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Macroevolutionary Trends in the Defense of Milkweeds against Monarchs

Latex, Cardenolides, and Tolerance of Herbivory

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Theory predicts that plants will increase their defenses against herbivores over time; however, several milkweed defenses have declined as the genus *Asclepias* has diversified. We review the evolutionary history of milkweed defense and provide new findings from an experiment on 53 *Asclepias* species. As predicted, monarch performance increased on more recently evolved species, and latex was primarily responsible for this effect. We next conducted focused analyses of eight *Asclepias* species, spanning early diverging and recently evolved species. We hypothesized that a decline in latex and cardenolides may be favored if (1) chemical defenses lose effectiveness due to sequestration, and (2) milkweeds evolve tolerance to herbivory in place of resistance traits. Monarch sequestration did not decline on derived *Asclepias* because they concentrated cardenolides from mid- to low cardenolide plants; nonetheless, derived milkweed species were more tolerant of herbivory because of enhanced investment in roots and the potential for clonal growth. The costs of producing latex and cardenolides, costs of sequestration for monarchs, and overall herbivore attack rates likely contributed to these macroevolutionary patterns.

INTRODUCTION

The availability of molecular phylogenies is transforming our ability to address classic questions in chemical ecology and coevolution (Wink 2003; Futuyma and Agrawal 2009; Kursar et al. 2009). In particular, some of our most long-standing coevolutionary hypotheses relate to the determinants of insect herbivore host shifts (Dethier 1941; Becerra 1997; Murphy and Feeny 2006), relationships between plant defense traits and diversification in plants and insects (Ehrlich and Raven 1964; Farrell et al. 1991; Agrawal et al. 2009a), and tradeoffs and synergies among plant defenses (Feeny 1976; Rudgers et al. 2004; Agrawal and Fishbein 2006). Many of the original hypotheses were based in comparative biology, and yet only recently has a phylogenetically informed comparative approach been advocated and implemented (Agrawal 2007). Because milkweeds and monarchs have been so important in the devel-

opment of the research fields of chemical ecology and coevolution (Malcolm 1995), and a recent molecular phylogeny of *Asclepias* has been produced (Fishbein et al. 2011), the time is ripe to summarize what we know about the evolutionary history of milkweeds (Figure 4.1) as it relates to monarch biology.

PHYLOGENETIC PATTERNS

Our laboratories have been studying the defenses and evolutionary history of New World milkweeds (*Asclepias* spp.) for the past two decades. Several interrelated phylogenetic trends can be summarized briefly as follows: as American *Asclepias* has diversified, there have been consistent increases or declines in particular trait values (Figure 4.2), with a decelerating rate toward the present (Agrawal et al. 2009a). These directional trends have been particularly apparent in the defensive traits of milkweeds,

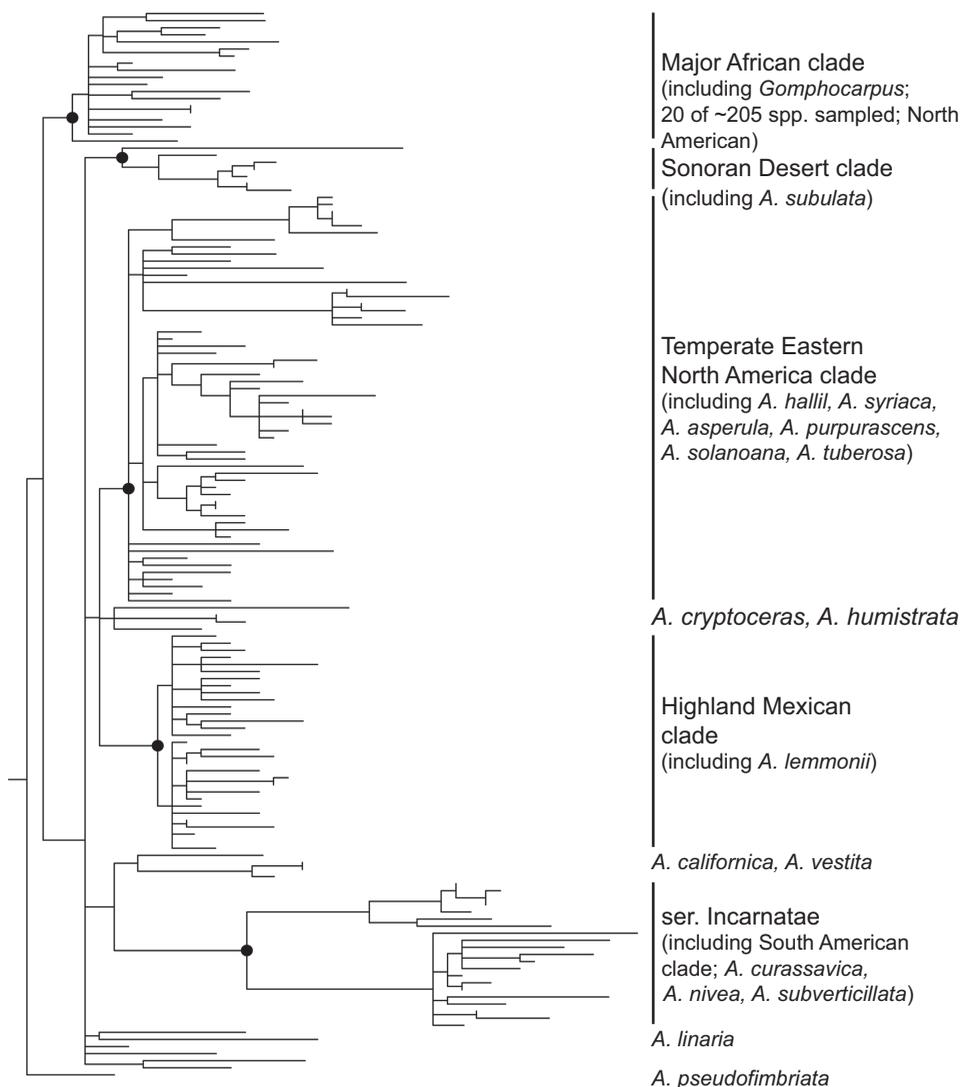


Figure 4.1. Maximum likelihood phylogeny of *Asclepias* (after Fishbein et al. 2011), in which branch lengths are proportional to expected substitutions per site in noncoding plastid DNA (introns and intergenic spacers at 3 loci). Major clades are indicated by large dots on the phylogeny and with names on the right, along with representative species discussed in the text. Other species names have been omitted for clarity. The earliest dichotomy represents the split between African *A. pseudofimbriata* and all other African and American species. The second dichotomy separates all other sampled African species (which have been classified as *Asclepias* and as many as 20 other genera) and all American *Asclepias*, which form a single clade. Relationships among the major American clades are not well supported by these data, although a single origin of all South American species from within series *Incarnatae* is strongly supported.

but not in other vegetative and physiological traits unrelated to defense (Agrawal and Fishbein 2008; Agrawal et al. 2009a).

