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Evolution of latex and its constituent defensive chemistry in milkweeds (*Asclepias*): a phylogenetic test of plant defense escalation

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Abstract

A tremendous diversity of plants exude sticky and toxic latex upon tissue damage, and its production has been widely studied as a defensive adaptation against insect herbivores. Here, we address variation in latex production and its constituent chemical properties (cardenolides and cysteine proteases) in 53 milkweeds [*Asclepias* spp. (Apocynaceae)], employing a phylogenetic approach to test macroevolutionary hypotheses of defense evolution. Species were highly variable for all three traits, and they showed little evidence for strong phylogenetic conservatism. Latex production and the constituent chemical defenses are thus evolutionarily labile and may evolve rapidly. Nonetheless, in phylogenetically independent analyses, we show that the three traits show some correlations (and thus share a correlated evolutionary history), including a positive correlation between latex exudation and cysteine protease activity. Conversely, latex exudation and cysteine protease activity both showed a trade-off with cardenolide concentrations in latex. We also tested whether these traits have increased in their phenotypic values as the milkweeds diversified, as predicted by plant defense escalation theory. Alternative methods of testing this prediction gave conflicting results – there was an overall negative correlation between amount of evolutionary change and amount of latex exudation; however, ancestral state reconstructions indicated that most speciation events were associated with increases in latex. We conclude by (i) summarizing the evidence of milkweed latex itself as a multivariate defense including the amount exuded and toxin concentrations within, (ii) assessing the coordinated evolution of latex traits and how this fits with our previous notion of 'plant defense syndromes', and finally, (iii) proposing a novel hypothesis that includes an 'evolving community of herbivores' that may promote the escalation or decline of particular defensive strategies as plant lineages diversify.

Introduction

Latex production is remarkably common in plants. Nearly 8% of all plant species have pressurized canal systems from which latex is exuded upon damage, and this defense trait has evolved repeatedly among many plant taxa (Lewinsohn, 1991). In addition, latex has been implicated as a key innovation that has spurred adaptive radiation in plants. Clades with latex-bearing plants are significantly more species-rich than sister clades lacking latex (Farrell et al., 1991). Functionally, latex has no known role in primary metabolism (resource acquisition and allocation) and has been strongly implicated as a defense against chewing herbivores (Figure 1) (Dussourd & Eisner, 1987; Dussourd & Denno, 1991, 1994; Zalucki & Malcolm, 1999; Zalucki et al., 2001; Agrawal, 2005).

Although the defensive function of latex has historically been ascribed to the physical action of coating the insect and gumming up its mouthparts, there is some evidence for potent chemical defenses in latex (Shukla & Krishna Murti, 1971; Nelson et al., 1981; Konno et al., 2004; Stepek et al., 2005). For example, many milkweeds [*Asclepias* spp. (Apocynaceae)] have concentrations of cardenolides

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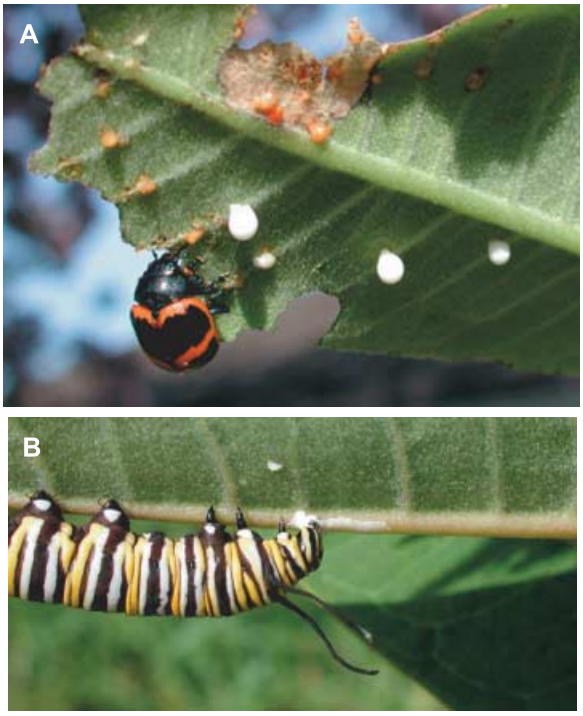

Figure 1 Common milkweed, *Asclepias syriaca*, being fed upon by (A) the milkweed leaf beetle, *Labidomera clivicollis* (Coleoptera: Chrysomelidae) and (B) a larva of the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Nymphalidae). Note the latex exuding from where the beetle has severed the veins and where the caterpillar is notching the mid-vein. Photos taken by Pete Van Zandt.

(steroids that disrupt cardiac ATPase function) that are orders of magnitude higher in their latex compared to their leaves (Nelson et al., 1981; Zalucki et al., 2001). In addition, the latex of many species, including milkweeds, contain cysteine proteases (Arribere et al., 1998; Trejo et al., 2001; Liggieri et al., 2004; Stepek et al., 2005), which have recently been implicated as toxins that digest the insect peritrophic membrane (Pechan et al., 2002; Konno et al., 2004). Regardless of the specific mode of action, latex has been widely demonstrated to affect the behavior (Dussourd & Eisner, 1987; Dussourd & Denno, 1991; Dussourd, 1997), performance (Zalucki & Malcolm, 1999; Zalucki et al., 2001; Van Zandt & Agrawal, 2004), and abundance of herbivores (Agrawal & Van Zandt, 2003; Agrawal, 2005).

Despite the impact of latex on herbivores, nearly all of the chewing herbivores of milkweed are specialists and have some mechanism to disarm the latex defense (although very few *Asclepias* species have been well-studied in this respect). Even when insects behaviorally deactivate latex, they often consume quite a bit and are physically exposed to it. This is further complicated by the fact that many milkweed herbivores sequester cardenolides. For example, Dixon et al. (1978) wrote in passing that fifth instar monarchs sometimes drink latex from plant wounds. One of us (Agrawal) has also observed larvae imbibing latex, but this has only been while very carefully notching a leaf (Figure 1B), presumably to depressurize the leaf. When laticifers are severed on leaves, larvae do not notch them or imbibe latex oozing from wounds (Dussourd & Eisner, 1987; AA Agrawal, pers. obs.). We thus interpret the latex imbibing behavior as a 'necessary cost' associated with fully deactivating the laticifers. Indeed, a substantial amount of evidence indicates that, despite adaptations of milkweed feeding insects, both latex and cardenolides reduce the performance or abundance of these herbivores. For example, when laticifers are severed, monarch larvae have a higher probability of survival compared to that on controls (Zalucki & Malcolm, 1999; Zalucki et al., 2001). We have also shown that genetic families of *Asclepias syriaca* L. that have higher latex exudation also have greater resistance to several insect species (Agrawal & Van Zandt, 2003; Van Zandt & Agrawal, 2004; Agrawal, 2005).

