was significant. For univariate analyses, we employed SAS PROC MIXED using Site as a random effect and Treatment as a fixed effect. Site was dropped from the model when not significant. As suggested by the SAS Institute (1999), we employed the likelihood-ratio χ^2 test for tests of the random effects. The likelihood-ratio χ^2 tests the hypothesis that the variation due to the random effect is greater than 0, and is a one-sided, single degree of freedom test.

In a separate field experiment, we measured the internode lengths of plants with and without grass neighbours ($n = 66$ of each treatment) because plants experiencing light competition typically have longer internode lengths than uncompeting plants (Dudley & Schmitt 1996). Milkweed plants were grown from seed in a common garden with or without 10 grass seedlings (*Bromus inermis*) surrounding each focal plant. In the second year of growth, we measured the distance between the first and second produced leaves. Internode lengths from plants with or without grass neighbours were compared using a t -test.

EFFECTS OF WEEVIL ATTACK ON PLANT MORTALITY

To determine if herbivory by weevils could affect plant fitness, we experimentally imposed weevil attack on plants in natural and experimental populations of milkweed in two experiments. In the first experiment, we bagged 117 natural stems of milkweed with spun polyester sleeves, and then introduced three weevils to half of the plants, allowing them to damage plants and oviposit for 3 days. At the same time, we imposed the

same treatment on 120 plants in a common garden. The common garden was established by planting 1-month-old plants (grown in a growth chamber). Plants in natural populations have well-developed root systems and are likely to be connected to other plants underground, while the plants in our common garden were single-stemmed, recently established individuals. We employed the Cochran-Mantel-Haenszel test of independence to determine the effect of weevil damage on stem mortality, while controlling for differences between the two sites (Stokes *et al.* 2000).

To more specifically test for the quantitative effects of weevil damage on stem mortality and also the effects of stem thickness on mortality, we conducted a second experiment. Across two natural patches of milkweed, we tagged 34 control plants (undamaged), 40 plants naturally damaged by weevils, and 35 plants that were undamaged but upon which we imposed weevil damage and oviposition as described above. We measured stem thickness at the base of the plants using callipers. As in all of our experiments, enclosing three weevils on each plant for 3 days imposed a realistic level of damage and oviposition and did not differ from the plants that were naturally colonized (mean \pm SE cm oviposition scar length per stem: naturally damaged plants = 56.1 ± 5.7 , plants treated with weevils = 62.5 ± 6.8 ; $F_{1,74} = 0.73$, $P = 0.40$). To establish how stem thickness influenced the probability of plants to die, we first examined the effect of stem thickness on mortality in the control plants using logistic regression. Then we asked how stem thickness and length of oviposition scars affected the probability of mortality in the naturally and experimentally damaged plants. Maximum likelihood estimates were generated in PROCLOGISTIC in SAS (Stokes *et al.* 2000).

Results

In natural patches of milkweed we observed a positive correlation between stem diameter and weevil attack ($n = 80$, $r = 0.362$, $P < 0.001$), but we observed no association between stem height and attack ($r = 0.058$, $P = 0.611$) (Fig. 2), despite the fact that stem diameter and plant height were themselves correlated ($r = 0.634$, $P < 0.001$). In our common garden experiment, we found strong evidence that stem width varied by genetic family ($F_{25,516} = 3.170$, $P < 0.001$). This variation in stem width showed positive phenotypic ($n = 330$, $r = 0.293$, $P < 0.001$) and genetic ($n = 21$, $r = 0.518$, $P = 0.016$) correlations with attack by weevils (Fig. 3). Although we found a weak phenotypic correlation between stem height and weevil attack ($n = 330$, $r = 0.122$, $P = 0.027$), there was no genetic correlation ($n = 21$, $r = 0.173$, $P = 0.453$). Among our three putative resistance traits, only latex production showed a negative correlation with attack (Table 1, Fig. 4). Latex production was not strongly phenotypically or genetically correlated with stem thickness or the other resistance traits (data not shown, all P s > 0.05). In the no-choice experiment,

