J Chem Ecol
DOI 10.1007/s10886-009-9719-0

Induced Responses to Herbivory and Jasmonate in Three Milkweed Species

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Received: 20 August 2009 / Revised: 9 October 2009 / Accepted: 25 November 2009

Abstract We studied constitutive and induced defensive traits (latex exudation, cardenolides, proteases, and C/N ratio) and resistance to monarch caterpillars (*Danaus plexippus*) in three closely related milkweed species (*Asclepias angustifolia, A. barjoniifolia* and *A. fascicularis*). All traits showed significant induction in at least one of the species. Jasmonate application only partially mimicked the effect of monarch feeding. We found some correspondence between latex and cardenolide content and reduced larval growth. Larvae fed cut leaves of *A. angustifolia* grew better than larvae fed intact plants. Addition of the cardenolide digitoxin to cut leaves reduced larval growth but ouabain (at the same concentration) had no effect. We, thus, confirm that latex and cardenolides are major defenses in milkweeds, effective against a specialist herbivore. Other traits such as proteases and C/N ratio additionally may be integrated in the defense scheme of those plants. Induction seems to play an important role in plants that have an intermediate level of defense, and we advocate incorporating induction as an additional axis of the plant defense syndrome hypothesis.

Keywords Multiple defenses · Secondary metabolites · Latex · Cardenolides · Monarch (*Danaus plexippus*) · Proteases · *Asclepias*

Introduction

Several lines of evidence highlight a matrix-like interaction among different types of plant defenses against herbivorous insects (Romeo et al. 1996; Rasmann and Agrawal 2009). Chemical and physical modes of defense can interact synergistically, often provoking an effect that is stronger than a single defense by itself. Alternatively, phytochemical diversity may be redundant, with different compounds providing the same impact, presumably as a protection against the failure of any one defense. In a classic study, Duffey and Stout (1996) showed that a variety of toxic compounds in tomato (alkaloids, phenolics, proteinase inhibitors, and oxidative enzymes) act together, affecting herbivores during ingestion, digestion, and metabolism to a higher degree than if each compound was ingested separately.

It is thought that plasticity in the deployment of plant defenses has evolved because they are costly (Karban and Baldwin 1997). Instead of expressing constitutively high defenses, many plant species express resistance traits in response to damage, thus resulting in fitness benefits (e.g., Agrawal 1998). For example, there are numerous changes in tomato foliage after caterpillar feeding, including increases in the levels of several proteinase inhibitors and in the activities of the oxidative enzymes polyphenol oxidase, peroxidase, and lipoxygenase, as well as marked changes in phenolic metabolism (Stout et al. 1996). These chemical changes are correlated with an increase in the resistance of tomato plants to several arthropod herbivores (Stout and Duffey 1996; Stout et al. 1998). Many chemical responses are controlled at the transcriptional level by the octadecanoid signaling pathway that includes jasmonic acid (Constabel et al. 1995). Exogenous jasmonic acid can be used to elicit many of the responses and to elicit resistance against herbivores (Thaler et al. 1996).

Milkweeds of the genus *Asclepias* (Apocynaceae) are a well-defended group of plants with a diverse array of resistance traits (Malcolm 1991; Agrawal and Fishbein 2008; Rasmann et al. 2009). All milkweeds have the ability

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Published online: 11 December 2009

to produce latex, which serves as a physical barrier and a source of concentrated toxins effective against chewing herbivores (Dussourd and Eisner 1987; Zalucki and Malcolm 1999; Zalucki et al. 2001b; Agrawal 2005; Agrawal and Konno 2009). Latex accumulates under pressure in special cells (laticifers), which quickly release the sticky substance upon damage. By partially severing a leaf's petiole or cutting the midrib, the flow of latex to distal tissues in that leaf essentially can be stopped. This treatment substantially improved the performance of monarch caterpillars on four latex-rich milkweed species, but had relatively minimal effect on caterpillars feeding on four milkweed species that produce less latex (Zalucki and Malcolm 1999; Zalucki et al. 2001a, b). In addition, genetic families of *Asclepias syriaca* L. that exude more latex also have enhanced resistance to several insect species in feeding assays and in the field (Agrawal and Van Zandt 2003; Van Zandt and Agrawal 2004; Agrawal 2005).