In other words, milkweed defenses have tended to either increase or decrease as new species evolved. Such trends are a remarkable feature of evolutionary biology (Farrell et al. 1991; Vermeij 1994; Jablonski 2008; Futuyma and Agrawal 2009), although

their causes are difficult to disentangle (Agrawal et al. 2009c). One interpretation of this pattern is that directional selection for changes in plant defense are realized only at the time of speciation (Futuyma 1987; Vermeij 1994; Pagel 1999), while another interpretation is that particular defensive phenotypes have promoted speciation (Paradis 2005; Madisson et al. 2007; Freckleton et al. 2008). These two

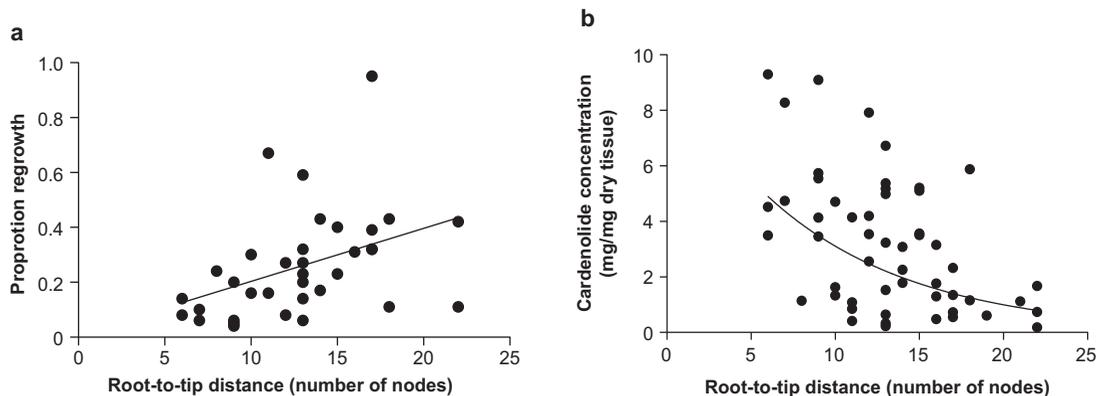


Figure 4.2a. Directional trends in the macroevolution of the defense traits in *Asclepias*; root-to-tip distance indicates phylogenetic distance measured as the number of speciation events or nodes on a phylogeny. Each point is the mean of an *Asclepias* species. (a) A positive and linear relationship between trait values and the extent of phylogenetic divergence, shown for regrowth of plants after defoliation (a measure of tolerance); data based on Agrawal and Fishbein 2008. (b) A decelerating decline in cardenolide values (also known for cardenolide diversity and latex exudation); based on Agrawal et al. 2009a. These relationships demonstrate an association between speciation events and trait evolution. When a directional trend is accelerating or decelerating (as in b), this is considered a signature of adaptive radiation because the greatest change occurred during the initial stages of the lineage diversification.

alternatives suggest that defensive traits are either a consequence of evolution or the cause. Despite the difficulty in resolving the direction of causality, such patterns allow us to infer that particular traits in the lineage change directionally, and thus are important for inferring drivers of evolution. Given that monarchs and milkweeds share a long evolutionary history (A.V.Z. Brower and Jeansonne 2004), such temporal trends provide insight into the importance of monarchs (and milkweed herbivores more generally, see Plate 3) in plant evolution.

EVOLUTIONARY HISTORY OF *ASCLEPIAS*

Although milkweeds are common, an understanding of *Asclepias* diversification has begun to emerge only recently. A basic question that has long vexed taxonomists concerns the relationships among the three geographic centers of milkweed diversity: Africa (ca. 250 spp.), North America (ca. 130 spp.), and South America (ca. 9 spp.). At the turn of the twenty-first century, the prevailing view held that, while African and North American milkweeds are closely related, these continents possess independent evolutionary lineages, and many African milkweeds have been placed in as many as 20 other genera (Bullcock 1952; Goyder 2001a). No unique traits characterize either African or North American milkweeds

as a whole, however, and progress in classifying African species in genera other than *Asclepias* has stalled (Goyder 2009). The accommodation in *Asclepias* of the few species endemic to South America, however, has been uncontroversial. For convenience, we refer to all African milkweeds as *Asclepias*, including those formerly placed in *Gomphocarpus* and other segregate genera.

Comprehensive phylogenetic study of milkweeds based on noncoding chloroplast DNA sequences (Fishbein et al. 2011) has largely supported the conjecture that African and American milkweeds represent distinct, but closely related evolutionary lineages. We (Fishbein et al. 2011) sampled all but a handful of the American species and found that all American species form a single clade to the exclusion of all sampled African species (Figure 4.1); moreover, all South American species belong to one lineage that represents a single dispersal event from North American ancestors. American and African milkweed lineages appear to consist of sister clades stemming from a single common ancestor; however, one African species, *A. pseudofimbriata*, was placed outside of these two major milkweed clades as an early diverging lineage independent of the major African diversification (Figure 4.1). This result is not explained by any unusual morphological feature of the species (Goyder 2001b), and the unexpected placement of *A. pseudofimbriata* has been supported

by subsequent phylogenetic study of African milkweeds (D. Chuba and M. Fishbein, unpublished data). Deeper phylogenetic relationships suggest that the ancestor of African and American milkweeds originated in Africa, and that the genus expanded its range to the Americas via spread across Atlantic or Beringian land bridges, or through long-distance dispersal (Fishbein et al. 2011). Because there is no fossil record for *Asclepias* (or any other milkweed genus), the timing of origin, dispersal to the Americas, and colonization of South America remain shrouded in mystery. Based on relative molecular branch lengths of the *Asclepias* phylogeny, it seems clear that the origin of the South American species has been quite recent, perhaps explaining the low diversity of this clade.