In this study, we examine the evolution of latex production and the concentrations of cardenolides and cysteine proteases in 53 milkweed species. Employing a phylogenetic approach, we address the following specific questions: (i) How much variation is there in plant-produced latex and associated chemical toxins? (ii) Are the levels of these three properties of latex correlated across species, and if so, have the correlations arisen independently of shared evolutionary history (Felsenstein, 1985)? and (iii) Is there evidence for escalation in these defensive traits as milkweeds have diversified (Ehrlich & Raven, 1964; Berenbaum, 1983; Futuyma, 1987; Vermeij, 1994)? We specifically define escalation in plant defense as a directional trend for increased anti-herbivore traits during the macroevolution of a lineage.

Materials and methods

Plants and growth

Asclepias in the narrow sense includes about 130 species in North America, including Mesoamerica and the Caribbean, and six additional species endemic to South America (Woodson, 1954; Bollwinkel, 1969; M Fishbein, unpubl.). Seeds of 53 species (51 *Asclepias* species and two species in the genus *Gomphocarpus*, which belongs to the African sister group of *Asclepias*) were collected by the authors and their colleagues or purchased from native plant nurseries (sources are given in the Acknowledgements section). Plants were grown in a glasshouse and are maintained in a permanent collection with periodic repotting and pruning of the plants. Although replication

varied slightly, we had an average of five plants per species; plants were completely randomized in the glasshouse.

Latex measurement

We measured latex from 51 of the species (Appendix 1) by cutting the tip off (0.5 cm) of the youngest fully expanded intact leaf and collected the exuding latex onto a pre-weighed 1-cm disc of filter paper. Latex stopped flowing after ~10 s, all latex was absorbed on the filter paper, and this disc was placed in a pre-weighed microcentrifuge tube. Tubes were then weighed to estimate wet latex exudation per plant, opened and dried at 60 °C, and finally re-weighed to estimate dry mass of latex. Wet and dry latex masses were highly correlated ($r = 0.887$, $P<0.001$) and thus we only report on results for wet latex production. Our method is a repeatable assay for determining latex exudation, it likely reflects what chewing insects must contend with, and has been shown to negatively correlate with the growth or preference of milkweed herbivores (Agrawal & Van Zandt, 2003; Van Zandt & Agrawal, 2004; Agrawal, 2005). We also removed the leaf that was clipped and measured its area to assess the relationship between leaf size and latex exudation.

Latex chemistry

The chemistry of the latex was assessed on a subset of the 53 species because a few species either did not produce sufficient latex for analysis (e.g., *Asclepias tuberosa*) or were senesced or in poor health at the time of these chemical analyses. Cardenolide content of the latex from 37 species (Appendix 1) was assessed by pipetting 5 µl of latex from a freshly cut leaf into 1 ml 95% ethanol. We then used a standard spectrophotometric microplate assay, employing detection of cardenolides using the tetranitrodiphenyl reaction (Brower et al., 1972; Agrawal, 2004) with digitoxin as the standard and absorbance read at 620 nm.

To measure the cysteine protease activity in the latex (36 species; Appendix 1), we employed a modification of the spectrophotometric protocol developed by Konno et al. (2004). Briefly, we made a 0.6% latex solution by diluting 5 µl latex in 833 µl sodium phosphate buffer. A reaction buffer with casein as the substrate was incubated for 30 min and stopped with trichloroacetate. After precipitating the undigested casein, the supernatant was read at an absorbance of 280 nm.

Statistical analysis

Interspecific variation in components of latex investment (latex amount, cardenolide concentration, and cysteine protease activity) were analyzed with one-way ANOVA in JMP (version 6, SAS Institute, Cary, NC, USA). Pairwise associations between the trait levels measured in each species were performed with Pearson product-moment correlations; these correlations do not distinguish the contributions of shared ancestry and independent adaptation and will henceforth be referred to as 'raw' correlations. Phylogenetically independent contrast (PIC) analyses were conducted using a pruned phylogeny of *Asclepias* (M Fishbein, unpubl.) (Figure 2). Briefly, the phylogeny was estimated using Bayesian inference implemented in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) from a data set of 145 samples, including nearly all species of *Asclepias* (which is exclusively American in the strict sense), 20 representatives of the African sister group, and representatives of four genera forming the outgroup. Sequence data were obtained from three non-coding regions of the chloroplast genome (*rpl16* intron, *trnC-rpoB* intergenic spacer, and *trnS-trnG* spacer/*trnG* intron); this data set is expanded from that used by Agrawal & Fishbein

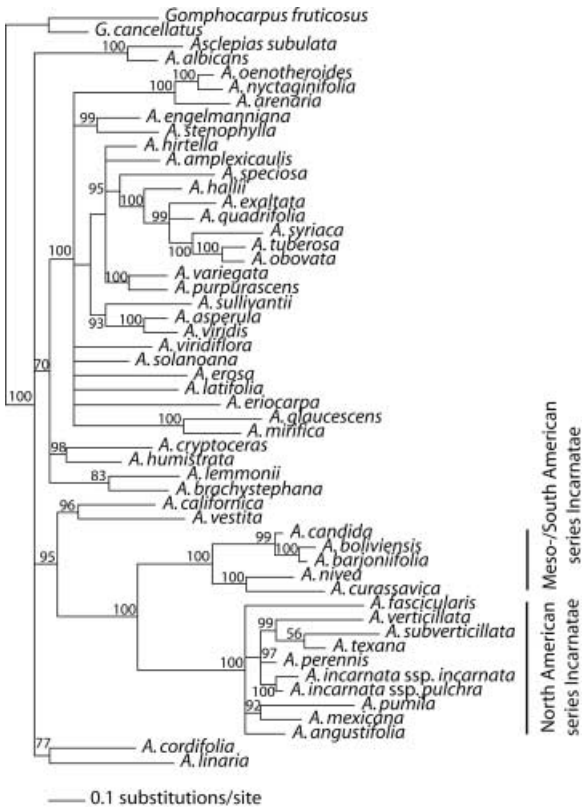

Figure 2 Phylogram of 51 species of new world *Asclepias* and two species of *Gomphocarpus* (from Africa) examined in this study, pruned from a comprehensive phylogeny of *Asclepias*. The complete phylogeny was the 50% majority rule consensus of trees sampled from the stationary distribution of a Bayesian analysis of three non-coding cpDNA regions sampled from 145 taxa; numbers indicate Bayesian posterior probabilities (M Fishbein, unpubl.).

(2006). Branch lengths were estimated by maximum likelihood on the 50% majority-rule consensus of trees sampled from the stationary distribution of a Markov Chain Monte Carlo exploration of tree space. This tree (a phylogram, in which branch lengths represent expected nucleotide substitutions per site) was converted to a chronogram (in which branch lengths represent time) using penalized likelihood implemented with r8s (Sanderson, 2002, 2003), with cross validation to optimize the rate smoothing parameter. Trees were modified to include only those taxa for which phenotypic traits were measured by pruning terminal branches leading to taxa for which trait data were unavailable.

To test for correlations among defense traits using PICs, we employed two gradual and one speciational model of trait evolution: (i) a gradual model, in which rates of change in defense traits are proportional to rates of molecular evolution (using the phylogram); (ii) a gradual model, in which rates of change in defense traits are proportional to the temporal duration of lineages between speciation events (using the chronogram); and (iii) a speciational model, in which rates of change in defense traits are proportional to the number of speciation events in a lineage (using a cladogram, in which branch lengths are of equal length and the topology of relationships is identical to the phylogram and chronogram). These three models correspond to distinct assumptions about the mode of evolution of latex traits, for which we have no a priori knowledge; hence, our approach permits us to explore how assumptions about the mechanism by which traits evolve impact conclusions about the causes of the observed variation among species.

