

Specificity and trade-offs in the induced plant defence of common milkweed *Asclepias syriaca* to two lepidopteran herbivores

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Summary

1. We studied induced defences in common milkweed (*Asclepias syriaca*) to two specialist caterpillars (*Danaus plexippus* and *Euchaetes egle*) to test several hypotheses about co-evolution and defence allocation.

2. Latex, a potent defence of milkweed, showed nearly fivefold variation in the amount exuded among genetic families and more than doubled production in response to herbivory by *D. plexippus*; this induced latex response was fourfold stronger than the latex response to *E. egle* herbivory. In contrast, *D. plexippus* and *E. egle* equivalently induced foliar cardenolide concentration (an average increase of 26%).

3. We found broad-sense heritable variation for the induced plant responses, and there was a genetic correlation between the responses induced by the two herbivores for cardenolides, but not for latex. We found no genetic correlation between investment in latex and cardenolides in any of the treatments.

4. Using a bias-corrected Monte Carlo procedure, we found strong evidence for a trade-off between constitutive and induced cardenolides, but not for latex.

5. *Synthesis.* As natural selection by specific herbivores varies in space and time, we expect that these defence traits in *A. syriaca* will evolve independently, with the expression of cardenolides showing less specificity, as well as being constrained by a trade-off between constitutive and induced defence.

Key-words: *Asclepias syriaca*, cardenolides, chemical ecology, *Danaus plexippus*, *Euchaetes egle*, induced resistance, latex, plant defence theory, plant–insect interactions, trade-offs

Introduction

Defence is central to plant survival and reproduction, and plants have evolved diverse strategies to resist herbivory (Rasmann & Agrawal 2009). Most plants employ a combination of constitutive (always present) and induced (facultatively expressed following attack) defences. In some cases, the same induced defence may be triggered by a variety of stimuli and affect a range of associated species; alternatively, plant responses and their effects can be highly specific to particular cues (Karban & Baldwin 1997). Plant responses to herbivory may exhibit either specificity of elicitation, in which the change in plant phenotype depends on the identity of the initial damaging herbivore, or specificity of effect, where two or more herbivores show different responses to a particular induced plant phenotype (Stout *et al.* 1998). While there are some examples

of generalized induced responses that result from many stimuli, most studies that have compared responses induced by different herbivores find strong evidence for specificity (Hartley & Lawton 1991; Stout *et al.* 1998; Van Zandt & Agrawal 2004a,b; Voelckel & Baldwin 2004).

In this study, we tested several predictions regarding the evolutionary ecology of specificity in induced defences and pre-requisites of plant–herbivore co-evolution. The first key prediction is that phenotypically (or phylogenetically) similar herbivores should induce similar responses (Karban & Baldwin 1997). While sometimes supported by comparisons of plant responses to herbivores in different feeding guilds (e.g. Walling 2000; De Vos *et al.* 2005), this prediction is often not supported when comparing closely related species. Studies that have compared induction by closely related species of spider mites (Karban & Carey 1984), lepidopterans (Stout *et al.* 1996; De Moraes *et al.* 1998) and beetles (Viswanathan, Narwani & Thaler 2005; Rasmann & Turlings 2008) have shown that

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congeners or confamilials frequently induce different responses in the same plant. For example, in *Solanum dulcamara*, damage by three chrysomelid beetles induced highly divergent chemical responses with differing ecological effects (Viswanathan, Narwani & Thaler 2005).

Despite substantial evidence for specificity of elicitation, there has been little exploration of how such specificity could evolve by natural selection and whether it can be considered adaptive (Agrawal 2005a). Many studies have identified a heritable basis for constitutive and induced defence traits to one herbivore (Agrawal 1999; Havill & Raffa 1999; Underwood *et al.* 2000; Agrawal *et al.* 2002; Stevens & Lindroth 2005). In general, these studies report significant levels of genetic variation in both constitutive and induced responses, with as much as 30-fold variation among plant genotypes in their induced effects on herbivores (Underwood *et al.* 2000). Similarly, heritability of herbivore-specific induced responses is an important pre-requisite for pairwise co-evolution and specific defensive responses to evolve, yet the heritability of such response specificity has previously not been investigated.

To address how specific plant responses could evolve, it is also essential to study genetic correlations between responses to different herbivores and different traits of the plant, when attacked by different herbivores. For example, where negative genetic correlations exist between responses to different herbivores, evolution of optimal levels of defence for a particular herbivore may be constrained. Where responses are positively associated with high correlation coefficients, a plant's response to one herbivore will tend to reinforce its response to another, resulting in generalized elicitation. Conversely, plant defence traits that are genetically independent allow for response specificity to evolve, especially where there is a history of strong pairwise interactions between a plant and a specific herbivore.

An additional prediction from co-evolutionary theory is that plants that invest in defence traits in the absence of herbivores are hypothesized to be at a selective disadvantage compared to plants that do not invest (and *vice versa*; Whittaker & Feeny 1971). Given that defence is thought to be costly, it has been predicted that different modes of defence (i.e. constitutive vs. induced) or defences targeted at different attackers, should trade-off (Brody & Karban 1992; Zangerl & Bazzaz 1992). In particular, if a defence trait is constitutively expressed at a high level, it is predicted that induction is not needed; alternatively, if induction is a cost saving strategy, it should primarily be employed by genotypes with a low constitutive allocation (Zangerl & Rutledge 1996). Despite these long-standing predictions, data have not strongly supported trade-offs (reviewed by Koricheva, Nykanen & Gianoli 2004), and methodological issues have plagued tests for such trade-offs (Morris, Traw & Bergelson 2006).

In this study, we investigated the heritability of two aspects of induced defence in the common milkweed *Asclepias syriaca*: the broad-sense heritability of the induced traits themselves and that of the responses to two different herbivores. Milkweed exhibits several inducible traits that have been shown to impact the performance of specialist herbivorous insects. These traits include latex, which exudes from wounds follow-

ing damage, and cardenolides, which are found in all plant tissues (Agrawal 2005b; Rasmann *et al.* 2009). Latex functions as a physical barrier to feeding, gums up insect mouth parts, and often contains concentrated defence compounds (Agrawal & Konno 2009). Cardenolides act by disrupting sodium and potassium flux in cells and are toxic to most animals (Malcolm 1991). Both the amount of latex exuded upon damage and tissue cardenolide concentration are known to be heritable and to vary in response to different herbivores (Van Zandt & Agrawal 2004b; Agrawal 2005b; Mooney, Jones & Agrawal 2008). Here, we focus on the specificity of induced latex and cardenolide responses of *A. syriaca* to two leaf chewing lepidopteran herbivores, caterpillars of the monarch butterfly *Danaus plexippus* and the milkweed tussock moth *Euchaetes egle*.