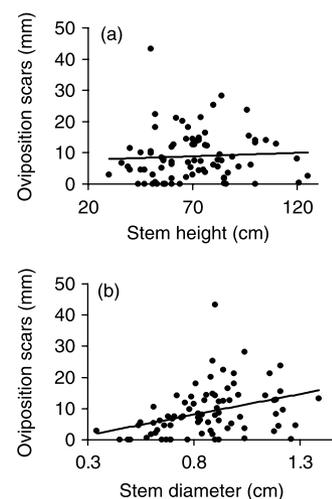


Fig. 2 Correlation between milkweed (a) height or (b) stem diameter with attack by weevils in natural populations at Jokers Hill.

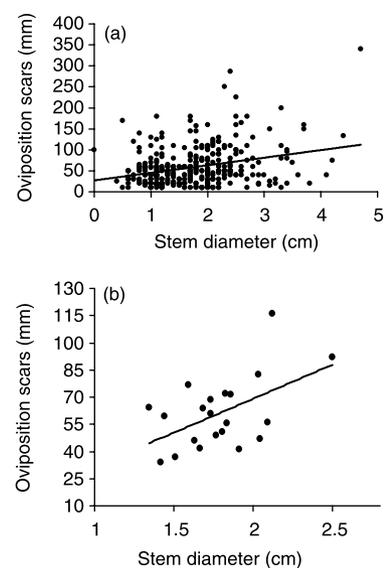


Fig. 3 (a) Phenotypic and (b) genetic correlations between milkweed stem diameter and attack by weevils in a common garden plot.

Table 1 Phenotypic ($n = 330$) and genetic ($n = 21$) correlations for effects of latex, leaf trichomes and leaf toughness on weevil attack on milkweeds in a common garden. The corrected alpha value after Bonferroni adjustment due to correlations with multiple traits is 0.017

	Phenotypic		Genotypic	
	r	P	r	P
Latex*	-0.055	0.320	-0.521	0.015
Trichomes*	0.014	0.800	-0.118	0.610
Toughness	0.012	0.813	0.172	0.456

Note: *next to the plant trait indicates significant genetic variation was found for this trait using family as the main effect in a one-way ANOVA (analyses not shown).

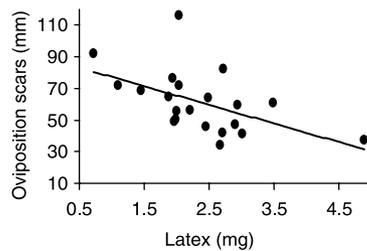


Fig. 4 Genetic correlation between milkweed latex production and attack by weevils in a common garden plot.

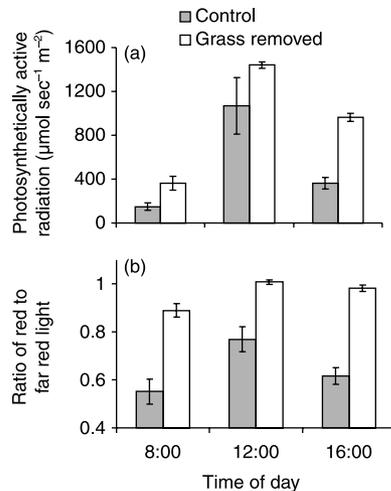


Fig. 5 The effects of grass removal surrounding milkweed plants in natural populations on the (a) quantity and (b) spectral quality of light reaching focal milkweeds. Measurements were taken at three time points throughout the day. The unobstructed ratio of sunlight on a clear day is *c.* 1. Bars represent mean \pm SE.