To assess independent, correlated evolution of latex attributes, we used phANOVA (version 1.1, available from www.herbivory.com, under 'lab members, Lajeunesse'). phANOVA uses the generalized least squares method for calculating independent contrasts (given that PICs are a special case of this statistical framework; Rohlf, 2006). The generalized least squares method codes the phylogeny as a variance-covariance matrix (**V**) to account for the correlated relationships between species (e.g., all off diagonals of the matrix contain the shared branch length distances between each species; Martins & Hansen, 1997). Because our best estimate of the phylogeny is not fully bifurcating (Figure 2), the degrees of freedom for statistical tests involving PICs were conservatively adjusted down by the number of polytomies (following Purvis & Garland, 1993).

We tested for directional trends in latex evolution in three ways. For two of these methods, we employed regression involving observed trait values. To test the hypothesis of a directional trend, in which traits exhibit an escalation in expression at each speciation event (see model 3, above, for calculating PICs), we employ a simple method advocated by Harvey & Pagel (1991). Their method predicts that the number of speciation events (i.e., number of intervening nodes) from the ancestor of a clade to species tips will correlate with trait values in those species. There are various arguments in the literature for why there may be such speciational trends (Futuyma, 1987; Grant, 1989; Harvey & Pagel, 1991; Mooers et al., 1999), and at the most basic level, the hypothesis being tested is that speciation itself is associated with directional change in phenotypic traits. We tested for speciational trends separately for each trait by regressing the raw trait values on the number of intervening nodes between each taxon and root of Asclepias, determined using the full phylogeny of the genus (not shown). Second, we performed a corresponding analysis that tests a gradual, directional model of trait evolution (see model 1, above, for calculating PICs) by regressing the raw trait values on the summed branch lengths from the root to each terminal node. Note that a test for directional trends in trait evolution cannot implement the assumptions in model 2 for calculating PICs, as the temporal durations from the root to all terminal taxa are equivalent. These approaches examine the overall amount of change between the root and the phenotypic values of extant species, but not the intervening steps (Figure 3).

Third, we also tested for directional trends in latex evolution by reconstructing ancestral states for each trait separately at all nodes of the pruned phylogeny, and enumerating the direction of change in the trait at each node (Baker & Wilkinson, 2001). This method also implements a speciational model of trait evolution, in which all changes in trait values are assumed to occur only at speciation events (i.e., nodes on the phylogeny). The hypothesis being tested using this method is that evolutionary change is directional, in as much as there is a preponderance of increases (or decreases) across speciation events. Note that this method does not address the magnitude of phenotypic change, but only the direction of change at individual nodes (Figure 3).

Ancestral states were reconstructed for each character under the assumption of linear parsimony (Swofford & Maddison, 1987) as implemented in MacClade 4.08 (Maddison & Maddison, 2005). Each species was valued by its mean trait value (Appendix 1). Because linear parsimony may not provide accurate estimates of ancestral states under some conditions, we limit our interpretations to the overall direction of changes at nodes and not to specific states. Linear parsimony requires a fully bifurcating phylogeny, which is problematic due to several polytomies in our best estimate of the phylogeny (Figure 2). Thus, we randomly resolved polytomies in MacClade and investigated the sensitivity of our conclusions by repeating analyses on 50 randomly resolved topologies.

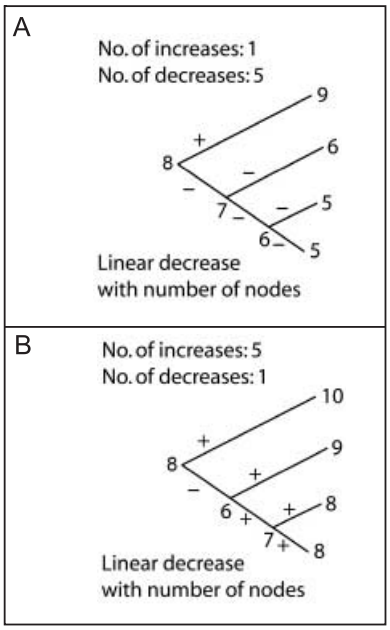

Figure 3 Schematic diagrams indicating two approaches to testing for evolutionary trends with (A) the same or (B) differing outcomes. Numbers at branch tips indicate a phenotypic value for extant species, and numbers at nodes indicate hypothesized ancestral trait values. The first approach is to count the number of speciation events (i.e., intervening nodes), or estimate the branch length, from the ancestor to the tips and to assess the correlation of these measures of evolution with trait values in those species. In the second approach, we reconstructed ancestral states for each trait separately at all nodes of the pruned phylogeny, and enumerated the direction of change in the trait at each node. Symbols represent a positive or negative change from each hypothesized ancestor. As discussed in the text, these two methods test slightly different hypotheses.

Because regression and ancestral state approaches use different techniques for assessing the existence of evolutionary trends, it is possible for these approaches to yield conflicting results (Figure 3). Conflicting results highlight the need for explicit formulation of hypotheses about evolutionary trends, a point that we explore in the Discussion.

Results

Trait variation

Production of latex varied over 1 000-fold across the 51 milkweed species (range 0.04–46.72 mg wet mass; coefficient of variation (CV) = 1.90, one-way ANOVA: $F_{50,162} = 6.043$, $P<0.001$), and latex exudation did not correlate with leaf area ($r = 0.182$, $P = 0.201$; $n = 51$). Thus, our measure of latex production is not simply a function of leaf size, which may be influenced by various environmental factors. The level of variation in latex exudation was substantially higher in magnitude than that of its constituent chemicals (measured in equal amounts of latex), with cardenolides varying over 18-fold across 37 species (range 0.07–1.28 optical density units; CV = 0.79, $F_{36,111} = 2.339$, $P<0.001$) and cysteine proteases varying 24-fold across 36 species (range 0.01–0.24 optical density units, CV = 0.59; $F_{35,136} = 10.737$, $P<0.001$).

Trait correlations

Raw correlations between latex production, cardenolide concentration, and cysteine protease activity were all positive in direction, but correlation coefficients were very small (all <0.07) and none were statistically significant (Table 1). However, after accounting for patterns of shared evolutionary history, many of the trait correlations became significant (Table 1). The strongest relationship among the three pairwise correlations is the negative association between cardenolides and proteases found with both gradual methods of calculating independent contrasts (Table 1, Figure 4). In contrast, the non-significant positive correlation using raw trait values was mirrored by a marginally significant positive correlation between PICs calculated assuming speciational evolution of the traits. There were significant correlations between latex production and the amounts of the two chemicals, when PICs were calculated using either of the gradual models – a positive correlation with cysteine protease activity and a negative correlation with cardenolide quantity (Table 1). Both correlations were positive, but not significantly so, when PICs were calculated with the speciational model.