Using a field experiment, we addressed the following questions: (i) is there specificity in induced responses and are there genetic correlations between responses to *D. plexippus* and *E. egle*, (ii) is there a trade-off between constitutive and induced allocation to these traits or between responses induced by the two herbivores, and (iii) do broad-sense heritabilities of the plant traits or the genetic correlations between the traits differ between herbivore damage treatments?

Materials and methods

STUDY ORGANISMS

Common milkweed (*A. syriaca*) is a perennial plant native to eastern North America and occurring in open habitats. It reproduces both clonally and sexually and is largely self-incompatible. The pollen of milkweed is contained in pollinia, which are transported as a unit during pollination; although it is possible to have fertilization by multiple pollinia, this is exceedingly uncommon for *A. syriaca* in the field, and progeny within a fruit have shared paternity (i.e. are full-sibs, Gold & Shore 1995).

Asclepias syriaca employs a variety of heritable defence traits, including the production of cardenolides, latex and leaf trichomes (Agrawal 2005b). Cardenolides increase systemically in concentration after damage to the plant (Malcolm & Zalucki 1996) and this induction was previously shown to be specific, with strongest induction following *D. plexippus* caterpillar herbivory and weaker induction following beetle damage (Mooney, Jones & Agrawal 2008). Latex also shows specificity of induction, with damage by beetles inducing a stronger response than *D. plexippus* caterpillars (Van Zandt & Agrawal 2004b). Despite this battery of defences, a variety of specialist herbivores with diverse feeding strategies use common milkweed (Agrawal & Malcolm 2002; Agrawal 2005b). These herbivores include foliage feeders such as *D. plexippus*, *E. egle*, and the milkweed leaf beetle (*Labidomera clivicollis*). Other insects feed on the stems (a weevil, *Rhyssomatus lineaticollis*), on roots (the red long-horned beetle larvae, *Tetraopes tetraophthalmus*), in leaf mines (a fly, *Liriomyza asclepiadis*), on sap (*Aphis asclepiadis*, *Aphis nerii*, and *Myzocallis asclepiadis*) and on seeds (*Lygaeus kalmii* and *Oncopeltus fasciatus*).

COMMON GARDEN STUDY

We used 20 full-sib families of *A. syriaca* collected near Ithaca, NY, USA, during the fall of 2007. Families were established from seeds of

single fruits collected from different parent plants in a single field. In April 2008, seeds from each family were germinated in petri dishes and seedlings were grown in 500 mL plastic pots with Pro-Mix BX soil and fertilized weekly with a dilute fertilizer (NPK 21:5:20, 150 ppm N). We fully randomized the plants (one per pot) in a growth chamber (400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light, 16 h/8 h light/dark cycle, 28 °C/24 °C temperature cycle) for over 1 month and then transplanted them to a ploughed field near Ithaca (total $n = 300$, mean of 15 plants per family). Plants were again randomized and enclosed in spun polyester mesh cages to prevent herbivory.

We randomly assigned one-third of the plants from each family ($n = 4-6$) to the following treatments: (i) unmanipulated control, (ii) herbivory by *D. plexippus*, and (iii) herbivory by *E. egle*. *Danaus plexippus* caterpillars were raised in the laboratory (on *A. syriaca* foliage) from locally collected adults. *Euchaetes egle* caterpillars were obtained from field collections and maintained in the laboratory until needed. We acknowledge that the two caterpillar species were reared under slightly different conditions before the experiment (field vs. laboratory collection), but we attempted to equalize rearing conditions by placing them together on cut *A. syriaca* foliage in the laboratory before treatments commenced. We imposed treatments in mid-July. We placed one or two third instar *D. plexippus* larvae on the upper leaves of each plant and allowed them to consume c. 15% of the leaf area (see Results). Because our *E. egle* larvae were smaller (second instar), we placed 2–5 larvae on the upper leaves of each plant. Although this approach varied the number of herbivores on plants, it allowed us to impose similar levels of damage in both herbivore treatments within a comparable time frame. Both caterpillar species were removed within 5–7 days of initiating feeding. After the caterpillars were removed, we visually assessed the location of and percentage damage for all plants. To assess differences in the distribution of damage, we recorded the median leaf position of all damaged leaves for each plant.

PLANT DEFENCE TRAITS

We measured plant traits 5 days after herbivores were removed from plants. We noted from field observations that *D. plexippus* fed primarily near the plant apex, while the *E. egle* primarily fed on lower leaves. Based on this observation, we incorporated a spatial component to our design by collecting data on defence traits from both upper and lower leaves.

To obtain data from upper leaves, we measured latex exudation on all plants by cutting the tip off the youngest, fully expanded, undamaged leaf and collecting the latex on a pre-weighed 1-cm filter paper disc. After absorbing all the latex, we placed the disc into a pre-weighed microcentrifuge tube. These tubes were stored in a freezer until they could be reweighed to estimate the amount of wet latex collected. This method is a repeatable measure of latex exudation and the amount of latex corresponds to resistance to herbivores (Van Zandt & Agrawal 2004b; Agrawal 2005b). A similar method was used to collect latex from the lower leaves, except here we used the oldest undamaged leaf to measure latex exudation. The order of collection (latex from lower vs. upper leaves) was randomized.

Immediately following latex collection, all the above-ground plant tissue was collected, separated into the top, middle and bottom third of the plant in large coin envelopes, and subsequently dried at 45–50 °C and weighed (we did not analyse trait variation for the middle tissues). Plants at this stage had > 20 leaves and were > 40 cm tall. Cardenolides were assessed by high performance liquid chromatography (HPLC) using methods modified from Malcolm & Zalucki (1996). Briefly, 50 mg dried leaf and stem tissue from each of the top

and bottom third of each plant were ground using a mixer-mill in 2-mL grinding vessels with steel balls. We added 1.8 mL methanol (MeOH) to each sample, spiked each sample with 20 μg digitoxin as an internal standard, and sonicated for 20 min at 55 °C in a water bath. After centrifugation, the supernatant was collected, dried, resuspended in 1 mL MeOH, and filtered through a 0.45- μm syringe-driven filter unit. Then, 15 μL of extract was injected into an Agilent Technologies (Santa Clara, CA, USA) 1100 series HPLC and compounds were separated on a Gemini C18 reversed phase column (3 μm , 150 \times 4.6 mm; Phenomenex, Torrance, CA, USA). Cardenolides were eluted on a constant flow of 0.7 mL min^{-1} with an acetonitrile–0.25% phosphoric acid in water gradient as follows: 0–5 min 20% acetonitrile, 20 min 70% acetonitrile, 20–25 min 70% acetonitrile, 30 min 95% acetonitrile, 30–35 min 95% acetonitrile. UV absorbance spectra were recorded from 200 to 400 nm by diode array detector. Peaks with absorption maxima between 217 and 222 nm were recorded as cardenolides and quantified at 218 nm. Concentrations were calculated and standardized by peak areas of the known digitoxin concentration; total cardenolide concentration was calculated as the sum of all individual cardenolide peaks and reported per mg dry mass tissue. We analysed the top third of all 300 plants for cardenolide concentrations. In addition, we sampled cardenolide chemistry by HPLC from the bottom third of 60 plants, one from each treatment of each family.