Within the American lineage, our results provide insights into the delimitation of major milkweed clades and patterns of morphological evolution, although their apparently rapid radiation leaves the details of the diversification yet to be fully resolved (Figure 4.1). Woodson (1954) proposed a classification of North American milkweeds into nine subgenera (the largest of which was further subdivided) based on flower characteristics. The implicit assumption was that the evolution of these complex flowers was conservative. Phylogenetic analysis showed this assumption to be unfounded, in that just one of Woodson's (1954) species groups is monophyletic, and that growth form and leaf morphology are perhaps more indicative of relationships (Fishbein et al. 2011). The phylogeny of *Asclepias* also exhibits a strong geographic component, in that major clades are largely distributed regionally, such as the Mexican highlands, the eastern temperate forests and plains, and the Sonoran Desert (see Figure 4.1) (Fishbein et al. 2011). Although we found strong support for these major clades based on chloroplast sequences, relationships among and within them were not supported. It should be noted that phylogenies estimated from chloroplast data alone may not accurately represent species-level relationships if incomplete lineage sorting or introgressive hybridization have occurred in milkweeds (Soltis and Kuzoff 1995; Maddison and Knowles 2006). Current efforts at resolving the *Asclepias* phylogeny are employing whole chloroplast genome sequences and multiple, independent nuclear markers (Straub et al. 2011, 2012). Results are promising, as whole genome sequences provide almost complete reso-

lution of the relationships among major milkweed clades (S. Straub, A. Liston, M. Fishbein, R. Cronn, unpublished data). However, data from whole chloroplast genomes, complete nuclear ribosomal DNA cistrons, and mitochondrial DNA fragments show strong intergenomic conflict in the Sonoran Desert milkweeds, and the genus as a whole is probably characterized by a complex history of diversification that includes both hybridization and incomplete lineage sorting (Straub et al. 2012).

DEFENSIVE TRAITS OF MILKWEEDS

Milkweeds take their name from the characteristic latex that exudes following tissue damage (Figure 4.3). An important feature of this trait is that latex has no known function in a plant's primary metabolism (resource acquisition and allocation) and has been strongly implicated as a defense against chewing herbivores such as monarchs (Agrawal and Konno 2009). Although the defensive function of latex has historically been ascribed to the physical action of coating and gumming up the insect's mouthparts, there is also evidence for potent chemical defenses in latex. For example, many milkweeds have tremendously high concentrations of cardenolides (steroids that disrupt cellular ATPase function) in their latex (Nelson et al. 1981; Zalucki et al. 2001a, Agrawal et al. 2012b). Cardenolides are also produced throughout the plant, even in nectar and roots, which are plant parts that lack latex (Rasmann et al. 2009; Agrawal et al. 2012b; Manson et al. 2012). Like latex, cardenolides have no known primary function and have been strongly implicated in the defense against insect herbivores (Agrawal et al. 2012b). Because the chemical mode of action and ecological impacts of cardenolides have been well-reviewed, we do not elaborate on these subjects here (Seiber et al. 1983; Malcolm 1991; Agrawal et al. 2012b).

In addition, the latex of many plants, including milkweeds, contain cysteine proteases (Arribere et al. 1998; Trejo et al. 2001; Liggieri et al. 2004; Stepek et al. 2005; Agrawal et al. 2008), which have recently been implicated as toxins that degrade an essential part of the insect's gut, the peritrophic membrane (Pechan et al. 2002; Konno et al. 2004). Beyond these particular chemical defenses, milkweeds have diverse traits that may also contribute to defense. For example, saponins, pregnanes, phenolics, and

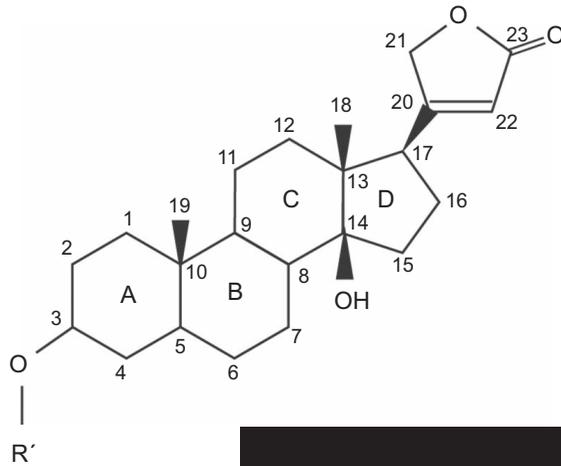


Figure 4.3. Cardenolides and latex are the two most well-studied defensive traits of milkweeds. (a) The skeleton structure of a cardenolide, composed of a core steroid (four fused rings), an oxygenated lactone group, and a glycoside group typically attached at position 3 (R'). R' represents one or more sugars in glycosides or H in genins. Cardenolides function by binding to the critical animal sodium-potassium pump, a ubiquitous cellular enzyme. (b) A nearly mature monarch larva beginning to notch the midrib of an *Asclepias syriaca* leaf. In this case, the larva was met by a large droplet of pressurized latex. It proceeded to move the latex out of the way by wiping it on either side of the midrib. Occasionally, the larva will even imbibe such a large droplet of latex, presumably to remove it, and continue cutting the latex-delivering canals. Photo by Ellen Woods.

alkaloids have been reported from *Asclepias* species (Malcolm 1991; Agrawal et al. 2012b). Leaf hairs (trichomes), low nutritional quality, and volatile organic compounds that are induced by herbivory are also produced by *Asclepias*, and may impact insect herbivores (Agrawal and Fishbein 2006; Agrawal et al. 2009b; Wason et al. 2013). Given this diversity of plant defensive strategies, it is no wonder that the bulk of insect species that consume milkweed are specialists that have evolved to cope with these defenses (Agrawal and Konno 2009; Agrawal et al. 2012b; Dobler et al. 2012).

In the rest of this chapter we focus on latex and cardenolides because these have been the best studied defense traits of milkweed, and many of their impacts and interactions with monarchs have been elucidated over the past decades (e.g., Zalucki et al. 2001a).