Table 1 Pairwise correlations among latex production and amounts of cardenolides and cysteine proteases found in latex, across species of *Asclepias*. Raw and phylogenetically independent correlation (PIC) coefficients (assuming three models of evolution) are shown and significance (P<0.05) is indicated in bold and by: †(P<0.1), *(P<0.05), **(P<0.001). Sample sizes were n = 37 (latex-cardenolide), n = 34 (latex-protease), and n = 32 (cardenolide-protease)

	Raw	PIC-phylogram	PIC-chronogram	PIC-cladogram
Latex-cardenolides	0.069	–0.312†	–0.342*	0.206
Latex-proteases	0.021	0.301†	0.338*	0.208
Cardenolides-proteases	0.034	–0.435*	–0.475**	0.345†

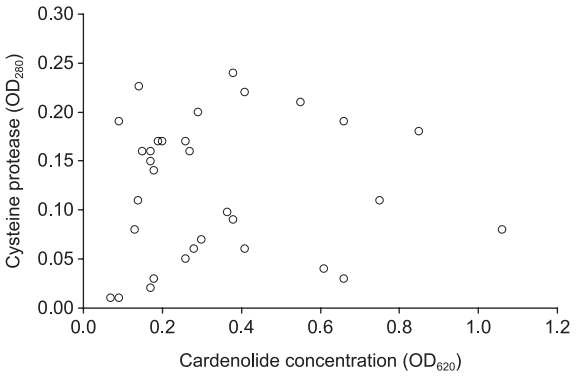

Figure 4 Raw correlation between cardenolide concentration and cysteine protease activity across 32 species of milkweed (*Asclepias*). OD = optical density difference between sample and control at the subscripted wavelength. Assuming gradual models of evolution, this relationship shows a significant trade-off between investment in cardenolides and cysteine proteases (Table 1).

Macroevolutionary escalation of defensive phenotypes

We used three methods to test the hypothesis of defense escalation as *Asclepias* diversified. First, we regressed the traits values (separately) for latex, cardenolides, and cysteine proteases on the number of intervening nodes between the ancestor of *Asclepias* and each species. The analyses were conducted using raw trait values. Contrary to theory, we found a significant 'deceleration' or decline in latex production as *Asclepias* diversified (Table 2, Figure 5). This result was consistent when employing the speciational and gradual model, although the gradual model explained the most variance (Table 2). The result for the speciational model was only marginally significant without the removal of an outlier, *Asclepias lemmonii* Gray, which exhibited extraordinarily high latex production (Table 2, Appendix 1). Regardless of method, phylogenetic distance explained a relatively small proportion of variation (<0.20) in latex production (Figure 5). No significant trends were found for cardenolides or cysteine proteases.

We also tested for trends in trait evolution using ancestral trait reconstruction by recording the direction of trait changes at each speciation event and testing for bias against the expectation of equiprobable increases or decreases. For all three traits, there were numerous cases in which there were equally parsimonious reconstructions of ancestral states, making the inference of the direction of change at these nodes ambiguous; thus, only unambiguous changes were tallied. Using this approach, we found a trend toward increasing latex production (Figure 6). The number of evolutionary increases exceeded the number of decreases for 37 of the 50 randomly resolved topologies, the converse was true for only four of the topologies, and for the remaining trees (nine) increases and decreases were tied. If the randomly resolved topologies can be considered a random sample of the possible phylogenies of *Asclepias*, the absence of a trend in latex production can be rejected (sign test: $n+ = 37$, $n- = 4$; $P<0.001$). The preceding result was obtained with the extreme latex producer, *A. lemmonii*, omitted; if this species is included, the result is greatly strengthened with all random resolutions showing a preponderance of evolutionary increases. Note that this statistical test indicates that the pattern of more increases is robust to uncertainty regarding the proper resolution of the phylogeny of *Asclepias*; the strength of this pattern (i.e., how many more increases than decreases), however, was not statistically compared. This evidence for a trend toward increasing latex appears to conflict with that obtained by regressing latex production on the number of speciation events or total branch lengths, which found a negative relationship between speciation events and latex production (Table 2). We further explore the conflicting results for latex production in the Discussion (see also Figure 3).

For cardenolides, there was weaker evidence for a trend toward increasing investment in defense. For the 50 randomly resolved topologies, 26 reconstructions of ancestral states

Table 2 Regression tests for evolutionary trends for latex production and its constituent chemical defense traits (cardenolides and cysteine proteases) across milkweed (*Asclepias*) species. See text for explanation of statistical methods. Significant effects are shown in bold. Latex production is analyzed twice; in the second analysis (*), one outlier species (*Asclepias lemmonii*) is removed because it produced nearly 250% more latex than the next highest species (see Appendix 1). Regressions are calculated on number of nodes to root (speciational) or total branch length to root (gradual)

		Speciational				Gradual			
Trait	d.f.	F	P	Slope	R^2	F	P	Slope	R^2
Latex	1,49	2.846	0.098	–0.925	0.055	5.973	0.018	–12.308	0.109
Latex (*)	1,48	6.736	0.012	–0.824	0.123	11.894	0.001	–9.913	0.198
Cardenolides	1,35	1.910	0.176	–0.037	0.051	0.761	0.389	–0.197	0.021
Proteases	1,34	1.710	0.200	0.009	0.048	0.236	0.630	0.027	0.007

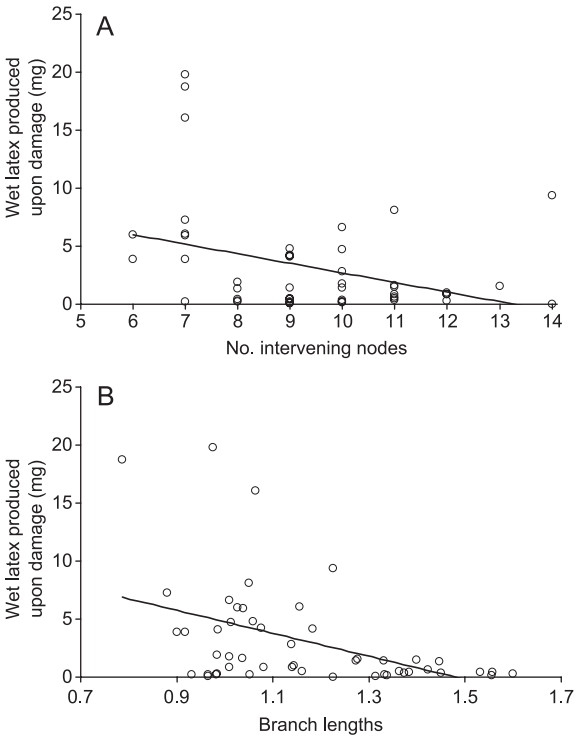

Figure 5 Phenotypic decline in the production of latex (in mg) as the milkweed genus *Asclepias* diversifies (n = 50). Trait values for each species are regressed on (A) the number of nodes between the species and the ancestor of *Asclepias*, determined from the unpruned, comprehensive phylogeny of *Asclepias* (M Fishbein, unpubl.) or (B) the total branch length between the species and the ancestor of *Asclepias*, determined from the unpruned, comprehensive phylogeny of *Asclepias* (M Fishbein, unpubl.). The outlier (*Asclepias lemmonii*) has been removed from this graph, which has a nodal value of 9 (branch length of 1.043) and produces a whopping 47 ± 16 mg of latex (Table 2, Appendix 1).

showed a greater number of unambiguous increases than decreases (sign test: n+ = 26, n– = 14; P = 0.08). In contrast, only 14 resolutions showed a preponderance of decreases (they were equal in 10 cases). For cysteine protease activity, there was similarly weak evidence for a trend toward increasing investment in defense. In this case, 25 of the randomly resolved topologies showed a preponderance of increases, 13 showed a preponderance of decreases, and they were equal for 12 (sign test: n+ = 25, n– = 13; P = 0.07).