STATISTICAL ANALYSIS

We employed mixed-model ANOVA using restricted maximum likelihood (REML) estimation in JMP (Version 7, SAS Institute Inc., Cary, NC, USA, 2007). In the full model, treatment and leaf position were fixed effects, and family and all interaction terms with family were random effects. For random effects, significance was tested using the likelihood ratio test (Littell *et al.* 1996). Many subsequent analyses were conducted with reduced statistical models (e.g. where traits were not measured on both plant positions) or on reduced data sets (e.g. when estimating responses and heritabilities specific to one of the herbivores).

Genetic variances and broad-sense heritabilities within each treatment were calculated from variance components obtained through REML with models including only family. Full-sib heritabilities (H^2) were calculated by dividing two times the variance component of family by the total variance (Roff 1997); significance was tested by the P -value of the genetic family term. To estimate H^2 of plasticity (i.e. the induced responses to *D. plexippus* or *E. egle*), we estimated variance components from models including treatment as a fixed effect and used two times the family \times treatment variance component divided by the total variance (Relyea 2005); significance was tested by the P -value of this interaction term. We interpret these genetic effects with caution because full-sib analyses include maternal effects, dominance and epistasis in estimates of heritability and genetic correlations.

We also estimated the coefficient of genetic variation or 'evolvability' (CV) as $\text{CV} = (\text{variance} \times 2)^{1/2} / \text{mean trait value}$. Evolvability, a measure by which to compare genetic variation between traits, standardizes genetic variation to the mean rather than to the total phenotypic variation, and as such has been argued to be a better comparative metric than H^2 (Houle 1992). It may also be more informative than H^2 in studies such as the present one, where maternal effects can lead to inflated H^2 (Houle 1992). To calculate CV for the induced response to *D. plexippus* and *E. egle*, we used the square root of two times the variance component for the interaction term divided by mean induction (induced minus constitutive) by each herbivore.

For each defence trait, family means were determined for each of the three treatments. Family means were used for genetic correlations among traits within and across treatments. The metric used for induction was the difference in the trait (latex or cardenolides) between induced and control plants (Morris, Traw & Bergelson 2006). When calculating the genetic correlations between plant responses to *D. plexippus* and *E. egle*, we randomly allocated half of the controls to the *D. plexippus* treatment and half to the *E. egle* treatment. This allowed us to calculate independent induction metrics for both herbivores. Finally, to test for a trade-off between constitutive and induced responses, we used a bias-corrected Monte Carlo procedure which accounts for sampling variation, measurement error and induced susceptibility (Morris, Traw & Bergelson 2006).

Results

SPECIFICITY OF INDUCED RESPONSES

The distribution of damage imposed by the two caterpillar species differed substantially; *E. egle* damaged plants primarily at their base, while *D. plexippus* damaged near the apex (caterpillar species effect on median leaf position damaged: $F_{1,19,28} = 153.9$, $P < 0.001$, Fig. 1). Feeding by both *E. egle* and *D. plexippus* increased latex exudation compared to controls on apical leaves, while lower leaves showed no response to herbivory (Table 1, Fig. 2a). Control levels of latex were equivalent on upper and lower leaf positions, indicating that there was differential induction of latex depending on leaf position. Averaged across genetic families, latex exudation was also significantly stronger in response to feeding by *D. plexippus* (113% increase compared to controls) than to that by *E. egle* (28% increase; Fig. 2a); nonetheless, we found that families varied in their response to treatments (Tables 1 and 2).

Feeding by *E. egle* and *D. plexippus* induced cardenolides to a similar level (c. 26% increase) compared to controls on apical leaves, while lower leaves again showed no response to herbivory (herbivore treatment on apical leaves: $F_{2,37,91} = 4.16$, $P = 0.023$, on low leaves: $F_{2,39} = 1.23$, $P = 0.304$, Table 2, Fig. 2b). In addition, there was a strong family \times herbivore treatment interaction for cardenolides induction on the apical leaves ($\chi^2 = 75.50$, $P < 0.001$), but only a family effect for the lower leaves ($\chi^2 = 14.5$, $P < 0.001$).

Since we were unable to precisely control the amount of damage imposed by the two herbivore species, we tested for a

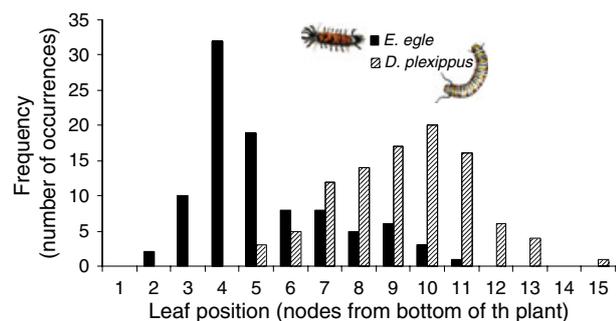


Fig. 1. Absolute frequencies for the distribution of median feeding damage by leaf position for *Euchaetes egle* and *Danaus plexippus* on common milkweed *Asclepias syriaca*.

Table 1. Overall mixed-model ANOVA testing for the effects of herbivory treatment (control, damage by *Euchaetes egle* or by *Danaus plexippus*), plant position and full-sib genetic family on latex exudation of *Asclepias syriaca*

Effect	d.f.	F -ratio or χ^2	P -value
Treatment	2,36.67	25.48	< 0.001
Position	1,19.06	21.74	< 0.001
Treatment \times Position	2,38.85	18.84	< 0.001
Family*	1	0.24	0.312
Family \times Treatment*	1	0.12	0.364
Family \times Position*	1	9.97	0.011
Family \times Treatment \times Position*	1	5.22	< 0.001

Treatment and position were fixed effects, while family and all interaction terms were random effects.