A BRIEF REVIEW OF PHYLOGENETIC TRENDS IN *ASCLEPIAS*

Previously, we reported that the production of total cardenolides and latex has declined during

milkweed diversification (Agrawal and Fishbein 2008; Agrawal et al. 2009a), while regrowth ability and production of phenolics has been enhanced (Agrawal and Fishbein 2008; Agrawal et al. 2009c). Because total cardenolide concentration and diversity of distinct cardenolide compounds show a strong evolutionary correlation (Rasmann and Agrawal 2011), it is clear that as cardenolide amounts declined, so too did the diversity of compounds. Nonetheless, we still do not know enough about the structure-function relationships of most cardenolides, and thus it may be too early to conclude that declines result in reduced toxicity. Additionally, the means by which latex is delivered is not fully understood. Latex is produced and delivered in elongated cells called laticifers. While most *Asclepias* have nonarticulated laticifers that consist of long multinucleate cells, some species such as *A. curassavica* have articulated laticifers comprising multiple cells that branch and rejoin. These two delivery methods may provide different means of defense (S. Malcolm, pers. comm.).

A further piece of evidence that the evolution of defenses promoted milkweed diversification comes from an analysis of the tempo and mode of trait evolution (Agrawal et al. 2009a). In particular, species-rich lineages of *Asclepias* underwent a proportionately greater decline in latex and cardenolides relative to species-poor lineages, and the rate of trait declines was most rapid early in milkweed diversification. Our interpretation of these results is that reduced investment in defensive traits accelerated diversification, and disproportionately so in the early adaptive radiation of milkweeds. While the declines in some traits, especially in latex and cardenolides, were surprising to us (and in opposition to theory), the statistical significance of the pattern has led us to speculate on the causes of this pattern.

First, we hypothesized that because most *Asclepias* herbivores are specialists, with adaptations to cope with latex and cardenolides (Dussourd and Eisner 1987; Holzinger and Wink 1996), the plants may be tending toward tolerance of herbivory (van der Meijden et al. 1988). In other words, given that several milkweed herbivores sequester cardenolides (Agrawal et al. 2012b), high levels of plant-produced cardenolides could backfire, as specialist herbivores may enjoy protection from natural enemies (e.g., Brower et al. 1967; Malcolm 1995; Sternberg et al. 2012). Second, if a plant cannot prevent herbivory

because the herbivores are specialists, a strategy of tolerance could be favored (Strauss and Agrawal 1999).

In the rest of this chapter, we describe three experiments that help us understand whether the evolutionary trends in defensive traits of milkweeds actually impact one of the major herbivores of *Asclepias*, the monarch caterpillar. The experiments address five questions: (1) Does caterpillar performance improve on progressively derived *Asclepias* spp., as was predicted from our previous phylogenetic analyses of plant defensive traits? (2) Which defensive traits are associated with impacts on monarch performance? (3) Do monarch caterpillars have enhanced sequestration when feeding on early diverging *Asclepias* compared with that on more derived species? (4) Are early diverging *Asclepias* less tolerant of monarch herbivory than more derived species? And, (5) is tolerance of herbivory explained by patterns of allocation to roots or shoots, and correlated with a particular life-history trait, such as the extent of clonal reproduction?

Experiment 1, monarch larval performance on 53 milkweed species

Here we report previously unpublished data on the performance of monarch larvae fed 53 different *Asclepias* spp. for which we had both phylogenetic and defense strategy data (see Figure 4.4, experimental details provided in Agrawal et al. 2009a; Rasmann and Agrawal 2011). Note that monarchs appear able to complete development on all *Asclepias* species, encounter many if not most species in the wild, and show little local adaptation because of their panmictic populations (Pierce et al., this volume, Chapter 23). We grew plants from seed in growth chambers, and when plants had grown for 30 days, we introduced a single freshly hatched monarch larva to each of 3–6 plants per species. Plants were fully randomized within the growth chambers and caterpillars were allowed to feed for 5 days, after which caterpillar mass was recorded. This time period resulted in some caterpillars being in the first instar while others were in the second, potentially contributing to variation in the data; nonetheless, we maintained a single harvest point in order to follow the mass gain for each caterpillar over a fixed amount of time, thereby integrating performance as growth per unit time.