Table 2 Effects of grass removal around focal milkweed plants on photosynthetically active radiation (PAR) and the ratio of red to far red light (R : FR) at three times of day. A separate variance *t*-test was performed for each measure at each time of day. Separate variance *t*-tests generate fractional degrees of freedom; *n* = 12–18 for each treatment-time combination. The corrected alpha value after Bonferroni adjustment due to multiple samples of the same replicates is 0.008

	Time of day	<i>t</i> -value	d.f.	<i>P</i>
PAR	8	3.047	18.5	0.007
	12	1.432	17.5	0.170
	16	9.288	19.7	< 0.001
R : FR	8	5.695	19	< 0.001
	12	4.462	18.2	< 0.001
	16	9.846	15.7	< 0.001

where weevils were restricted to single plants, we again found a positive correlation between stem diameter and weevil scars (*n* = 41, *r* = 0.351, *P* = 0.025).

In natural old-field populations, neighbouring vegetation drastically limited the photosynthetically active radiation (PAR) reaching milkweed plants (Table 2, Fig. 5). Plants with neighbours clipped

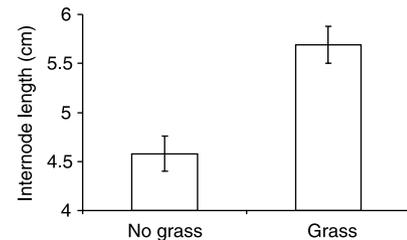


Fig. 6 The effect of grass neighbours on the internode length of milkweed plants in a common garden plot. Bars represent mean \pm SE.

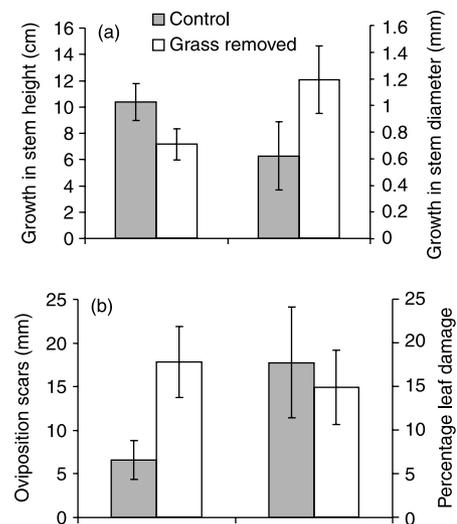


Fig. 7 The effects of grass removal surrounding milkweed plants in natural populations on (a) morphological growth and (b) attack by herbivores on focal milkweed plants. Bars represent mean \pm SE.

received between 34% and 167% more PAR than controls, depending on the time of day. Similarly, the ratio of red to far red light, the cue used by plants to perceive light competition, was between 30% and 60% higher in plants with their neighbours removed compared with controls. Not surprisingly, the differences between control and clipped plots were smallest at noon, when neighbouring vegetation intercepts the least amount of light (Table 2, Fig. 5).

The presence of grass neighbours strongly influenced plant morphology. We found that internode lengths were 20% shorter in milkweed plants growing without grass neighbours compared with plants growing next to grass (Fig. 6, *t* = 4.242, d.f. = 130, *P* < 0.001). In our overall MANOVA analysis, we found highly significant effects of neighbour removal (Wilks' Lambda = 0.846, *F*_{4,82} = 3.739, *P* = 0.007) and milkweed site (Wilks' Lambda = 0.516, *F*_{12,217} = 5.138, *P* < 0.001) on plant morphology and herbivory. Milkweed plants with their grass neighbours removed grew 30% less in height and 90% more in stem thickness compared with controls (Table 3, Fig. 7).

Clipping of grass neighbours also resulted in nearly 2.7 times the oviposition damage by stem weevils.

Table 3 Univariate analyses testing for effects of grass removal around milkweed plants on growth and herbivore attack on focal milkweeds. Two-tailed probabilities generated by SAS Proc Mixed are reported

Effect	d.f.	<i>F</i>	<i>P</i>
Height growth*	1	3.72	0.057
Error	85		
Diameter growth	1	2.819	0.092
Error	88		
Weevil oviposition	1	2.96	0.089
Error	88		
Leaf damage	1	0.25	0.620
Error	88		

*Site effect was significant using the likelihood-ratio χ^2 test for random effects.