*For random effects, the F -ratio is replaced with chi-square values obtained from the difference between the -2 log likelihoods with that factor included vs. excluded from the model (Littell *et al.* 1996). We further separated the impacts of the different herbivores in (Table 2).

correlation between the amount of damage and the induced plant responses. While mean percentage damage by *D. plexippus* was higher than that by *E. egle* (mean \pm SE percentage leaf damage, $15.4 \pm 1.6\%$ vs. $10.7 \pm 0.6\%$, respectively, $t = 2.786$, $P < 0.05$), we found no correlation between the amount of damage and the amount of latex produced from apical leaves for either herbivore species ($r = 0.103$, $P = 0.457$ and $r = 0.002$, $P = 0.962$ for *E. egle* and *D. plexippus*, respectively), nor between the amount of damage

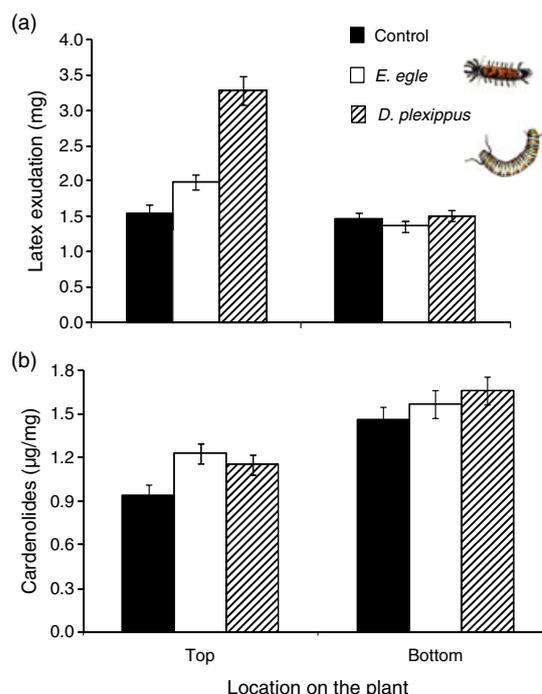


Fig. 2. Effects of damage by two specialist caterpillar species (*Euchaetes egle* and *Danaus plexippus*) on plant defence induction of common milkweed *Asclepias syriaca*. Shown are means \pm SE of: (a) latex exudation and (b) cardenolide concentration.

Table 2. Factorial mixed-model ANOVA testing for the effects of herbivory treatment and full-sib genetic family on latex exudation and cardenolides of *Asclepias syriaca*; analyses are from the top part of the plants and separated by the specific herbivore species

	d.f.	F-ratio or χ^2	P-value
<i>Danaus plexippus</i> – latex			
Treatment	1,19.09	36.45	< 0.001
Family*	1	3.39	0.033
Family \times Treatment*	1	6.08	0.007
<i>Euchaetes egle</i> – latex			
Treatment	1,19.09	11.32	0.004
Family*	1	6.63	0.005
Family \times Treatment*	1	0.45	0.251
<i>D. plexippus</i> – cardenolides			
Treatment	1,19.01	3.11	0.090
Family*	1	5.14	0.012
Family \times Treatment*	1	62.46	< 0.001
<i>E. egle</i> – cardenolides			
Treatment	1,18.72	10.90	0.004
Family*	1	1.42	0.117
Family \times Treatment*	1	27.44	< 0.001

Treatment was a fixed effect, while family and the interaction terms were random effects.

*For random effects, the F-ratio is replaced with chi-square values obtained from the difference between the $-2 \log$ likelihoods with that factor included vs. excluded from the model (Littell *et al.* 1996).

and apical cardenolide concentration [$r = 0.014$, $P = 0.899$ and $r = 0.190$, $P = 0.07$, for *E. egle* and *D. plexippus*, respectively, see (Fig. S1) in Supporting Information].

We found evidence for genetic variation in the induction (family \times treatment interaction) of latex and cardenolides, when we separated the treatments according to herbivore species (Table 2). With regard to latex, families responded distinctly to *D. plexippus* herbivory [16–175% increases across the 20 families; see (Fig. S2)]. In contrast, the interaction between family and *E. egle* herbivory was not significant for latex (Table 2). For cardenolides, we found strong family level variation for responses to both herbivores: the range for *D. plexippus* response was 290% increase to 50% decrease, while the range for *E. egle* was 198% increase to 17% decrease (Table 2, Fig. S2).

Differences in plant ontogeny or growth rate could potentially explain differences among genetic families for their investment in defensive traits or induction. However, all of our plants were at the same ontogenetic stage, having germinated at the same time and grown for the same amount of time. We also found no relationship between plant biomass and constitutive or induced levels of cardenolides or latex ($n = 20$, genetic family correlations, all $P \gg 0.1$).

BROAD-SENSE HERITABILITIES, GENETIC CORRELATIONS AND SPECIFICITY

Full-sibling heritabilities for latex exudation depended on the identity of the herbivore imposing damage (Table 3). In partic-

ular, the heritability for latex was low in the control ($H^2 = 0.301$) and *E. egle* ($H^2 = 0.207$) treatments, but was significantly higher in the *D. plexippus* treatment ($H^2 = 0.820$, comparison of heritabilities: $t > 2.03$, $P < 0.05$). Broad-sense heritabilities for cardenolides were significant and less variable across the three treatments (0.967–1.07, all $P < 0.001$, Table 3). Heritabilities for induction *per se* varied from zero (latex response to *E. egle*) to 1.67 ($P < 0.0001$; cardenolide response to *E. egle*; Table 3). Note that estimates of $H^2 > 1$ are possible with our full-sib design because we are estimating genetic variance in the presence of maternal effects, dominance and epistasis, and the suggested methods include doubling the family variance components (Roff 1997; Relyea 2005). Evolvabilities (CVs, Table 3) showed a similar pattern as broad-sense heritabilities and reinforce the evolutionary potential of the traits.

Genetic correlations for the strength of induction by the two lepidopteran herbivores differed for the two defence traits. When family means for latex induction by *D. plexippus* were plotted against those for *E. egle*, there was no correlation ($n = 20$, $P = 0.275$, Fig. 3). In other words, plant families that responded strongly with latex induction to *D. plexippus* did not necessarily show strong latex induction to *E. egle*. In contrast, we found a positive genetic correlation for plant responses to the two herbivores for cardenolides ($n = 20$, $r = 0.660$, $P = 0.001$, Fig. 3).