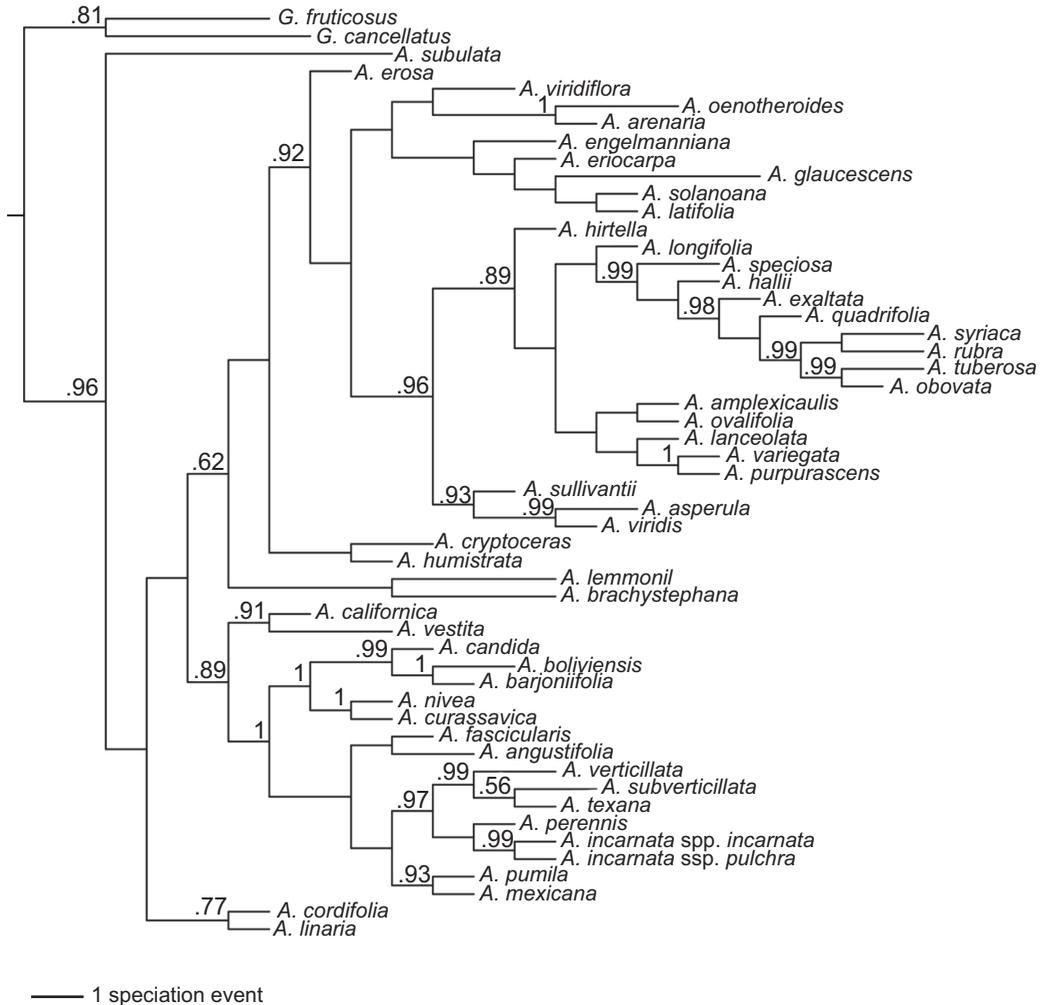


Figure 4.4. A pruned phylogeny of milkweed (53 species) based on the all-compatible consensus of trees sampled in a Bayesian analysis of the complete data set of 155 samples. Branch lengths are drawn in proportion to the number of speciation events between nodes; sister lineages may not be of equivalent length because multiple speciation events in the complete phylogeny may occur on a single branch of the pruned phylogeny. Modified from Agrawal et al. 2009.

We evaluated monarch performance (mean mass of the larvae on each milkweed species) as a function of phylogenetic root-to-tip distance, measured by the number of intervening nodes (Figure 4.2, see Figure 4.4 for the pruned phylogeny with branch lengths scaled to this measure) using Bayes-Traits (Pagel 1999). Phylogenetic signal (Pagel's λ) was estimated using maximum likelihood and tested against fixed models of $\lambda = 0$ and $\lambda = 1$. A λ value of 1 indicates phylogenetic conservatism consistent with a random walk model of larval performance as milkweeds diversified (i.e., similarity in performance is directly proportional to the extent of shared milk-

weed evolutionary history). A λ value of 0 indicates no influence of shared ancestry on performance (i.e., phylogenetic independence). Further details of our analyses can be found in published studies (Agrawal and Fishbein 2008; Agrawal et al. 2009a).

Short-term monarch growth varied more than 10-fold across the milkweed species, and caterpillars feeding on species that are more derived showed proportionally greater mass (Figure 4.5, likelihood ratio test, maximum likelihood estimate of $\lambda = 0$, LR = 4.112, $P = 0.043$). We next used a phylogenetically informed multiple regression to predict monarch mass using three predictors: cardenolides, latex

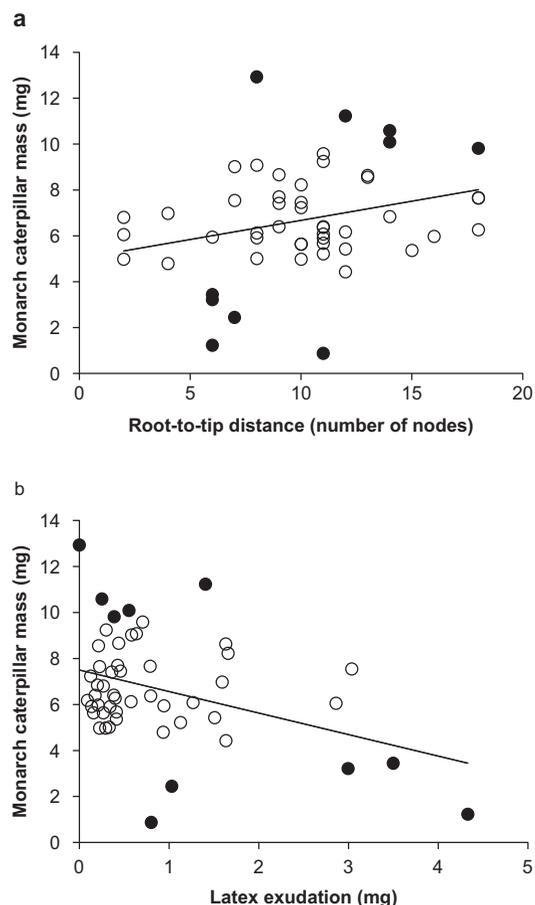


Figure 4.5. Impacts of evolutionary history and latex on short-term monarch growth. The five best- and five worst-quality milkweed species used for qualitative comparisons (Table 4.1) are shown in filled symbols. (a) More derived *Asclepias* species (those with a greater root-to-tip distance, see Figure 4.1) supported greater monarch larval growth. (b) In a phylogenetically informed analysis, latex was the only significant predictor of monarch growth. These data are consistent with earlier results suggesting phylogenetic declines in milkweed defenses.

exudation, and trichome density measured on the same plants (methods are given in Agrawal et al. 2009a). Briefly, foliar cardenolides were measured by high performance liquid chromatography, latex was measured as wet mass exuded from a 2 mm cut leaf tip on filter paper, and trichomes were measured as the number of leaf hairs along a transect under a dissection microscope. Our full model explained a modest 16% of the variation in monarch mass, and only latex was a significant predictor (Figure 4.5b, $\lambda = 0$, LR = 7.201, $P = 0.007$). Thus, phylogenetic declines in latex improve monarch performance,

while declines in cardenolides or trichome density were not implicated in promoting monarch growth in this experiment.

To characterize the biology of the best and worst food plants for monarchs, we next examined the top five and bottom five species for monarch growth based on our experiment that included 53 species. On average, the top five species supported monarchs that grew nearly five times the mass of those fed the bottom five species (Figure 4.5, Table 4.1). The phylogenetic position of our best food species for monarchs was decidedly derived (mean \pm SE nodes from root-to-tip, 13.2 ± 1.3 compared with our worst species: 7.2 ± 1.3). Again, our best predictor of monarch mass was latex (mean \pm SE latex exudation in mg, best species, 0.52 ± 0.52 compared with our worst species: 2.53 ± 0.52 , Table 4.1). Despite the fact that our poorest quality plant species had, on average, nearly five times the trichome density and nearly 80% higher cardenolides than our best quality *Asclepias*, these two variables were not significantly different among the two extreme plant categories (Table 4.1). Nonetheless, we note two interesting points relating to deployment of plant defenses in milkweeds as a suite of synergic traits. First, latex and trichomes show positive correlated evolution (Agrawal and Fishbein 2006; Agrawal et al. 2009b), suggesting that, on average, species with high latex also have dense trichomes. Second, latex delivers a concentrated dose of cardenolides, often more than 10-fold that of leaves per se. Thus, these traits are not necessarily acting independently, and we still have much to learn about the combined effects of latex, cardenolides, and trichomes (see also Zalucki et al. 2001a, 2012).