Discussion

Within plant taxa that typically produce latex, there is still substantial variation in the amounts produced following tissue damage. This variation is hierarchical: from phenotypic plasticity within a species, to heritable genetic variation within a species, to across-species variation. For example, following damage by herbivores, some species of *Asclepias* can more than double latex exudation (Van Zandt & Agrawal, 2004; S Cook, A Erwin & A Agrawal, unpubl). Genetically based variation within *A. syriaca* accounted for up to four-fold variation in latex production across full-sibling families in a common field environment (Agrawal & Van Zandt, 2003; Agrawal, 2005). Finally, as demonstrated in this study, latex production can vary from nearly zero to upwards of 46 mg exuded upon minor tissue damage.

Latex production showed some evidence of phylogenetic conservatism, yet was also remarkably labile. For example, in the North American subclade of *Asclepias* series Incarnatae (as emended by M Fishbein, unpubl.; Figure 2), all species produced very low latex amounts (all under 0.5 mg) (Appendix 1). In contrast, in the mostly South American subclade of the same series (Incarnatae) (Figure 2), there was over 20-fold variation in latex production. Similarly, some sister taxa, such as *A. tuberosa* and *A. obovata*, have dramatically diverged in latex production, with the former showing an almost complete loss of latex. Given the heritable variation in latex production within a species and some evidence for natural selection acting on this trait (Agrawal, 2005), it is not surprising that closely related taxa can strongly diverge. However, any adaptive

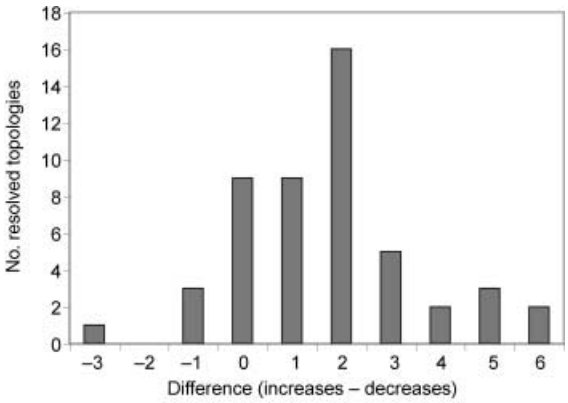

Figure 6 A trend toward increasing latex production in milkweed (*Asclepias*) (n = 50). Frequency distribution of the difference between the number of speciation events in which latex production is inferred to have increased and the number in which it is inferred to have decreased, for 50 randomly resolved, pruned phylogenies of *Asclepias* (see Figure 2). The null hypothesis of an equal tendency for latex production to increase or decrease is represented by zero on the *x*-axis and is rejected (see text). The preponderance of positive values for the difference indicates support for a trend toward increasing latex production.

role of latex in divergence and speciation has yet to be demonstrated.

Latex as a multivariate defense

Although plant defenses have traditionally been studied as single-trait weapons, a fuller understanding of plant defense ecology and evolution can be gained by simultaneous, integrative studies of suites of traits (Feeny, 1976; Agrawal & Fishbein, 2006; Fine et al., 2006). Clearly, latex functions through multiple modes of action, including physical barriers to consumption and toxicity. Thus, the level of resistance provided should depend, not only on the dose of latex, but also on the concentration of toxins within it. We show an evolutionary trade-off (after accounting for phylogenetic relatedness) between cardenolides and both proteases and latex production, with a concomitant positive correlation between latex production and proteases (Table 1). Positively correlated evolution of latex production and cysteine protease activity is consistent with the finding that the evolution of anti-herbivore defenses in milkweeds is characterized by suites of traits that are expressed in concert (Agrawal & Fishbein, 2006). This result prompts a modification of the proposed 'high edibility/high physical defense' syndrome of Agrawal & Fishbein (2006), as it now appears that expression of a chemical defense (cysteine protease) is correlated with two 'physical' defenses (latex exudation and trichome density) in *Asclepias*. Despite long-standing hypotheses of trade-offs in anti-herbivore defenses, such trade-offs are rare (Steward & Keeler, 1988; Agrawal & Fishbein, 2006; Agrawal, 2007). The trade-offs that we have documented between cardenolide quantity (in latex) and both latex production and cysteine protease activity (Table 1) are the first for milkweed defenses, and contributes to the syndromes view of coordinated defense strategies (Agrawal & Fishbein, 2006, Table 1).

The alternative models of trait evolution used to calculate PICs for testing trait correlations produced differing results. The results were somewhat stronger using the two models employing estimated branch lengths, with the result employing a chronogram being the strongest. Of the three models, we favor the gradual model employing a chronogram to account for shared evolutionary history. In this model, branch lengths estimate the temporal duration between speciation events. If rates of defense trait evolution are approximately constant over time, the chronogram most accurately depicts shared opportunities for evolutionary change among related species. We find the gradual model producing a phylogram, where branch lengths are scaled by molecular evolution (i.e., expected rates of nucleotide substitution in non-coding DNA sequences), to be a less intuitive approach. Here, there is no a priori reason to assume that rates of evolution in defense traits should be predicted well by rates of nucleotide substitution. It is reassuring that the results from these differing models are similar, which provides robustness to our preference for using the chronogram. However, the results from employing a speciational model to calculate PICs are quite different. With this model, there are equivalent opportunities for trait evolution at every speciation event, and none of the resulting correlations among defense traits were significant. Gradual models result in the inference of a significant positive correlation (independent of phylogenetic relationship) between the evolution of latex production and cysteine protease activity, along with a significant negative correlation between these two traits and the evolution of cardenolides; the speciational model results in the inference of independent (uncorrelated) evolution between latex production and the other two chemical defenses, along with a marginally significant positive correlation between the activity of cardenolide and protease. As in other studies of the phylogenetic component of character evolution (e.g., Moen, 2006), we do not have a rigorous a priori basis for assessing whether gradual or speciational models of character change are most appropriate for the evolution of latex.