Investment in latex and cardenolides was not genetically correlated in the control treatment or when damaged by either herbivore species (Fig. S3). In other words, there was no treatment dependence for the genetically based allocation to the two traits.

TRADE-OFFS BETWEEN CONSTITUTIVE AND INDUCED DEFENCE

Using the Monte Carlo procedure developed by Morris, Traw & Bergelson (2006), we found no genetic correlation between

Table 3. Variance components (V_{fullsib}), evolvabilities (CV_{fullsib}) and full-sib (or broad sense) heritabilities (H^2) within treatments and for inducibility by each herbivore

Trait	V_{fullsib}	CV_{fullsib}	$H^2 \pm \text{SEM}$
Latex			
Control	0.208	26.960	0.301 \pm 0.176
<i>Danaus plexippus</i>	1.812	57.880	0.820 \pm 0.208
<i>Euchaetes egle</i>	0.116	24.339	0.207 \pm 0.172
Inducibility (<i>D. plexippus</i>)	0.557	61.009	0.310 \pm 0.199
Inducibility (<i>E. egle</i>)	-0.048	0.000	-0.077 \pm 0.115
Cardenolides			
Control	0.0685	39.423	0.995 \pm 0.199
<i>D. plexippus</i>	0.0852	36.118	0.967 \pm 0.203
<i>E. egle</i>	0.1068	37.605	1.070 \pm 0.197
Inducibility (<i>D. plexippus</i>)	0.1222	296.029	1.567 \pm 0.121
Inducibility (<i>E. egle</i>)	0.1363	165.225	1.669 \pm 0.096

All values are significant except *E. egle* inducibility for latex. Significance was determined from likelihood ratio tests for the family or family \times treatment effects (Table 2).

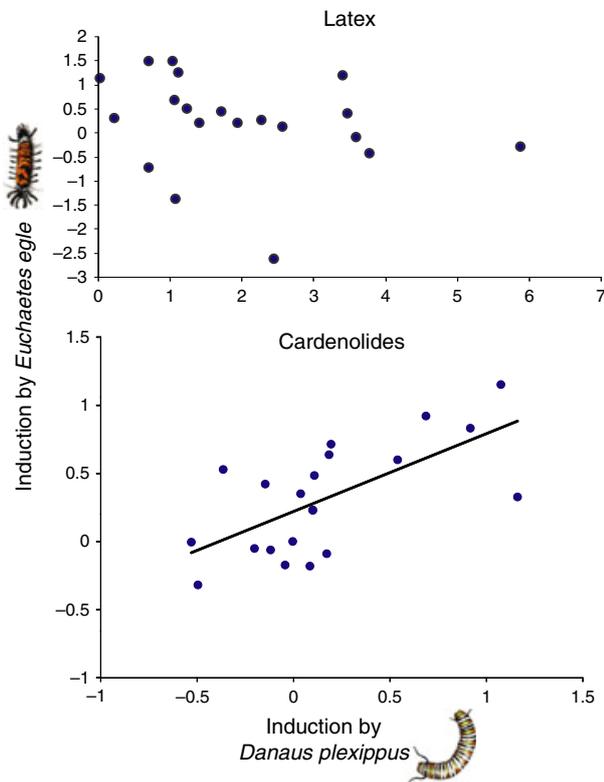


Fig. 3. Genetic correlations between induction (induced minus constitutive values) of latex and cardenolides for 20 full-sib families of *Asclepias syriaca* damaged by *Danaus plexippus* and *Euchaetes egle*. Separate control plants were used to calculate family means for induction by the two herbivores.

constitutive and induced (damage minus control) latex production ($r = 0.158$, lower fifth percentile of the bootstrap distribution of the correlation coefficient = -0.50 , Fig. 4). The probability of seeing a correlation as small (or smaller) was high ($P = 0.875$). For cardenolides, however, there was strong evidence for a trade-off between constitutive and induced allocation for both herbivores (*D. plexippus*, $r = -0.7511$, lower fifth percentile of the bootstrap distribution of the correlation coefficient = -0.4060 , $P < 0.001$, (Fig. 4); *E. egle*, $r = -0.4956$, lower fifth percentile of the bootstrap distribution of the correlation coefficient = -0.3818 , $P = 0.023$). In other words, those *A. syriaca* genetic families that showed strong constitutive production of cardenolides invested proportionally less in the induced cardenolide response.

Discussion

There is growing evidence that plants use highly specific cues to trigger physiologically distinct responses (Kessler & Halitschke 2007), however, most of this research is highly mechanistic and does not investigate how this specificity may evolve. In this paper, we provide the first report of heritable variation in response to two different herbivores. Our key findings were: (i) there was a genetic component to the induced responses of *A. syriaca* to herbivory by different herbivores; latex induction

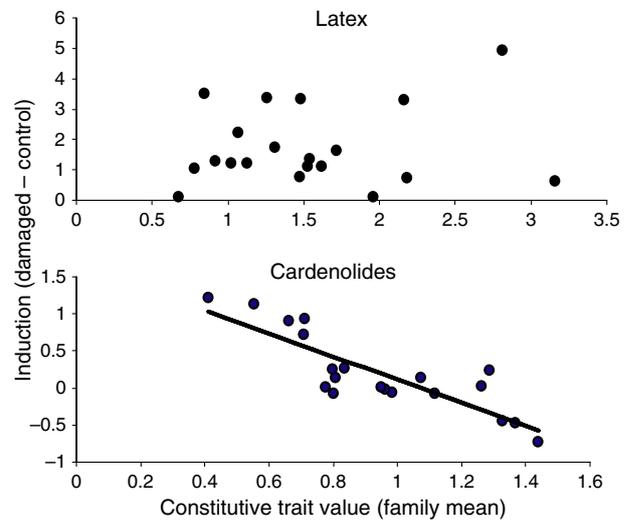


Fig. 4. Genetic correlations between constitutive and induced defence (by monarch *Danaus plexippus* feeding) for latex exudation and cardenolide concentration. Data points represent means for 20 full-sib families of *Asclepias syriaca*. Raw data are shown, although analyses are corrected for measurement error, sampling variation and induced susceptibility (Morris, Traw & Bergelson 2006).

was more variable in response to herbivory by *D. plexippus* than to herbivory by *E. egle*, while variation in cardenolide induction by the two species was similar; (ii) both latex and cardenolide induction were dependent on leaf position, with both occurring only near the plant apex; (iii) the latex responses to the two herbivores were not correlated, while the cardenolide responses were correlated; (iv) the latex response was independent of the cardenolide response under all conditions tested; and (v) there was a strong trade-off between constitutive and induced responses for cardenolides, but not for latex.