All milkweed species also contain cardenolides, with concentrations varying over several orders of magnitude across species and among organs. Cardenolides inhibit Na^+/K^+-ATPases, which are important for maintenance of membrane potential in most animal cells. Cardenolides are toxic to a wide array of animals (Malcolm 1991). Differences in structure and polarity have been linked to differential absorption by animals, resulting in differential toxicity (Seiber et al. 1980; Malcolm 1991; Frick and Wink 1995). For example, the non-polar compound digitoxin is almost completely absorbed, irrespective of how it is administered to insects; conversely, ouabain, a highly polar cardenolide, is absorbed slowly from the gastrointestinal tract (Malcolm 1991). The Na^+/K^+ ATPase from specialist herbivores, such as the monarch butterfly (*Danaus plexippus*) has a slightly modified cardenolide binding site, thus reducing cardenolide toxicity (Holzinger and Wink 1996). Specialist herbivores of milkweed also may sequester cardenolides that are used for their own defense (Brower et al. 1972; Malcolm et al. 1989).

Other secondary metabolites that occur in milkweeds are cysteine proteases. Proteases, found in all living organisms, cleave proteins. Various types are found in the latex of plants that belong to a diversity of phylogenetic clades. For example, cysteine proteases are reported in latex of families such as Caricaceae, Moraceae, and Apocynaceae (Light et al. 1964; Sgarbieri et al. 1964; Arribére et al. 1998), and serine protease in Moraceae, Euphorbiaceae, Apocynaceae, Convolvulaceae (Arima et al. 2000; Tomar et al. 2008). Direct evidence for the involvement of cysteine proteases in plant resistance against herbivores came from experiments in which the toxicity of wild fig (*Ficus virgata*) against two generalist herbivores disappeared when E-64, a specific cysteine protease inhibitor, was painted on leaf surfaces (Konno et al. 2004). In previous work, we found that cysteine protease activity showed a 24-fold variation across the latex of 36 milkweed species (Agrawal et al. 2008).

It has been proposed that the carbon/nitrogen ratio (C/N ratio) of plant tissues is a significant regulator of susceptibility to leaf-eating insects (Behmer 2009). In particular, nitrogen may be the limiting factor for herbivores (Mattson 1980). Changes in C/N ratios in leaves might, therefore, be seen as an additional line of defense against folivorous insects.

The orchestration of multiple types of defenses in response to herbivore attack seems to be the key to 'plant immunity' (Howe and Jander 2008). Jasmonic acid plays a central role in coordinating the induction of the multivariate responses (Chini et al. 2007; Thines et al. 2007). Yet, we still know remarkably little about how different defensive traits are altered by herbivory, which traits are the most important for resistance, or how trait expression is coordinated. Closely related species that show divergent defensive strategies are especially interesting to compare because they can give us insight into how traits and their importance in resistance vary and potentially how they evolve.

In this study, we assessed variation of three plant defense related traits (latex, cardenolides, and proteases), one primary metabolism trait (C/N ratio), and one measure of resistance (larval growth of specialist monarch butterfly caterpillars) among three milkweeds (*Asclepias spp.*). We examined induction by monarch larvae or by treatment with jasmonic acid. Additionally, we sought to disentangle the impacts of two potentially potent resistance components, latex and cardenolides, on larval growth.

Methods and Materials

Plants and Growth Milkweeds of the genus *Asclepias* include about 130 species in North America, including Mesoamerica and the Caribbean, and six additional species endemic to South America (Woodson 1954; Fishbein et al. 2009). Within *Asclepias* is a monophyletic clade, series Incarnatae, with about 21 species (Fishbein et al. 2009) that we have been studying with regard to the evolution of latex and cardenolides (Rasmann et al. 2009). Preliminary experiments within this clade permitted us to choose three species (*A. angustifolia, A. fascicularis*, and *A. barjoniifolia*) that vary in their latex production, cardenolide production, and inducibility (Rasmann et al. 2009; Agrawal et al. 2009). *Asclepias angustifolia*, found mainly in Arizona (USA) and northern Mexico, and *A. fascicularis,* found throughout the west coast of the United States and Northern Mexico (Woodson 1954), exude relatively little latex and produce low levels of cardenolides. Latex production is induced in the latter following monarch herbivory. *A. barjoniifolia*, principally found on hot dry slopes in inter-Andean

valleys of Bolivia and Northern Argentina (David 2007), produces more latex and higher levels of cardenolides constitutively. Cardenolides (Rasmann et al. 2009), but not latex (S.C. Cook et al., unpublished) are induced by monarch herbivory.