Although the univariate effects of neighbour removal on morphology and weevil attack were only marginally significant, one-tailed interpretations of the analyses would be statistically significant and are appropriate given our a priori hypotheses. Thus, plants with neighbours removed were shorter, thicker stemmed, and were more heavily attacked by weevils, compared with controls.

In the first experiment examining effects of weevils on stem death (senescence of the above-ground parts of a ramet), mortality was low in the natural population, with 5% of stems dying in the weevil damaged treatment and no mortality in the controls. However, in our common garden, 23% of weevil-attacked stems died, whereas, again, there was no mortality in controls (Cochran-Mantel-Haenszel test: $Q_{MH} = 18.687$, d.f. = 1, $P < 0.001$). In the second experiment we examined the quantitative effects of stem thickness and oviposition scars on mortality. Here again, in the absence of weevil damage (controls), mortality was relatively low (14.7%) and was not affected by stem thickness ($\chi^2 = 0.04$; $P = 0.84$). In contrast, in our analysis of weevil-damaged plants, both stem thickness and weevil damage were associated with plant mortality (Table 4, Fig. 8). The inclusion of the marginally significant interaction term between weevil damage and stem thickness affected the significance of the main effect of

stem thickness (Table 4). As can be seen from Fig. 8, however, the interaction between stem thickness and length of oviposition scars was not strong, indicating that different sized stems generally experienced the same weevil-induced mortality. The logistic regression analysis revealed that the consequences of our manipulation for weevil damage were not different from the effects of natural weevil damage, and that the likelihood of stem death increased 2.4% with every additional centimetre of weevil damage (one to two eggs) and decreased 45% with every additional millimetre of stem thickness (Fig. 8).

Discussion

THE POTENTIAL FOR COEVOLUTION

Feeding by stem-attacking weevils has strong negative consequences for milkweed plants. By the end of the growing season, many milkweed stems are filled with weevil frass and many other attacked stems have died. Because milkweeds grow clonally, weevil-induced stem senescence may not represent death of the genet, but certainly represents reduced reproductive performance and an energetic cost to the genet. Although the mechanisms by which weevils detrimentally affect milkweeds are unknown, other stem feeding weevils such as the sunflower stem weevil (*Cylindrocopturus adspersus*) reduce plant fitness by increasing the probability of stem breakage, facilitation of pathogen attack, and damage to the vascular tissue (Charlet *et al.* 1997).

Because *Rhyssomatus lineaticollis* only eats milkweeds, it is tempting to speculate that the two have been involved in a pairwise coevolutionary interaction. In order for coevolution to occur, there must be reciprocal fitness impacts of the species on each other and there must be a genetic basis to the defensive and counter-defensive traits. Our initial observations implicated stem thickness as an important determinant of weevil attack. Stem thickness may be correlated with the level of consumable pith tissue for the weevil larvae or with protection from predators and parasitoids (i.e. enemy-free space *sensu* Stamp 2001). Given the positive genetic correlation between stem thickness and weevil

Table 4 Logistic regression analysis for effects of treatment (naturally colonized or manipulated weevil damaged), plant patch, weevil damage (length of oviposition scars) and stem thickness on stem mortality

Parameter	Estimate	Standard error	Wald χ^2	<i>P</i>
Intercept	−0.988	2.607	0.144	0.705
Treatment	0.172	0.333	0.269	0.604
Patch	−1.644	0.497	10.946	0.001
Weevil damage	0.102	0.045	5.198	0.023
Stem thickness	−0.211	0.266	0.628	0.428
Weevil damage × Stem thickness	−0.008	0.004	3.326	0.068

Note: When the interaction between weevil damage and stem thickness is removed, stem thickness has an estimate of −0.607, SE 0.212, Wald $\chi^2 = 8.195$, and $P = 0.004$. All other parameters remain qualitatively the same.