In this study, latex induction in common milkweed differed in response to herbivory by two leaf-feeding lepidopteran herbivores. Van Zandt & Agrawal (2004b) reported similar results for *A. syriaca* in response to herbivory by swamp milkweed beetles (*L. clivicollis*) and *D. plexippus*; in that case, the stronger response was elicited by the beetles. It is possible that oral secretions underlie these differences as has been proposed for *Nicotiana attenuata* (Voelckel & Baldwin 2004). However, it could also be that plants are responding to the different feeding locations of the insects (Orians 2005) as opposed to characteristics of the herbivores themselves, as was reported by Halitschke *et al.* (2000). In the latter case, the volatile response in *N. attenuata* was strongest when the second fully expanded leaf was damaged. Although the feeding location of the two herbivore species could be responsible for their specific latex responses in the current study, the cardenolide responses to the two herbivores showed no such pattern. It is also conceivable that the different amounts of damage imposed by the two herbivores could explain the different latex responses, as in other studies that reported a positive correlation between damage amount and induction response (Stout, Workman & Duffey 1994; Agrawal & Karban 2000); however, we found no such relationship for either herbivore or plant trait (Fig. S1).

EVOLUTIONARY ECOLOGY OF SPECIFIC INDUCED RESPONSES

Early studies of induced plant defences reported specificity of induction to different herbivore species or types of leaf damage (Karban & Carey 1984; Hartley & Lawton 1987; Turlings, Tumlinson & Lewis 1990; Tallamy & Raupp 1991; Stout, Workman & Duffey 1994). Nonetheless, whether such specificity is adaptive for the plant or the damaging herbivore remains highly controversial (Fowler & Lawton 1985; Geervliet *et al.* 1997; Dicke 1999; Mithofer, Wanner & Boland 2005). A criticism of specificity has been that any two herbivores, even siblings of the same species, may induce slightly different plant responses because of myriad differences in their vigour, gender, rearing history, presence of microbes, etc. Even if plants have general responses to damage, slight differences in the damaging cues may simply induce slightly different responses. How then can we test for evidence that specific plant responses may be shaped by natural selection?

A specific correspondence between the damaging herbivore and the impact on that herbivore would indicate that the plant response is potentially adaptive. For example, Takabayashi *et al.* (1995) demonstrated specificity in induced responses of corn plants damaged by either early or late instars of *Pseudaletia separata*; only plants damaged by the early instars induced volatile responses that attracted parasitoids (and only early instars are susceptible to parasitism). Of course, because some herbivores are highly specialized and adapted to particular plants, they may differentially induce plants for their own benefit. Cases of plant responses that do not negatively affect or even benefit some herbivores abound (Agrawal 2000; Fordyce 2003; Van Zandt & Agrawal 2004a; Viswanathan, Narwani & Thaler 2005). For example, Viswanathan, Narwani & Thaler (2005) reported strong asymmetric effects of previous herbivory by two leaf-feeding chrysomelid beetles *Psylliodes affinis* and *Plagiometriona clavata*. Early season herbivory on *S. dulcamara* by *P. affinis* significantly reduced oviposition and emergence of *P. clavata*, but not *vice versa*. Indeed, perhaps the most general pattern in specificity of induction is that some herbivores appear to be quite strong inducers, while others are not independent of the level of damage they impose (Karban & Carey 1984; Agrawal 2000; Traw & Dawson 2002; Voelckel & Baldwin 2004).

Here, we took an alternative approach in studying the evolutionary ecology of specific induced plant responses by asking if specificity could potentially evolve. We found significant (broad sense) heritable variation for induction of latex and cardenolides by *D. plexippus* in *A. syriaca*, but only heritable variation for cardenolide responses to *E. egle*. Interestingly, the two traits showed substantially different potential for the evolution of specificity: cardenolide responses were genetically correlated for the two herbivores (and were of equal magnitude, thus, no specificity), while latex responses were not correlated (potential for specificity to evolve). Given that allocation to latex and cardenolides was not genetically correlated in any of the treatment environments (or in previous studies, Agrawal 2005b), we predict that induced latex responses may evolve

divergently, with specificity, from cardenolide responses. Both latex and cardenolides have been shown to negatively impact *D. plexippus* (Dussourd & Eisner 1987; Zalucki, Brower & Alonso 2001; Agrawal 2005b), although little data exists on the impacts of these traits on *E. egle*.

As described above, heritabilities provide an indication of whether traits can evolve, and genetic correlations can help us understand how suites of traits will respond to natural selection. We interpret our results in this regard with caution because our estimates of genetic effects included maternal effects, dominance and epistasis. In addition, as is evident from the results of the current study, heritabilities and genetic correlations can be variable from one environment to another or among traits. This influence of the environment on genetic architecture is widely appreciated, but still poorly understood and no consistent pattern for the effect of the environment on genetic variation has been found (Hoffmann & Merila 1999; Sgro & Hoffmann 2004). Much of this research has focused on comparing patterns of genetic variation under favourable vs. stressful conditions, and while there is a trend towards higher heritabilities under stressful conditions, conclusions based on the sum of studies are equivocal (Hoffmann & Merila 1999; Sgro & Hoffmann 2004). Our results for latex indicated substantially higher heritabilities in the treatment with the *D. plexippus* damage (which also showed the strongest induction) caused by increased expression of phenotypic variation in this environment (Fig. S2). Evolvabilities followed the same pattern as broad-sense heritabilities for latex and cardenolides, and indicate considerable evolutionary potential for the induction of latex by *D. plexippus* and the induction of cardenolides by both herbivore species.

TRADE-OFFS BETWEEN CONSTITUTIVE AND INDUCED RESPONSES

The idea of fitness trade-offs is central to most scenarios for the evolution of plant defence, and theory has predicted that different modes of defence such as constitutive and induced allocation should trade-off (Zangerl & Bazzaz 1992). In a meta-analysis, Koricheva, Nykanen & Gianoli (2004) concluded that while there was not strong evidence for a trade-off between investment in different types of defence traits (e.g. trichomes vs. chemical compounds), there was evidence for a trade-off between constitutive and induced defence in 11 of 15 data sets analysed. Nonetheless, the authors expressed caution as to the generality of this conclusion, and, as pointed out by Morris, Traw & Bergelson (2006), methodological issues have undermined previous tests of this relationship. One of the best examples of an evolutionary trade-off between constitutive and induced responses, that of sinigrin induction in black mustard (Traw 2002), did not hold up after applying necessary statistical corrections (M. B. Traw, pers. comm.). We corrected for sampling variation, measurement error and induced susceptibility in our data, and report a strong trade-off between induced and constitutive cardenolides in *A. syriaca*. We did, however, find that traits responded differentially, with cardenolides showing the trade-off, but not latex. Thus,

although cardenolides may evolve without specificity to the two lepidopterans studied, their allocation is strongly genetically constrained by the mode of deployment (constitutive vs. induced).