The defense escalation hypothesis

The concept of directional trends was derived from early work on plants (Grant, 1963; Ehrlich & Raven, 1964) and suggests that as lineages diversify, there are directional phenotypic changes in ecologically important traits. Although the existence of directional trends has been a topic of intense scrutiny by paleontologists (McNamara, 1990; Alroy, 2000), and the mechanisms of such trends have been debated (Futuyma, 1989; Grant, 1989) they have less often been tested using data obtained from extant species (e.g., Omland, 1997; Mooers et al., 1999; Baker & Wilkinson, 2001; Hibbett, 2004; Adamowicz & Purvis, 2006; Moen, 2006). One classic example is the work on plant defense, including Ehrlich & Raven's (1964) proposed 'escalation' of defense hypothesis. Both Berenbaum (1983) and Farrell & Mitter (1998) made initial attempts to test these ideas and found some evidence for escalation as plant lineages diversified (coumarins in the Apiaceae and cardenolides in *Asclepias*, respectively). In both of these initial studies, the focus was on biochemical diversification (to more potent forms) within the chemical class being investigated. However, these studies of plant defense escalation did not make explicit use of phylogeny and models of character evolution (Harvey & Pagel, 1991; Mooers et al., 1999; Whittall & Hodges, 2007).

We tested for directional trends associated with the production of latex and two constituent chemical defenses using two general approaches. In the first method, we

regressed the phenotypes of species against (i) the number of intervening nodes between each species and the hypothetical ancestor of *Asclepias* or (ii) the total branch length between ancestor and tips. These results were concordant, indicating that evidence for the decline in latex exudation was robust to assumptions regarding speciational vs. gradual models of evolution.

In the second approach, we reconstructed ancestral states and determined whether phenotypes tended to increase or decrease during speciation events. With this method, we found evidence for increases in all three traits, although the evidence for repeated increases in latex production was the strongest. Several issues complicate interpretation of the conflicting results between the two approaches to testing for directional trends. First, the methods differ in how phenotypic data are used to measure amounts of phenotypic divergence. The regression method uses only observed phenotypic data and implicitly considers simultaneously both the magnitude and direction of phenotypic change, whereas the ancestral state method uses inferred ancestral states and considers only the direction of change at internal nodes. Thus, the most likely cause of the conflicting results for latex production, for example, is that the ancestral states method discovered that more speciation events were associated with increases in latex production, but the regression method discovered that the overall magnitude of decreases in latex production per speciation event exceeded the magnitude of increases (cf. Figure 3). It is thus conceivable that there have been large drops in latex exudation that have occurred a few times during the diversification of *Asclepias*, but that subsequently these declines have been followed by many small increases in latex values. In this way, there may be an overall decline in latex exudation as the clade diversified, but there may be a preponderance of increases at speciation events.

Because these are correlative analyses, cause and effect are unclear. In fact, Ehrlich & Raven's (1964) hypothesis was based on the notion that the evolution of escalated traits spurred diversification; we have not specifically tested this hypothesis with *Asclepias*. Here, we have investigated a broader hypothesis, that there has been a dominant trend of directional change in phenotypes during the macroevolution of a lineage. The detection of a directional trend is consistent with a relationship between trait values and diversification, but the causal arrow may point from trait to diversification or vice versa.

Based on our findings, we suggest a hypothesis for evolutionary patterns that include both escalation and decline of particular defenses as plant lineages diversify. Our notion is based on a perspective including an 'evolving community of herbivores' and possible directional trends in plant defense that respond to herbivory. Where specialist herbivores dominate the contemporary fauna of a particular plant group, as is the case for milkweeds, there may be relaxed selection for particular defenses, especially those for which the specialist herbivores have evolved a mechanism for circumventing the defense. Latex, cardenolides, and trichomes are possible examples, as milkweed herbivores deactivate latex (Dussourd & Eisner, 1987), have altered ATPase physiologies (Vaughan & Jungreis, 1977; Moore & Scudder, 1986; Holzinger & Wink, 1996; Labeyrie & Dobler, 2004), and shave trichomes on leaves (Malcolm, 1995; Agrawal & Malcolm, 2002; Agrawal, 2007) to overcome these respective barriers to consumption. Nonetheless, as discussed above, these traits are still effective at reducing herbivory by some milkweed specialists. As classic qualitative defenses (Feeny, 1976), these traits are effective barriers against feeding by generalists, even at very low doses. Thus, depending on the costs of particular defenses, their effectiveness against particular groups of herbivores, and the consistency of selection in space and time, we may predict either an escalation or decline in the expression of defense through the plant diversification process.

Furthermore, the conflicting results between the regression and ancestral state approaches to evaluating evolutionary trends suggest that both escalation and decline are important aspects of the history of the evolution of latex defense in *Asclepias*. Infrequent, large declines in latex investment may be associated with a shift to an alternative defense syndrome (Agrawal & Fishbein, 2006), whereas smaller and more numerous increases in latex may be indicative of escalation within the same syndrome.

Some defensive strategies such as tolerance or re-growth following damage, low nutritive value, or certain toxins that cannot be overcome by specialists, should persist, if not escalate through diversification, as originally proposed by Ehrlich & Raven (1964). Thus, our view is a phylogenetic synthesis of some classic hypotheses, including apparency theory (Feeny, 1976), resistance-tolerance trade-offs (van der Meijden et al., 1988; Simms & Triplett, 1994), and plant responses to selection by generalists vs. specialist (Da Costa & Jones, 1971; Blau et al., 1978; van der Meijden, 1996; Lankau, 2007). We predict that there should be directional phenotypic changes as plant lineages diversify, but that changes in defense investment will depend on the specific defenses and particular guilds of herbivores attacking plants. We advocate the search for patterns of defense investment across taxa and refinements of defense theories in the context of phylogenetic history.

Conclusion

Here, we have shown highly variable investment in latex defense among milkweed species. We find preliminary

evidence for phylogenetic trends in latex exudation as *Asclepias* diversified, although the direction of these trends depends on the method of analysis (Figures 4 and 5). Our strongest results, supported by gradual models of evolution, demonstrate the repeated evolution of a positive association between latex production and cysteine protease activity, along with a significant negative correlation between these two traits and the evolution of cardenolides. Building on our past work on defense syndromes in *Asclepias* (Agrawal & Fishbein, 2006), here we have presented the first evidence for a trade-off between defenses in this system. Additionally, *Asclepias* species in the 'physical defense syndrome' outlined in our past work (with high levels of trichomes and latex) also have high activity of defensive cysteine proteases. Latex itself is thus a multivariate defense, with the amount exuded delivering distinct concentrations of defensive cardenolides and cysteine proteases. Our investigation is a first attempt to understand the role of evolutionary history in driving patterns of association between these traits and how they have evolved as the plant lineage has diversified.

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References

Adamowicz SJ & Purvis A (2006) From more to fewer? Testing an allegedly pervasive trend in the evolution of morphological structure. Evolution 60: 1402–1416.

Agrawal AA (2004) Resistance and susceptibility of milkweed: competition, root herbivory, and plant genetic variation. Ecology 85: 2118–2133.

Agrawal AA (2005) Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. Evolutionary Ecology Research 7: 651–667.

Agrawal AA (2007) Macroevolution of plant defense strategies. Trends in Ecology and Evolution 22: 103–109.

Agrawal AA & Fishbein M (2006) Plant defense syndromes. Ecology 87: S132–S149.

Agrawal AA & Malcolm SB (2002) Once upon a milkweed. Natural History 111: 48–53.

Agrawal AA & Van Zandt PA (2003) Ecological play in the coevolutionary theatre: genetic and environmental determinants of attack by a specialist weevil on milkweed. Journal of Ecology 91: 1049–1059.