For all experiments, seeds were germinated at room temperature after being stratified at 4°C on moist filter paper for 2 wk. Seedlings were randomized and grown in potting soil (10 cm diam pots) in a growth chamber (14 h daylight, 26°C day: 20°C night). Plants were fertilized (N:P:K 21:5:20, 150 ppm N [μg/g]) once, 1 week after planting.

We used larvae of the monarch butteriflies *Danaus plexippus plexippus*, a species that feeds almost exclusively on *Asclepias* spp. Monarchs are native to North and South America, and are split into a subspecies in South America, *D. plexippus erippus* (Ackery and Vane-Wright 1984). We assume that the three *Asclepias* spp. that we studied are experiencing herbivory from these two subspecies of monarchs regularly.

Induction Experiments After 4 wk of growth, plants from the 3 species were divided equally into 3 groups: 1) controls, 2) plants induced by addition of one first instar monarch caterpillar, and 3) plants sprayed with roughly 1.5 ml of 0.5 mM jasmonic acid (N=16 plants per treatment, per species). After 4 d, all larvae used for induction were removed from the treated plants, and half of the plants (N=8 plants per treatment, and per species) were sampled to determine latex exudation and chemical composition (see below). The remaining plants were left untouched for 5 d before inoculating each with one first instar monarch caterpillar. This set of larvae was weighed (fresh weight) after 5 d of feeding to obtain a measure of larval growth rate.

Latex Exudation We measured the amount of latex exuded onto a 1 cm diam pre-weighted filter paper, after cutting the first 5 mm off the tip of the youngest fully expanded and intact leaf. Latex stopped flowing after ≈10 s, and all latex was absorbed on the filter paper. This disc was placed in a pre-weighed microcentrifuge tube and weighed to estimate wet latex exudation per plant. Our method is a reproducible assay for determining latex exudation.

Cysteine Protease Activity To measure protease activity, we prepared a 0.6% latex solution by removing 1–3 leaves at the petiole of each plant to collect 1.5 μl of latex, which was mixed with 250 μl of 50 mM sodium phosphate buffer (pH 7). We employed a modification of the spectrophotometric protocol developed by Konno et al. (2004). Briefly, a reaction buffer with casein (Sigma, St. Louis, MO, USA) as the substrate was incubated for 30 min and stopped with trichloroacetic acid. After precipitating the undigested casein, absorbance of the supernatant at 280 nm was recorded.

HPLC Analysis of Cardenolides After collecting latex, we harvested all aboveground plant tissue and immediately froze it at −80°C. Collected plant material was oven-dried at 50°C for 3 d and ground. Fifty milligrams of ground material, spiked with 20 μg of digitoxin (Sigma) as internal standard, was extracted with 1.9 ml of 95% ethanol in a sonicating water bath at 55°C for 20 min. Ethanol was evaporated, and the residue was dissolved in 0.5 ml methanol. Samples were analyzed by HPLC using a Gemini C18 reversed phase column (3 μm, 150×4.6 mm, Phenomenex, Torrance, CA, USA) and an Agilent 1,100 series instrument with diode array detection. The 15 ul injection was eluted at a constant flow of 0.7 ml/min with a gradient of acetronile and 0.25% phosphoric acid in water as follows: 0–5 min 20% acetonitrile; 20 min 70% acetonitrile; 20–25 min 70% acetonitrile; 30 min 95% acetonitrile; 30–35 min 95 % acetonitrile. Peaks were detected by a diode array detector at 218 nm, and absorbance spectra were recorded from 200 to 400 nm. Peaks showing a characteristic symmetrical absorption band with a maximum between 217 and 222 nm were recorded as cardenolides (Malcolm and Zalucki 1996). Concentrations were calculated and standardized by using a standard curve that related peak areas to known digitoxin concentrations.

Above-ground tissue C/N was assessed with ≈3 mg of dried and powdered material at the Cornell University Stable Isotope Laboratory.

All statistical analyses were conducted by using JMP (Version 7. SAS Institute Inc., Cary, NC, 2007). First, we performed an overall MANOVA on the four response variables together (latex, cardenolides, C/N ratio, and proteases). Then, we performed a two-way ANOVA on each of the responses to assess effects of species, treatment, and their interaction.