A further assessment of the phylogenetic, geographic, and climatic attributes of these 10 “best” and “worst” species reveals some interesting patterns. First, the worst species are not strongly phylogenetically clustered (although *A. californica* and *A. vestita* are sister taxa), despite the fact that all were relatively early diverging species (Table 4.1, Figure 4.4); nonetheless, they are native to arid regions of the western United States and northern Mexico (although *A. lemmonii* is most abundant in wet pine-oak forest in the Sierra Madre Occidental). For *A. asperula*, it appears that a slightly different path has been taken, given that it is the most derived species in the well-defended group, and has maintained high cardenolides, despite having low latex.

Table 4.1. Characteristics of the five best and five worst *Asclepias* food plants from Experiment 1

	Monarch mass(mg dry)	Root-to-tip distance	Trichome index	Latex exudation (mg)	Cardenolide (mg/g dry mass)
Poorest host plants					
<i>A. asperula</i>	0.874	11	0.384	0.802	6.612
<i>A. lemmonii</i>	1.231	6	3.248	4.329	3.224
<i>A. cryptoceras</i>	2.446	7	0.489	1.030	1.029
<i>A. vestita</i>	3.215	6	117.82	2.995	3.098
<i>A. californica</i>	3.444	6	42.807	3.498	4.853
mean ± SE	2.24 ± 0.52	7.2 ± 0.97	32.95 ± 22.69	2.53 ± 0.69	3.76 ± 0.94
Best host plants					
<i>A. syriaca</i>	9.815	18	7.980	0.388	1.533
<i>A. hallii</i>	10.088	14	0	0.553	1.560
<i>A. purpurascens</i>	10.586	14	5.734	0.251	0.349
<i>A. solanoana</i>	11.230	12	19.195	1.406	2.720
<i>A. nivea</i>	12.930	8	1.6002	0	4.385
mean ± SE	10.93 ± 0.56	13.2 ± 1.62	6.90 ± 3.39	0.52 ± 0.24	2.11 ± 0.68
ANOVA	131.4**	10.1*	1.3^{ns}	7.5*	2.0^{ns}

Source: Data from Agrawal et al. 2009a.

Notes: Values to the right of each species name indicate the species means, usually based on 5 replicates (except the root-to-tip distance, which is simply based on the phylogenetic position of the species). The row in gray, directly below each group, represents the means ± standard error of that group; the final row, marked ANOVA, provides an analysis of variance result with an *F* value (df = 1,8 for all tests).

* < 0.05, ** < 0.001, ^{ns} = not significant

The best *Asclepias* species for monarchs are dominated by the eastern North American clade (except *A. solanoana*), and are highly variable in terms of geography and habitat. *Asclepias solanoana* occurs only on dry and barren serpentine soils in northern California. It is possible that it typically accumulates toxic metals from the soil that were not available in our common potting mix, or that this edaphic specialist typically escapes herbivores because of its geographic location or life in harsh environments. The other derived species are scattered from the eastern United States to the Rocky Mountains (and also include the Caribbean island endemic, *A. nivea*) and are more typical of open field, mesic habitats than the early diverging, poor food plant species; nonetheless, *A. purpurascens* and *A. nivea* can be abundant in open forest understories and edges, and *A. hallii* grows in seasonally wet but open riparian habitats.

Asclepias syriaca is in the “best” food group. In addition to having relatively modest levels of trichomes, latex, and cardenolides, it is by far the most common host plant of monarchs across North America. Indeed, it has been estimated that more than 90% of butterflies that migrate to the Mexican

overwintering grounds developed on *A. syriaca* (Malcolm et al. 1993). Thus, if monarchs are adapted to feeding on any milkweed, it is likely the common milkweed, *A. syriaca*.

In summary, there does not appear to be a strong phylogenetic constraint on the evolution of plant defenses against monarchs. Given the geographic and climatic characteristics of the best-defended species, it seems that defense trait convergence has occurred because of similar environments or the historical impact of herbivores in those regions. Some overlap exists between inhabiting relatively harsh habitats and early divergence in the milkweed phylogeny (with more derived forms having reduced resistance traits). As for the least defended species, they appear to be more diffuse in terms of the geographic or climatic conditions associated with relaxed expression of cardenolides and latex.

Experiment 2, monarch sequestration on eight milkweed species

In this experiment, we tested the hypothesis that phylogenetic trends can be observed in monarch

sequestration. One possible explanation for the macroevolutionary declines in total cardenolides could be that more derived species would support reduced sequestration, and thereby enhanced control by predators and pathogens. To address this, we grew eight species of *Asclepias* under controlled conditions as described previously (Agrawal and Fishbein 2008; Agrawal et al. 2009a). *Asclepias curassavica*, *A. humistrata*, *A. linaria*, and *A. subulata* were selected as early diverging species that span the root of the American milkweed clade while belonging to low-diversity subclades (Figures 4.2, 4.4), with root-to-tip distances of 9, 9, 6, and 9 nodes, respectively. *Asclepias purpurascens*, *A. solanoana*, *A. subverticillata*, and *A. syriaca* are more derived species representing species-rich clades, with root-to-tip distances of 17, 15, 14, and 22, respectively. Caterpillars were allowed to grow for 10 days after which we starved them to empty their guts for 24 hours, and then harvested both the insects and the plants ($n = 10$ plants per species). Cardenolides were assessed by HPLC following the methods of Rasmann and Agrawal (2011).

We first assessed the relationship between plant cardenolides and sequestration. Our eight species varied from barely detectable cardenolides (*A. subverticillata*) to the highly toxic *A. linaria*, with more than 7 mg/g dry mass cardenolide content (Figure 4.6). As previously summarized by Malcolm (1995,

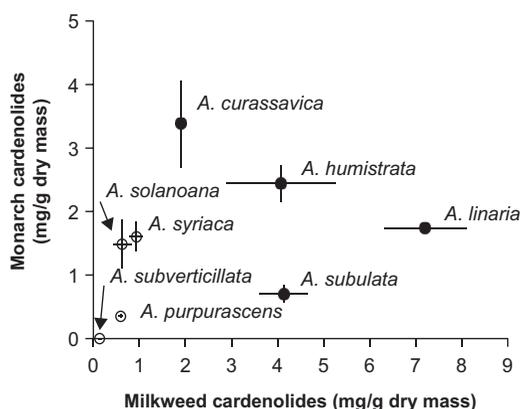


Figure 4.6. The relationship between foliar cardenolides and sequestered cardenolides in monarch larvae. Phylogenetically basal species are coded as closed circles, derived species as open circles. Note that basal plant species showed substantially higher cardenolides than derived species; however, this pattern did not translate into significant differences in larval sequestration. Shown are means \pm SE.