Speculation and Conclusion

The fact that herbivores in different feeding guilds (i.e. suckers vs. chewers) induce different plant responses has become dogma; yet there is still some controversy as to the importance of within-guild differences in induction caused by herbivores. Our results clearly provide evidence for within-guild specificity and some potential for such specificity to evolve by natural selection. Examining multiple plant traits is clearly important, as we have demonstrated that different traits exhibit differing levels of specificity. The remarkably strong latex response to *D. plexippus* is well matched spatially to its feeding and may be adaptive given its susceptibility to latex (Zalucki, Brower & Alonso 2001). Plasticity itself (i.e. the ability to induce plant responses) showed a range of heritabilities depending on the trait and inducing herbivores. Although the two lepidopterans we studied fed on different parts of the plant, both traits were only inducible near the apex, again indicating spatial complexity to the herbivore-specific induction process. Given their lower feeding location, *E. egle* appears to evade induced plant responses.

Cardenolides showed treatment-independent heritabilities (which were quite high), a general response to the two herbivores, and expression of induction that was constrained by constitutive allocations (i.e. a trade-off). These results are consistent with natural selection shaping cardenolide expression. In contrast, latex appears to have the potential to evolve specificity, is unconstrained by trade-offs between constitutive and induced responses, and shows variable (and lower) heritabilities depending on the environment. Although natural selection can indeed act on latex exudation in *A. syriaca* (Agrawal 2005a,b), it appears to be less constrained. Such variability in the genetic architecture of plant traits underscores that specificity not only occurs in the elicitation of defence, but also in the evolution of diverse defensive responses to even closely related herbivores.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. No relationship between proportion leaf damage and the latex or cardenolide response of common milkweed.

Figure S2. Reaction norm plots for the impact of *Danaus plexippus* herbivory on 20 full-sibling families of common milkweed for latex and cardenolides.

Figure S3. No correlation between latex and cardenolides in any of the herbivore treatments.

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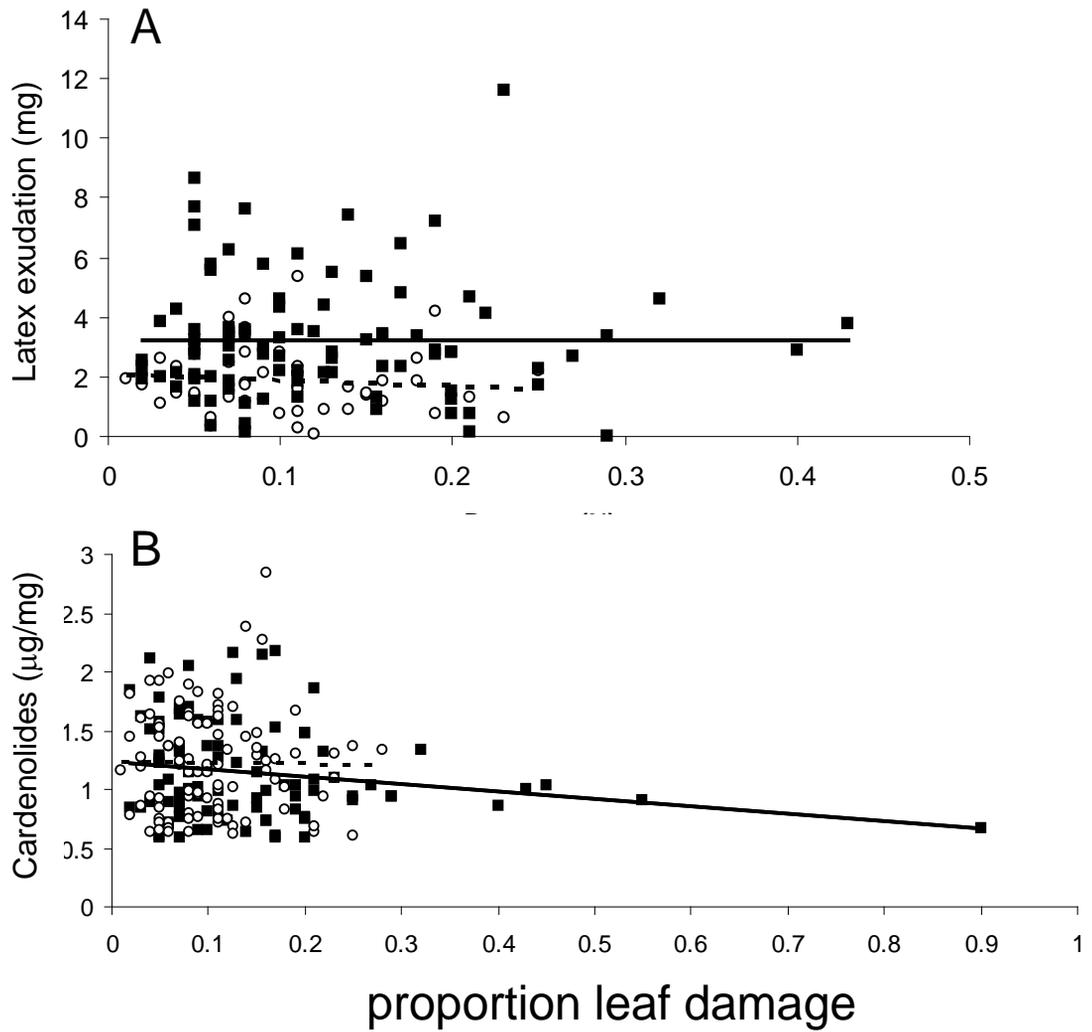


Figure S1: No relationship between proportion leaf damage and the A) latex or B) cardenolide response of common milkweed eaten by monarchs (solid circles) or *Euchaetes* (open circles). Control plants not shown.