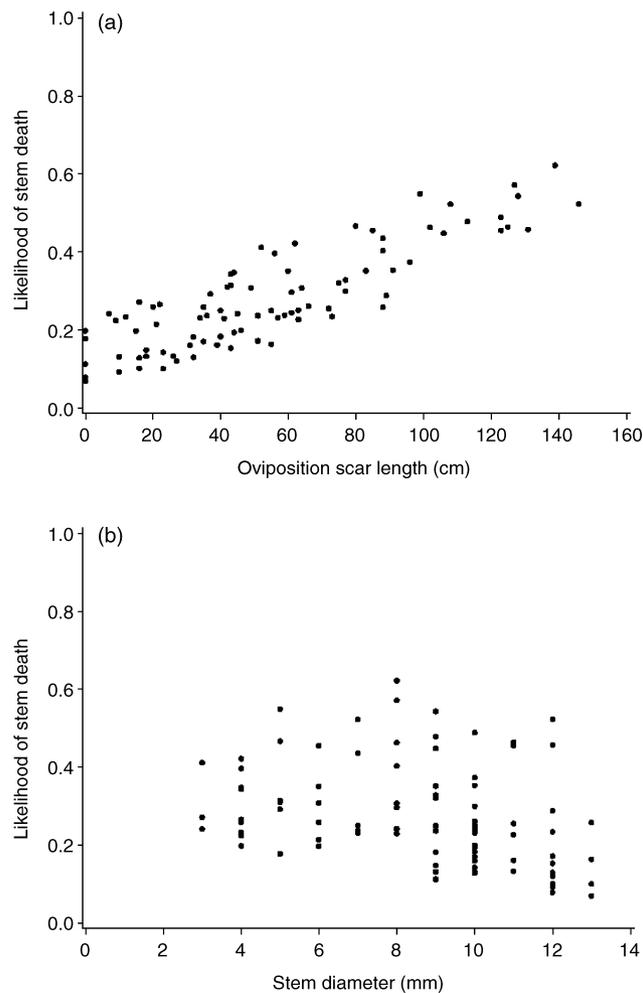


Fig. 8 The likelihood of stem mortality as caused by (a) oviposition scar length and (b) stem diameter as generated by maximum likelihood logistic regression. The scatter in each panel represents variation that is not accounted for by the other term.

attack that we found (Fig. 3), and the negative effect of weevils on plants, it is reasonable to conclude that weevils are important selective agents on plant morphology.

Several other plant traits, including latex production, trichomes, leaf toughness and cardenolide content, may influence attack by weevils. The foliar traits (trichomes and toughness) may only be relevant to adults because weevils typically feed on leaves before ovipositing in the stems, while larvae feed inside stems. However, we found no evidence that adult weevils were deterred by leaf trichomes or leaf toughness. Alternatively, latex production, which we found to be negatively correlated with attack, may be a physical barrier to feeding and oviposition by the weevils. Before feeding, adult weevils typically notch the leaves of milkweed in a similar process to the behaviour of monarch butterfly larvae (Dussourd & Eisner 1987; Fordyce & Malcolm 2000; Agrawal & Malcolm 2002). After notching, weevils feed on leaf tissue distal to the notch where latex is no longer released upon feeding. In addition, oviposition is a very slow process, usually taking well over an hour, that often involves the weevil

chewing into the stem, and carefully navigating around copious flows of latex (Agrawal & Malcolm 2002).