focusing on adults) we found a nonlinear pattern of cardenolide sequestration in larvae. Monarchs tend to follow one of three patterns of sequestration: (1) little to no cardenolides in monarchs fed plants with little to no cardenolides, (2) concentrated cardenolides, often double the concentration in monarchs fed plants of intermediate cardenolide concentrations (plants ranging ≈ 0.75 – 2.5 mg/g), or (3) saturated cardenolides, where monarch bodies contain no higher levels (or perhaps even reduced levels) compared with the very high foliar concentrations in some plant species. Indeed, in our study, *A. linaria* exhibited typically high cardenolide concentrations, but larvae contained intermediate levels and could barely grow or sequester on *A. linaria*; they achieved only 3 mg mass (and remained in the second instar) compared with larvae on most other species, which were more than 19 mg and in the third to fourth instar.

Although it was initially thought that monarchs were capable of accumulating the highest level of cardenolides from plants with the highest concentrations (Brower et al. 1972), we now recognize that monarchs seem particularly good at accumulating and concentrating cardenolides to levels much higher than those found in their host when their host plants contain moderate to low concentrations. Plants with very high cardenolides represent a saturation point for the monarchs, or may even decrease their ability to sequester cardenolides. Our data confirm this pattern. Why monarchs have difficulty feeding on some species (e.g., *A. linaria*, *A. perennis*, *A. asperula*) appears to be related to the extremely high cardenolide content, although further studies on the toxicity and sequestration of high levels of cardenolides are needed (see also Zalucki et al. 2001a; Agrawal et al. 2012b). We further speculate that the linear, tough leaves of *A. linaria* may be nearly impossible for monarchs to notch to cut off latex flow, which may make this species even more difficult to consume.

Monarch sequestration is a dynamic process. At least five factors may explain the amount of cardenolides a monarch will sequester: sex, quantity of cardenolides in a plant (including induced compounds), biomass consumed, polarity of cardenolides, and structural attributes independent of polarity. First, females sequester higher concentrations of cardenolides than do males when reared on plant species with similar cardenolide quantities (Nelson 1993).

This phenomenon and its ecological consequences are not well studied. Because the fat bodies of adult female butterflies contain nutritional reserves carried through pupation from larval feeding, and these are a major part of the resources available for somatic maintenance and gamete production, high levels of sequestration may be critical. Second, both quantitative and qualitative factors play a large role in cardenolide storage. Cardenolide quantity was discussed above. It may be less costly to store and sequester relatively polar compared to nonpolar cardenolides (Malcolm 1991). This hypothesis is supported by the observation that nonpolar cardenolides are converted into more polar forms during sequestration (Seiber et al. 1980; Brower et al. 1984; Martin et al. 1992), while polar cardenolides and those of intermediate polarity are directly stored (Frick and Wink 1995). Finally, structural attributes of cardenolides, especially the sugar groups (Figure 4.3), have a strong impact on their toxic potential. For example, genins (cardenolides lacking a sugar group) are less toxic than identical compounds with sugars (Malcolm 1991; G. Petschenka et al., unpublished).

In summary, we hypothesized that the most derived *Asclepias* species may benefit from having reduced cardenolide concentrations because of reduced sequestration by their herbivores. Indeed, cardenolide sequestration was remarkably low on the four most derived species tested. Other very low cardenolide species not tested here (i.e., the highly derived *A. tuberosa*), may provide no opportunity for monarch sequestration. Thus, although more work is needed to understand which cardenolides are most easily sequestered and are most effective as a defense against predators of monarchs, it is clear that the macroevolutionary trend toward reduced *Asclepias* cardenolides impacts sequestration and is likely to impact interactions with enemies.

Experiment 3, tolerance of herbivory in basal and derived milkweeds

We used the same set of eight species to test for differences in tolerance of herbivory between early diverging and more derived milkweeds. A previous study suggested that as milkweeds diversified and showed reduced investment in latex and cardenolides, their ability to regrow following damage was enhanced (Agrawal and Fishbein 2008). This led us to hypothesize that tolerance had evolved as an

alternative strategy to resistance, and that such a strategy may be particularly useful when plants are faced with highly specialized herbivores (Agrawal and Fishbein 2008). We further demonstrated that the ability to regrow following damage was dependent on relatively high investment in roots (i.e., a high root-to-shoot ratio) (Agrawal and Fishbein 2008); however, our previous work had two weaknesses. First, our measure of tolerance was limited to the regrowth capacity of plants in aboveground tissues relative to their previous growth and not to an undamaged control. Second, we imposed catastrophic damage by cutting the plants down to the soil, but we did not use real herbivores. Real vs. mechanical damage causes distinct responses in milkweed (Mooney et al. 2008). Here, we report the results of a new study designed to test tolerance of herbivory in eight milkweeds using both root and shoot measures.

Plants were grown from seed as described above. After they had grown for 21 days following germination, we measured total leaf area by spreading each leaf over square millimeter graph paper, tracing its outline, and calculating the corresponding area. Half the plants were then treated with a single freshly hatched monarch larva and the other half were left as undamaged controls ($n = 10$ plants per species per treatment). After 10 days, larvae were removed from plants, and all uneaten leaves were clipped off with scissors from plants of the treatment group. Thus, real herbivores attacked all the plants for an equal amount of time, followed by 100% leaf tissue removal for plants in the damaged treatment. This combination allowed for insect-specific signals to induce plants and also for equal (catastrophic) impacts on plants. Total leaf area was remeasured on all plants after 21 days of (re)growth. Finally, above- and belowground tissues were harvested and separated (roots were washed free of their soil), dried at 45 °C, and weighed. We report three measures of tolerance for each species: (1) the proportional reduction in root mass of damaged compared with undamaged plants (the difference in root mass of control plants and damaged plants divided by the root mass of control plants), (2) the proportional reduction in shoot mass (calculated as for roots), and (3) proportional leaf area recovery. This final measure was based only on defoliated plants (relative to their starting area); we divided the amount of new leaf area produced during the 21 day (re)growth period by the initial leaf area recorded just before damage was imposed.