Artificial Diet Experiment To test specifically for the impacts of latex and cardenolides, we focused on one species (*A. angustifolia*). This species was chosen because it exudes comparatively small amounts of latex and has low levels of cardenolides. The species is relatively poorly induced by herbivory (Rasmann et al. 2009). We investigated the role of latex by comparing monarch larval weight gain on 5–wk-old plants grown as described above (treatment *Intact*) to weight gain of larvae fed on cut leaves (treatment *Cut*) (i.e., no latex exudation). All cut leaves were placed on moist filter paper in 10 cm diam plastic boxes, with the petiole in moistened foam to reduce desiccation. Moreover, we tested the effect of two cardenolides (digitoxin and ouabain) on larval growth by placing one first instar monarch larva on a cut leaf that was painted with either 0.5% dry weight (DW) digitoxin (Sigma) in a methanolic solution (treatment *Digitoxin*), or 0.5% DW ouabain (Sigma) in a methanolic solution (treatment *Ouabain*), or pure

methanol (treatment *Methanol*). All five treatments were randomized on a laboratory bench. Larvae were allowed to feed for 2 d before recording their mass. To assess the amount of leaf tissue eaten during the trial, we scanned the leaves and quantified the area of tissue consumed using ImageJ 1.41 software (http://rsbweb.nih.gov/ij/). Area was plotted against a known leaf mass per area regression curve (r^2=0.969, leaf mass=0.027* leaf area—0.01, P<0.001) to obtain the fresh leaf mass eaten by the insect. The effect of different treatments was assessed with one-way ANOVA. We assessed differences within species and treatments using Student's *t post-hoc* tests.

Results

Induction Experiment Overall, we found strong variability across the four plant traits (latex, cardenolides, C/N ratio, and proteases) measured across three species, and three treatments (Fig. 1, Table 1, MANOVA, species: Wilks' λ=, $F_{16,263.37}$=31.618, P<0.001; treatment: Wilks' λ=, $F_{8,172}$= 3.979, P<0. 001, interaction: Wilks' λ=, $F_{32,318.75}$=1.877, P<0.001). *Asclepias angustifolia* was the best host plant for monarch larvae, supporting an average growth rate (over 3 treatments) that was five-fold higher than the average rate on *A. barjoniifolia*, and three-fold higher than the average rate on *A. fascicularis*. Of the three species, only *A. fascicularis* showed increased resistance after induction by monarchs or jasmonic acid, and larvae on induced plants grew 68 and 74% less, respectively, compared to larvae on controls. In contrast, on *A. angustifolia* we found induced susceptibility; larvae grown on monarch-treated plants were 61% heavier compared to control plants. This effect was not mirrored in the jasmonic acid treatment, which had no effect (Fig. 1, Table 1). *Asclepias barjoniifolia* was uniformly of poor quality, and larval growth rate was not affected by either type of induction.

Latex exudation largely mirrored the effects of treatments and species on larval mass. *Asclepias barjoniifolia* and *A. fascicularis* produced the same amount of latex, while *A. angustifolia* produced four-fold less. *Asclepias barjoniifolia* increased latex production 32% following the jasmonic acid treatment, but production diminished more than 90% after monarch herbivory. Interestingly, after monarch damage, *A. barjoniifolia* latex production was essentially the same as *A. angustifolia*. Induction of latex also was found for *A. fascicularis,* with plants exuding 30% more following jasmonic acid or monarch induction.

Cardenolides were two and five times more abundant in *A. fascicularis* and *A. barjoniifolia,* respectively, than in *A. angustifolia.* A significant (30%) increase in cardenolide prosecution was induced in *A. barjoniifolia* by either induction treatment Overall the level of cardenolides was correlated with the number of individual cardenolide peaks found in our HPLC analysis (Pearson correlation: N=9, r^2=0.997, P<0.001). We found more peaks in *A. barjoniifolia* (8-10) than in *A. angustifolia* (4-6) or *A. fascicularis* (4-6) (Fig. 2). More cardenolide peaks were recorded after jasmonic acid or monarch treatments in all species. Across species, cardenolide peaks eluted between 15 and 20 minutes. Only *A. barjoniifolia* produced more polar cardenolides that eluted before 15 minutes (Fig. 2).