Cardenolides, a family of well-known toxins from milkweeds that disrupt the sodium-potassium channels of animal cells, could also play a role in resistance to *R. lineaticollis* (Malcolm 1991; Fordyce & Malcolm 2000). Unlike many of the other herbivores of milkweed, *R. lineaticollis* is not aposomatically coloured (it is plain black) and does not sequester cardenolides (Nishio *et al.* 1983). Fordyce & Malcolm (2000) hypothesized that feeding on the pith tissue may be a mechanism by which the larvae of *R. lineaticollis* spatially avoid cardenolides. Although the pith does not contain latex, they found high levels of cardenolides in the pith tissue, thus rejecting their hypothesis. Nevertheless, lower amounts of cardenolides were found in plants that were naturally attacked compared with unattacked plants. Thus, weevils may either be choosing low cardenolide plants or may be inducing a plant response that lowers cardenolide content (Fordyce & Malcolm 2000). The current study clearly shows that weevils can be choosy and are affected by plant traits, but further research will be required to determine the

effects of cardenolides on weevil behaviour and performance.

Ultimately, the demonstration of coevolution in this system will require an understanding of the reciprocal fitness impacts and selection on specific defensive and counter-defensive traits. We conclude that each partner clearly has an influence on the other: weevils increase plant mortality while the plant strongly affects weevil behaviour. In addition, we have identified plant traits (stem thickness and latex) that may be targets of natural selection imposed by weevils. The gap in our understanding relates to selection on traits of the weevil that are being affected by these plant defences.

TRAIT-MEDIATED INDIRECT EFFECTS

While milkweed plants and weevils may have reciprocal fitness impacts on one another, trait-mediated indirect effects may limit the potential for pairwise coevolution. Although the effects we observed were relatively weak, our data are consistent with the hypothesis that neighbouring plants altered stem morphology and this change influenced attack by weevils. Thus, the presence of grass neighbours may alter the selective impact of weevils on plants. Two non-exclusive alternative hypotheses for stem thickness mediating this indirect effect of neighbours are that (i) neighbouring plants increased latex production and subsequently reduced weevil attack, or (ii) neighbouring plants physically obscured focal milkweed plants and made them less apparent to weevils. We reject both of these alternatives for the following reasons. First, evidence from another experiment showed no influence of grass competition on latex production (A.A. Agrawal, unpublished data), suggesting that the trait-mediated effect did not involve latex. We have, however, shown that herbivory by other species influences latex production, opening the door to the environmental influence of induced resistance on host selection by weevils (Van Zandt & Agrawal 2004). Secondly, in our no-choice experiment where weevils were restricted to single stems, weevils imposed less damage and laid fewer eggs on thin-stemmed plants compared with thick-stemmed plants, demonstrating that in the absence of neighbours, weevils prefer thicker stemmed plants. Thus, we conclude that neighbouring plants change the stem morphology of milkweeds and this change is likely to have caused the effects on subsequently colonizing weevils that we observed.

Studies on the effect of a plant's stem morphology on attack by insects have a long history. For example, classic work was conducted on the interaction between wheat and sawflies, where sawflies bored into wheat stems and thus reduced yield (Painter 1951; Holmes 1984). Early work conducted by Holmes indicated that increased 'solidness' of stems was correlated with resistance to herbivory. Reduced light levels, especially early in the season, caused an increase in the hollowness of stems and consequently higher rates of attack

(Holmes 1984). Experimental manipulations of the quantity and quality of light also caused similar responses. Thus, although plant competition was not directly manipulated, it is plausible that increased competition would decrease resistance of wheat through a trait-mediated indirect effect. Although this effect would be in the opposing direction to the one we describe (i.e. competition increases resistance), the stem traits that provide resistance appear to vary between the systems.

Plant competition may influence herbivory via several types of trait-mediated interactions (Cahill *et al.* 2002). For example, competitor-mediated reductions in defensive chemistry (Cipollini & Bergelson 2001) could have caused increased plant susceptibility observed in field plots of *Brassica napus* (Cipollini & Bergelson 2002). Opposing effects, where increased plant density increased resistance, were found for an unknown resistance trait of cultivated cotton in the laboratory (Karban *et al.* 1989); these effects were supported by experiments with wild cotton in natural populations (Karban 1993). Thus, although it seems clear that competing plants can influence herbivory, we still lack a framework to predict the magnitude and direction of the outcome. In addition, although competitors have strong influences on plant morphology (Morgan & Smith 1976; Ballare *et al.* 1994; Dudley & Schmitt 1996), to our knowledge no other work has been conducted on the community consequences of such morphological changes, despite their likely importance (Agrawal 2001; Callaway *et al.* 2003).