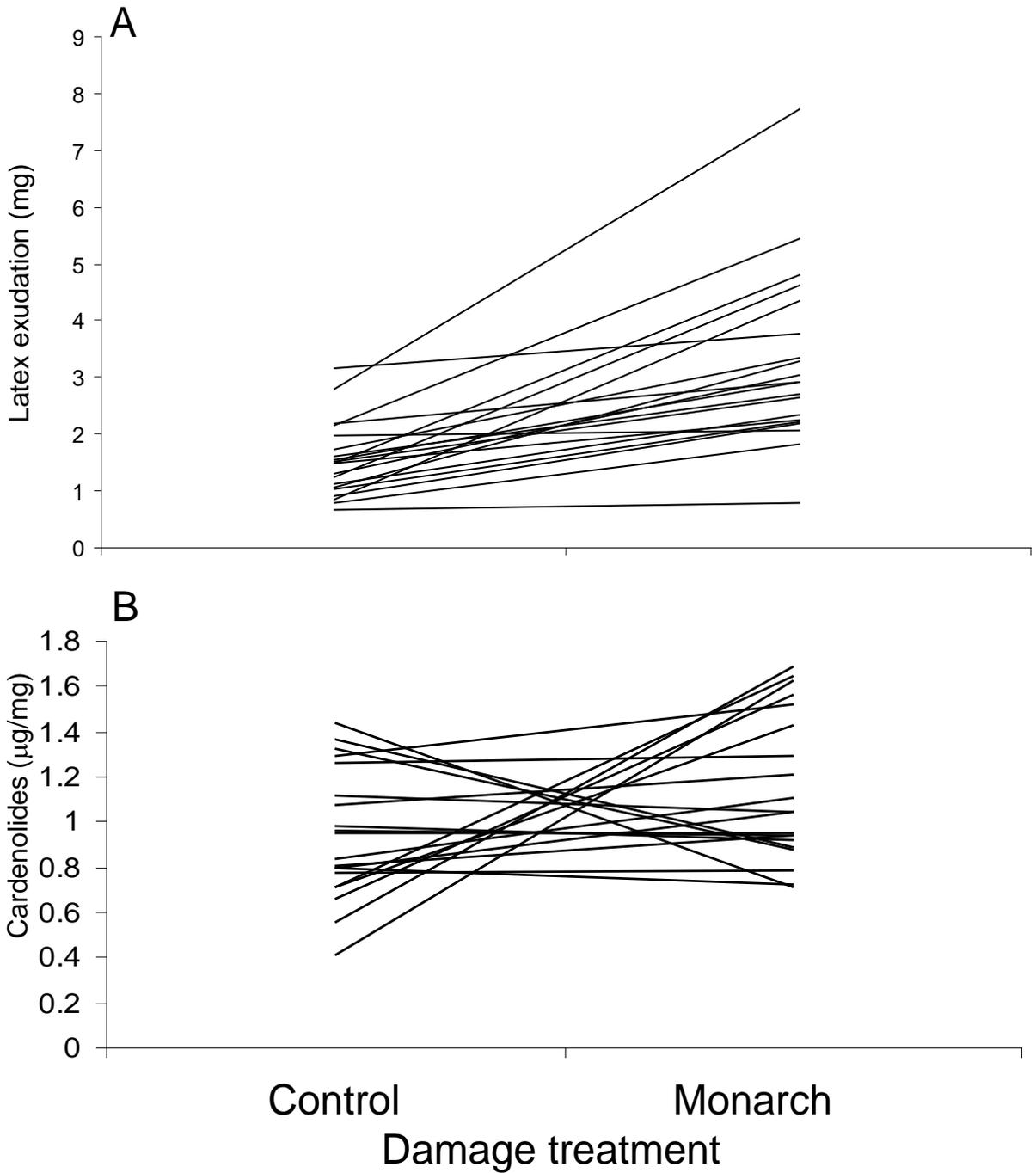


Figure S2: Reaction norm plots for the impact of monarch herbivory on 20 full-sibling families of common milkweed for A) latex and B) Cardenolides; the mean increase across families was >100% and >30% respectively (Figure 2)

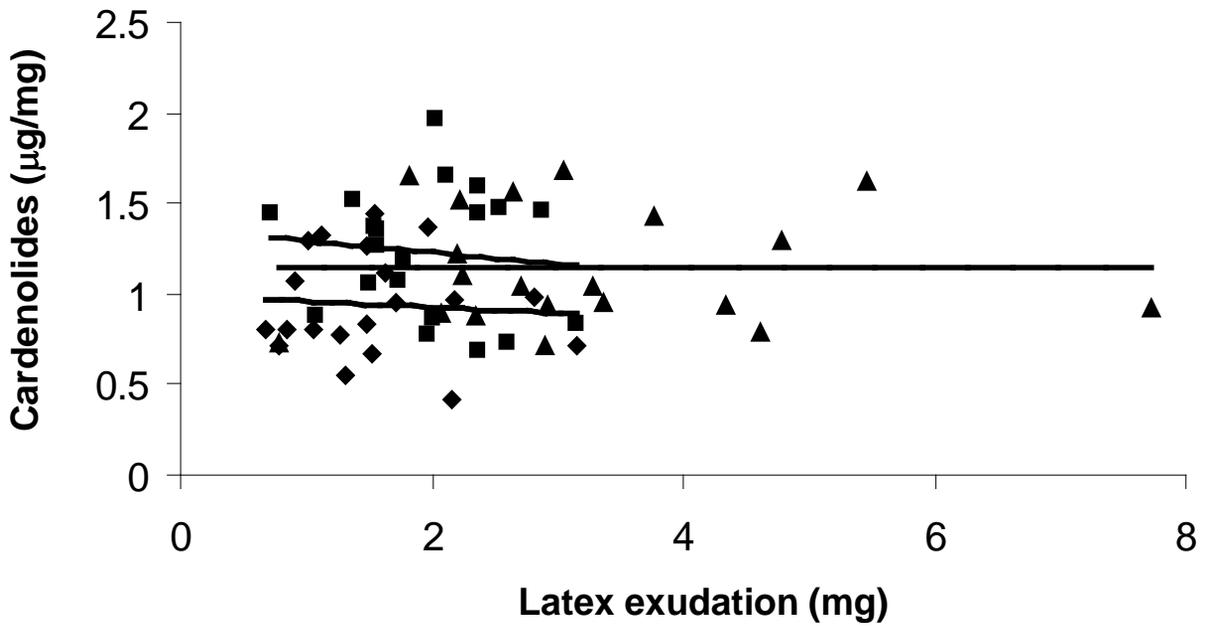


Figure S3: No correlation between latex and cardenolides ($n=20$ full-sibling families of common milkweed) in controls (diamonds), plants damaged by monarchs (squares), or plants damaged by *Euchaetes* (triangles).