Alroy J (2000) Understanding the dynamics of trends within evolving lineages. Paleobiology 26: 319–329.

Arribere MC, Cortadi AA, Gattuso MA, Bettiol MP, Priolo NS & Caffini NO (1998) Comparison of Asclepiadaceae latex proteases and characterization of *Morrenia brachystephana* Griseb. cysteine peptidases. Phytochemical Analysis 9: 267–273.

Baker RH & Wilkinson GS (2001) Phylogenetic analysis of sexual dimorphism and eye-span allometry in stalk-eyed flies (Diopsidae). Evolution 55: 1371–1385.

Berenbaum M (1983) Coumarins and caterpillars – a case for coevolution. Evolution 37: 163–179.

Blau PA, Feeny P, Contardo L & Robson DS (1978) Allylglucosinolate and herbivorous caterpillars: a contrast in toxicity and tolerance. Science 200: 1296–1298.

Bollwinkel CW (1969) A revision of the South American Species of *Asclepias* L. Southern Illinois University, Carbondale, IL, USA.

Brower LP, McEvoy PB, Williamson KL & Flannery MA (1972) Variation in cardiac glycoside content of monarch butterflies from natural populations in eastern North America. Science 177: 426–429.

Da Costa CP & Jones CM (1971) Cucumber beetle resistance and mite susceptibility controlled by the bitter gene in *Cucumis sativus* L. Science 172: 1145–1146.

Dixon CA, Erickson JM, Kellett DN & Rothschild M (1978) Some adaptations between *Danaus plexippus* and its food plant, with notes on *Danaus chrysippus* and *Euploea core* (Insecta: Lepidoptera). Journal of Zoology 185: 437–467.

Dussourd DE (1997) Plant exudates trigger leaf-trenching by cabbage loopers, *Trichoplusia ni* (Noctuidae). Oecologia (Berlin) 112: 362–369.

Dussourd DE & Denno RF (1991) Deactivation of plant defense: correspondence between insect behavior and secretory canal architecture. Ecology 72: 1383–1396.

Dussourd DE & Denno RF (1994) Host range of generalist caterpillars: trenching permits feeding on plants with secretory canals. Ecology 75: 69–78.

Dussourd DE & Eisner T (1987) Vein-cutting behavior: insect counterploy to the latex defense of plants. Science 237: 898–900.

Ehrlich PR & Raven PH (1964) Butterflies and plants: a study in coevolution. Evolution 18: 586–608.

Farrell BD, Dussourd DE & Mitter C (1991) Escalation of plant defense: do latex and resin canals spur plant diversification. American Naturalist 138: 881–900.

Farrell BD & Mitter C (1998) The timing of insect-plant diversification: might *Tetraopes* (Coleoptera: Cerambycidae) and

Asclepias (Asclepiadaceae) have co-evolved? Biological Journal of the Linnean Society 63: 553–577.

Feeny PP (1976) Plant apparency and chemical defense. Biochemical Interaction between Plants and Insects (ed. by JW Wallace & RL Mansell), pp. 1–40. Plenum, New York, NY, USA.

Felsenstein J (1985) Phylogenies and the comparative method. American Naturalist 125: 1–15.

Fine PVA, Miller ZJ, Mesones I, Irazuzta S, Appel HM et al. (2006) The growth-defense trade-off and habitat specialization by plants in Amazonian forests. Ecology 87: S150–S162.

Futuyma DJ (1987) On the role of species in anagenesis. American Naturalist 130: 465–473.

Futuyma DJ (1989) Speciational trends and the role of species in macroevolution. American Naturalist 134: 318–321.

Grant V (1963) The Origin of Adaptation. Columbia University Press, New York, NY, USA.

Grant V (1989) The theory of speciational trends. American Naturalist 133: 604–612.

Harvey PH & Pagel MD (1991) The Comparative Method in Evolutionary Biology. Oxford University Press, Oxford, UK.

Hibbett DS (2004) Trends in morphological evolution in homobasidiomycetes inferred using maximum likelihood: a comparison of binary and multistate approaches. Systematic Biology 53: 889–903.

Holzinger F & Wink M (1996) Mediation of cardiac glycoside insensitivity in the monarch butterfly (*Danaus plexippus*): role of an amino acid substitution in the ouabain binding site of Na+/K+-ATPase. Journal of Chemical Ecology 22: 1921–1937.

Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y et al. (2004) Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. Plant Journal 37: 370–378.

Labeyrie E & Dobler S (2004) Molecular adaptation of *Chrysochus* leaf beetles to toxic compounds in their food plants. Molecular Biology and Evolution 21: 218–221.

Lankau RA (2007) Specialist and generalist herbivores exert opposing selection on a chemical defense. New Phytologist 175: 176–184.

Lewinsohn TM (1991) The geographical distribution of plant latex. Chemoecology 2: 64–68.

Liggieri C, Arribere MC, Trejo SA, Canals F, Aviles FX & Priolo NS (2004) Purification and biochemical characterization of asclepain c I from the latex of *Asclepias curassavica* L. Protein Journal 23: 403–411.

Maddison DR & Maddison WP (2005) MacClade, version 4.08. Sinauer Associates, Sunderland, MA, USA.

Malcolm SB (1995) Milkweeds, monarch butterflies and the ecological significance of cardenolides. Chemoecology 5/6: 101–117.

Martins EP & Hansen TF (1997) Phylogenies and the comparative method: A general approach to incorporating phylogenetic information into the analaysis of interspecific data. American Naturalist, 149: 646–667.

McNamara KJ (1990) Evolutionary Trends. University of Arizona Press, Tucson, AZ, USA.

van der Meijden E (1996) Plant defence, an evolutionary dilemma: contrasting effects of (specialist and generalist) herbivores and natural enemies. Entomologia Experimentalis et Applicata 80: 307–310.

van der Meijden E, Wijn M & Verkaar HJ (1988) Defense and regrowth, alternative plant strategies in the struggle against herbivores. Oikos 51: 355–363.

Moen DS (2006) Cope's rule in cryptodiran turtles: do the body sizes of extant species reflect a trend of phyletic size increase? Journal of Evolutionary Biology 19: 1210–1221.

Mooers AØ, Vamosi SM & Schluter D (1999) Using phylogenies to test macroevolutionary hypotheses of trait evolution in cranes (Gruinae). American Naturalist 154: 249–259.

Moore LV & Scudder GGE (1986) Ouabain-resistant Na,K-ATPases and cardenolide tolerance in the large milkweed bug, *Oncopeltus fasciatus*. Journal of Insect Physiology 32: 27–33.

Nelson CJ, Seiber JN & Brower LP (1981) Seasonal and intraplant variation of cardenolide content in the California milkweed *Asclepias eriocarpa*, and implications for plant defense. Journal of Chemical Ecology 7: 981–1010.

Omland KE (1997) Examining two standard assumptions of ancestral reconstructions: repeated loss of dichromatism in dabbling ducks (Anatini). Evolution 51: 1636–1646.

Pechan T, Cohen A, Williams WP & Luthe DS (2002) Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proceedings of the National Academy of Sciences of the United States of America 99: 13319–13323.