The C/N ratio was different across species, with *A. barjoniifolia* and *A. fascicularis* having a 22 and 14% higher ratio, respectively, than *A. angustifolia* (Fig. 1). Interestingly, jasmonic acid treatments decreased the ratio by 11%; but overall, monarch treatment increased it by 8%, resulting in a significant difference between these two treatments. Monarch treatment increased the ratio in *A. barjoniifolia* by 27%.

Proteases showed a significant species effect with *A. fascicularis* having almost twice the level found in *A. angustifolia,* and *A. barjoniifolia* having intermediate levels. (Fig. 1, Table 1). Jasmonic acid or monarch treatments generally decreased protease activity, with a dramatic 80% decrease in either jasmonic acid- or monarch-induced *A. barjoniifolia* compared to controls.

Artificial Diet Experiment Larval weight gain after two days of feeding was differentially affected by treatments ($F_{4,75}$= 4.68; P =0.002, Fig. 3a). Larvae were 1.7 times heavier on cut leaves, compared to those feeding on intact plants, indicating a role for latex in resistance to monarchs. However, larvae feeding on cut leaves painted with digitoxin performed as poorly as those feeding on intact plants. There was no effect of ouabain or the methanol control when painted on leaves, compared to control cut leaves.

The weight gain per amount of leaf mass eaten (food conversion efficiency) on digitoxin and on ouabain painted leaves was 38 and 36% lower than on control cut leaves, respectively ($F_{3,40}$=2.857, P=0.049, Fig. 3b). In other words, although conversion efficiency was decreased substantially for monarchs that consumed ouabain, they apparently were able to compensate for this reduction by consuming 53 % more tissue than larvae feeding on digitoxin.

Discussion

Variability of Responses Within plant taxa and across our three treatments, there was substantial variation in the constitutive and induced levels of several putative defense traits. Latex and cardenolides levels were species specific, and induction by monarchs and jasmonic acid had overlapping and distinct effects. Latex and cardenolides showed some correlation with larval performance. For instance, the