Community ecologists are increasingly studying trait-mediated indirect interactions, and although most studies have focused on the non-lethal effects of predators in simple food webs, a few studies have focused on effects of resources or non-trophic effects (i.e. light competition) on trait-mediated indirect interactions (Agrawal 2001; Werner & Peacor 2003). The case we studied involved a non-trophic, trait-mediated effect (*sensu* Werner & Peacor 2003) because a plant-competitor changed the phenotype of our focal species, and this change influenced a consumer. A few additional studies reviewed by Werner & Peacor (2003) found evidence for interactions between species that affect predators through changes in traits. For example, salamanders influenced a behavioural trait of isopods that caused the isopods to be more vulnerable to predation by fish (Huang & Sih 1990). More generally, such trait-mediated indirect interactions may result in apparent competition (Holt & Lawton 1994) or apparent mutualism (Abrams & Matsuda 1996) because the competitors may be, in part, influencing each other via an enemy. Indeed, most cases of apparent competition are asymmetric, with one party influencing the other more strongly than vice versa (Chaneton & Bonsall 2000). In our system we predict such an asymmetry because neighbours influence milkweed indirectly through attack by weevils, but weevils are monophagous, only eating milkweed and not the neighbours.

STUDYING COEVOLUTION IN A COMMUNITY
CONTEXT

One goal in evolutionary ecology is to merge the complexity of natural systems with studies of reciprocal evolutionary interactions. Historically, the term coevolution had been used promiscuously and this led to community ecologists rampantly classifying traits and interactions as being coevolved. Two papers in the early 1980s more rigorously defined coevolution and predicted that some coevolutionary interactions may be 'diffuse' because groups of species may be interacting and imposing natural selection on each other (Janzen 1980; Fox 1981). One approach to studying diffuse coevolution is to use quantitative genetics to assess the strengths of selection in the presence or absence of some environmental factor (Iwao & Rausher 1997; Stinchcombe & Rausher 2001, 2002; Tiffin 2002). Alternatively, we took the approach of studying the resistance mechanisms and how their function is modified by an environmental factor. Ultimately, the two approaches should be bridged, with benefits of the first, emphasizing measurement of selection, and the second, emphasizing natural history and ecological mechanisms, yielding the most accurate depiction of nature.

According to Thompson (1994, 1997, 1999), the term 'diffuse coevolution' has become a catchall phrase encompassing any multispecies interaction, and as such it limits our ability to make progress in understanding the coevolutionary process. In particular, Thompson has repeatedly suggested replacing the concept of diffuse coevolution with a geographical perspective on coevolution (Thompson 1994, 1997, 1999). Although a geographical perspective may be quite informative, it is unclear why the two perspectives are mutually exclusive (Stinchcombe & Rausher 2002). Recall that diffuse coevolution is simply the modification of the rate or direction of a reciprocal evolutionary interaction between two species by a third species. Thus, coevolution may vary geographically because of spatial variation in the presence of species that modify an interaction (Stinchcombe & Rausher 2002).

Although coevolution may be diffuse, two interacting species may still be evolving adaptations to each other. Additional species that modify the coevolutionary interaction do just that, modify the interaction, usually in terms of the rate of adaptation (e.g. Stinchcombe & Rausher 2002). We speculate that additional species that alter the direction of coevolution will be rare, but that alterations in the rate or strength of coevolution will be common. It may be that all coevolution is diffuse. Even if this is the case, however, the challenge now is to try and understand the influence of different factors (abiotic, community members, etc.) on the evolution of interactions. For weevils attacking milkweeds, although stem thickness and latex are genetically determined resistance traits, community members modify these traits, and thus any coevolution between the two cannot proceed in a simple pairwise fashion.

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