In the undamaged state, early diverging and derived milkweeds produced, on average, similar absolute amounts of root and shoot mass as well as leaf area (all P -values > 0.2). Thus, overall growth rates appear quite similar; nonetheless, the relative investment in roots (i.e., root-to-shoot ratio) per species was more than four times higher in the more derived compared with the early diverging species (mean \pm SE early diverging species, 0.304 ± 0.276 compared with more derived: 1.383 ± 0.276 , $F_{1,8} = 7.636$, $P = 0.033$). Reduction in root mass due to herbivory was strongly predicted by phylogenetic position, with more derived species showing progressively reduced proportional impacts of leaf defoliation (Figure 4.7a); however, there was no difference for shoot mass (Figure 4.7b) or leaf area growth ($F_{1,8} = 1.106$, $P = 0.334$). Thus, the early relative allocation to roots is higher in more derived milkweeds, and this seems to allow greater tolerance (i.e., reduced negative impacts of defoliation) in root tissues.

Interestingly, early diverging and more derived species recovered quite equally aboveground, and this apparently comes at a greater cost to early diverging species, since they apparently lose proportionally more root tissue. Although the specific importance of root reserves is unclear, we expect that the low tolerance of roots could lead to compromised fitness over several years of defoliation (all species of *Asclepias* are perennial). We caution that we examined only the short-term growth of young plants; thus, the degree to which the extensive “root” diversity among milkweed species (true roots, tubers, rhizomes, etc.; M. Fishbein, unpublished data) could contribute to differences in tolerance in mature plants is unknown and may not reflect patterns found during the early establishment phase.

As a final approach to understanding resistance and tolerance of milkweeds in the context of overall strategies to cope with both their biotic and abiotic environments, we consider the extent of clonality in milkweeds, which could be a proxy for tolerance by means of sprouting new ramets. As a measure of clonal potential, we examined the caudices (the underground bases of the stem) of all of the individuals from our previous study of 51 milkweed species (Agrawal et al. 2009a), and recorded the number of belowground buds, which are dormant meristems that can produce new ramets. Species known to grow as well-defined genets emerging from single root crowns (e.g., *A. humistrata*, *A. incarnata*, *A. linaria*, and *A. tuberosa*) showed very few, if any, underground stem buds, while highly clumped species known to grow in clonal patches (e.g., *A. pumila*, *A. subverticillata*, *A. speciosa*, and *A. syriaca*) produced high numbers of such underground buds. A phylogenetic analysis of directional trends revealed that progressively more derived species have enhanced clonal growth potential (Figure 4.8, LR = 12.4, $P < 0.001$). In this analysis, λ was estimated by maximum likelihood to be zero, indicating no phylogenetic signal for the clonal potential of *Asclepias*.

We emphasize that we have not attempted to disentangle the various drivers of root system evolution. Our primary conclusion is that more derived species are indeed more tolerant of herbivory, and that this tolerance is achieved by enhanced investment in the initial root system. It is also associated with the potential for clonal growth. Although it is tempting to ascribe these directional phylogenetic trends as causally correlated with the declines observed in

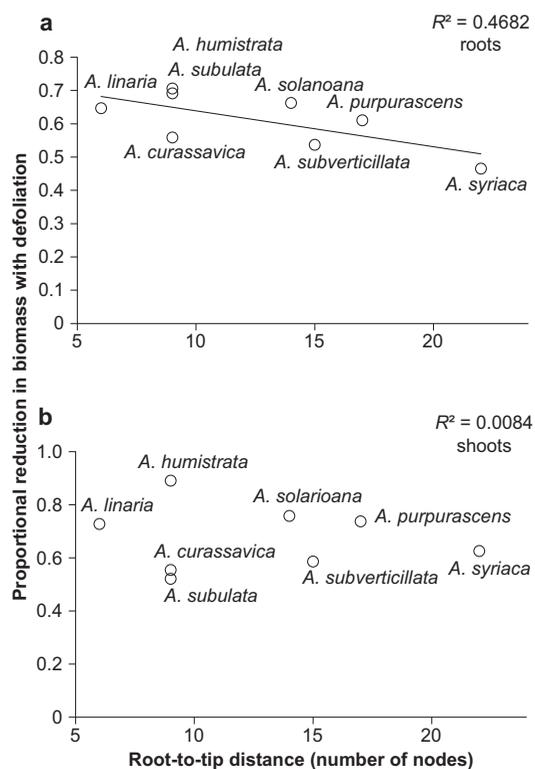


Figure 4.7. The relationship between phylogenetic position of milkweeds and tolerance to herbivory in (a) root and (b) shoot tissues. The identity of basal and derived species is given in the text under Experiment 2.

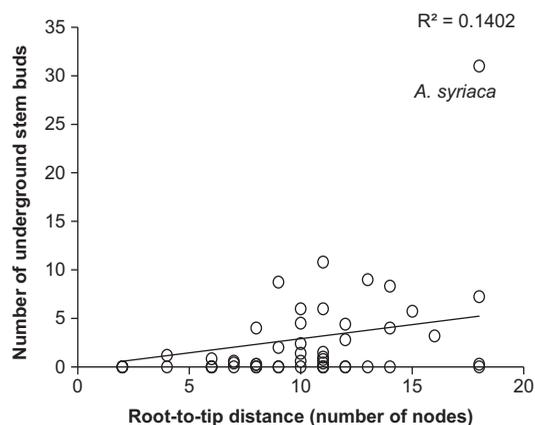


Figure 4.8. The relationship between phylogenetic position and production of stem buds produced on roots, an indicator of potential clonal growth for 51 milkweeds. Note that this relationship is significant with or without the single outlier (*Asclepias syriaca*) shown in the top right corner.

resistance traits (latex and cardenolides), we have yet to make this a strong link.

CONCLUSION AND OUTLOOK

We have been working under the assumption that monarchs and other specialist herbivores are major selective agents in milkweed evolution. Indeed, phylogenetic patterns suggest that milkweeds

have evolved greater levels of tolerance of herbivory and reduced levels of latex (which is abundantly implicated in resistance to monarchs). The details of cardenolide evolution are less clear cut. Although we have observed phylogenetic declines in cardenolides, the interpretation of this pattern is complicated by the fact that monarchs sequester cardenolides, which may protect them against predators and parasites. Nonetheless, we know remarkably little about the costs of producing the various milkweed defenses, the costs of sequestration for monarchs, and the rates of herbivore attack on the majority of *Asclepias* species in the field. All these factors have likely contributed to the macroevolutionary patterns we observe.

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