Purvis A & Garland T (1993) Polytomies in comparative analyses of continuous characters. Systematic Biology 42: 569–575.

Rohlf FJ (2006) A comment on phylogenetic correction. Evolution 60: 1509–1515.

Ronquist F & Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Molecular Biology and Evolution 19: 101–109.

Sanderson MJ (2003) r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. Bioinformatics 19: 301–302.

Shukla OP & Krishna Murti CR (1971) The biochemistry of plant latex. Journal of Scientific and Industrial Research (India) 30: 640–662.

Simms EL & Triplett J (1994) Costs and benefits of plant responses to disease: resistance and tolerance. Evolution 48: 1973–1985.

Stepek G, Buttle DJ, Duce IR, Lowe A & Behnke JM (2005) Assessment of the anthelmintic effect of natural plant cysteine proteinases against the gastrointestinal nematode, *Heligmosomoides polygyrus*, in vitro. Parasitology 130: 203–211.

Steward JL & Keeler KH (1988) Are there trade-offs among anti-herbivore defenses in *Ipomoea* (Convolvulaceae)? Oikos 53: 79–86.

Swofford DL & Maddison WP (1987) Reconstructing ancestral character states under Wagner parsimony. Mathematical Biosciences 87: 199–229.

Trejo SA, Lopez LMI, Cimino CV, Caffini NO & Natalucci CL (2001) Purification and characterization of a new plant

endopeptidase isolated from latex of *Asclepias fruticosa* L. (Asclepiadaceae). Journal of Protein Chemistry 20: 469–477.

Van Zandt PA & Agrawal AA (2004) Specificity of induced plant responses to specialist herbivores of the common milkweed, *Asclepias syriaca*. Oikos 104: 401–409.

Vaughan GL & Jungreis AM (1977) Insensitivity of lepidopteran tissues to ouabain – physiological mechanisms for protection from cardiac-glycosides. Journal of Insect Physiology 23: 585–589.

Vermeij GJ (1994) The evolutionary interaction among species: selection, escalation, and coevolution. Annual Review of Ecology and Systematics 25: 219–236.

Whittall JB & Hodges SA (2007) Pollinator shifts drive increasingly long nectar spurs in columbine flowers. Nature 447: 706–712.

Woodson RE (1954) The North American species of *Asclepias* L. Annals of the Missouri Botanical Garden 41: 1–211.

Zalucki MP & Malcolm SB (1999) Plant latex and first-instar monarch larval growth and survival on three North American milkweed species. Journal of Chemical Ecology 25: 1827–1842.

Zalucki MP, Brower LP & Alonso A (2001) Detrimental effects of latex and cardiac glycosides on survival and growth of first-instar monarch butterfly larvae *Danaus plexippus* feeding on the sandhill milkweed *Asclepias humistrata*. Ecological Entomology 26: 212–224.

Appendix 1 Milkweed (*Asclepias*) species sampled in this study and their means and standard errors (SE) for latex exuded upon tissue damage (mg), cardenolides in latex (optical density difference between sample and control at 620 nm), and cysteine protease activity in latex (optical density difference between sample and control at 280 nm). Dashes indicate no available data. Periods in the SE columns indicate that only one plant was sampled

	Latex		Cardenolides		Protease	
	Mean	SE	Mean	SE	Mean	SE
A. albicans	0.234	0.122	–	–	–	–
A. amplexicaulis	4.092	1.065	–	–	–	–
A. angustifolia	0.156	0.121	0.181	0.064	0.035	0.017
A. arenaria	4.144	1.556	0.085	0.040	–	–
A. asperula	0.840	0.338	0.659	0.048	0.190	0.031
A. barjoniifolia	1.453	0.473	–	–	–	–
A. boliviensis	0.641	0.076	–	–	–	–
A. brachystephana	4.692	1.754	–	–	–	–
A. californica	19.790	5.989	0.130	0.047	0.078	0.034
A. candida	1.384	0.330	–	–	–	–
A. cordifolia	3.846	.	0.074	0.021	0.005	0.017
A. cryptoceras	1.341	0.343	–	–	–	–
A. curassavica	0.063	0.024	0.851	0.054	0.177	0.016
A. engelmanniana	0.240	0.154	0.092	0.043	0.015	0.017
A. eriocarpa	6.028	2.256	0.295	0.061	0.073	0.015
A. erosa	16.059	6.476	0.547	0.346	0.208	0.039
A. exaltata	0.961	0.193	0.166	0.070	0.151	0.013
A. fascicularis	0.457	0.245	0.148	0.045	0.162	0.006
A. glaucescens	1.441	0.401	0.283	0.124	0.061	0.005
A. hallii	6.787	2.723	0.267	0.042	0.161	0.017
A. hirtella	–	–	–	–	0.159	0.013
A. humistrata	7.226	1.153	1.061	0.568	0.081	0.013
A. incarnate	0.385	0.066	0.166	0.153	0.016	0.018
A. latifolia	5.925	2.176	0.290	0.062	0.202	0.008
A. lemmonii	46.720	15.805	–	–	–	–
A. linaria	5.991	1.413	1.278	0.235	–	–
A. mexicana	0.436	0.126	–	–	–	–
A. mirifica	–	–			0.147	0.027
A. nivea	0.498	0.184	0.408	0.069	0.223	0.008
A. nyctaginifolia	0.461	0.136	0.380	0.168	0.242	0.013
A. obovata	9.364	2.524	–	–	–	–
A. oenotheroides	2.830	0.983	0.746	0.426	0.108	0.034

Appendix 1 *Continued.*

	Latex		Cardenolides		Protease	
	Mean	SE	Mean	SE	Mean	SE
A. perennis	0.068	0.016	0.626	0.110	–	–
A. pulchra	0.222	0.042	0.167	0.095	0.160	0.031
A. pumila	0.406	0.179	0.414	0.176	0.060	0.018
A. purpurascens	1.762	0.266	0.257	.	0.169	0.034
A. quadrifolia	0.839	.	0.388	.	–	–
A. solanoana	3.876	0.459	–	–	–	–
A. speciosa	0.819	0.359	0.094	0.071	0.193	0.023
A. stenophylla	1.931	.	–	–	0.011	0.028
A. subulata	0.228	0.082	0.608	0.148	0.045	0.012
A. subverticillata	0.277	0.092	0.205	0.056	0.167	0.026
A. sullivantii	4.518	1.458	0.187	0.169	0.174	0.028
A. syriaca	1.540	0.862	–	–	0.067	0.018
A. texana	0.339	0.102	0.136	0.020	0.108	0.006
A. tuberose	0.042	0.099	–	–	–	–
A. variegate	6.650	4.930	0.127	0.077	–	–
A. verticillata	0.149	0.030	0.259	0.081	0.051	0.020
A. vestita	4.766	1.610	0.364	0.071	0.098	0.029
A. viridiflora	0.187	.	0.141	.	0.226	0.018
A. viridis	1.616	0.963	0.200	0.005	0.141	0.024
G. cancellatus	18.722	2.896	0.382	0.088	0.085	0.015
G. fruticosus	0.261	0.050	0.657	0.124	0.028	0.023