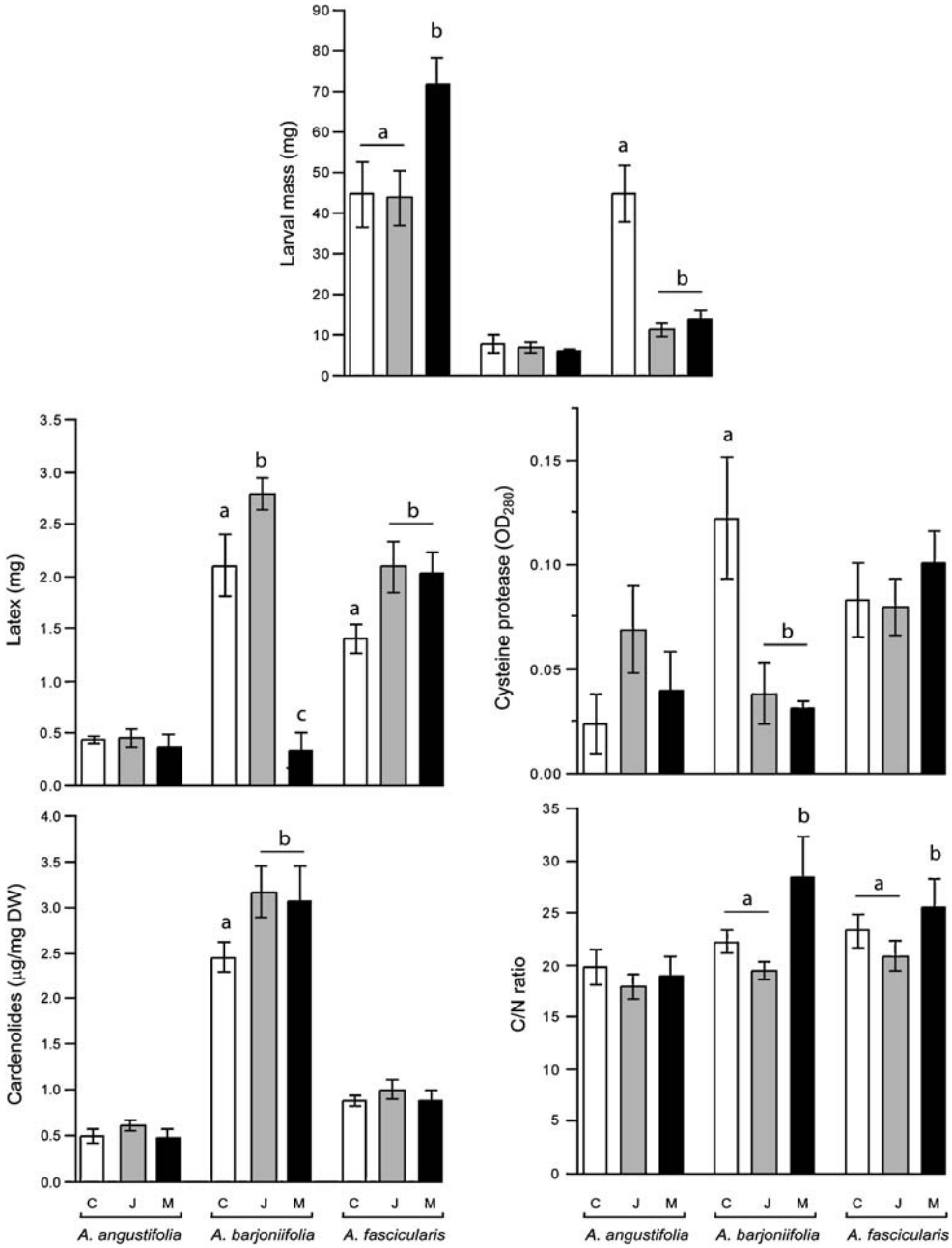

Fig. 1 Means ± SE of larval mass, latex, cardenolides, C/N ratio and proteases, in *Asclepias angustifolia*, *A. barjoniifolia*, and *A. fascicularis* and across the control (C), jasmonic acid (J), and monarch (M) treatments. Different letters above bars indicate significant differences among treatments within plant species ($P<0.05$)

low latex and low cardenolide species *A. angustifolia* supported more larval weight gain compared to high latex (*A. barjoniifolia* and *A. fascicularis*) or high cardenolide (*A. barjoniifolia*) species. The induction of latex but not cardenolides in *A. fascicularis* was accompanied by a decrease in larval weight gain (Fig. 1). In *A. barjoniifolia*, monarch induction did not alter larval growth because although cardenolides were higher after induction, latex production was diminished.

A high carbon to nitrogen ratio (C/N) could be caused by low levels of nitrogen or high levels of carbon-based compounds in the leaves, and in general indicates poor leaf quality. If a leaf is of a poor nutritive quality, it is predicted to be less defended than a high quality leaf (Coley 1983). In our case, we found species differences in the constitutive C/N ratios, but these were not mirrored by cardenolide levels. These differences in C/N may be more influenced by abiotic factors that shape plant ecophysiology (Agrawal et al. 2009) than by a response to herbivory. Matsuki and Koike (2006) showed that across seven species of deciduous broad-leaved trees that differed in their leaf life span, C/N correlated negatively with total N and herbivore survival, revealing that older leaves with low nitrogen content were less suitable for caterpillar growth. Following monarch feeding on *A. barjoniifolia* and *A. fascicularis*, C/N increased; this could be a result of metabolite reallocation and changes in

Table 1 Overall univariate anovas testing the effects of species (*asclepias angustifolia, a. fascicularis,* and *a. barjoniifolia*), treatments (control, jasmonic acid, and monarch), and their interaction for each of the tested traits sampled in the induction experiment

Source	Effect	DF	*F* ratio	*P*
Larval mass	Species	2,44	55.860	**<0.0001**
	Treatment	2,44	4.503	**0.017**
	SxT	4,44	7.748	**<0.0001**
Latex	Species	2,58	59.533	**<0.0001**
	Treatment	2,58	16.082	**<0.0001**
	SxT	4,58	13.845	**<0.0001**
Cardenolides	Species	2,53	145.161	**<0.0001**
	Treatment	2,53	3.760	**0.03**
	SxT	4,53	1.780	0.147
No. cardenolide peaks	Species	2,53	179.678	**<0.0001**
	Treatment	2,53	5.705	**0.006**
	SxT	4,53	2.076	0.097
C/N	Species	2,52	6.533	**0.003**
	Treatment	2,52	5.028	**0.011**
	SxT	4,52	0.969	0.432
Proteases	Species	2,53	3.884	**0.027**
	Treatment	2,53	0.682	0.510
	SxT	4,53	3.810	**0.009**

Bold indicates significant values ($P<0.05$).

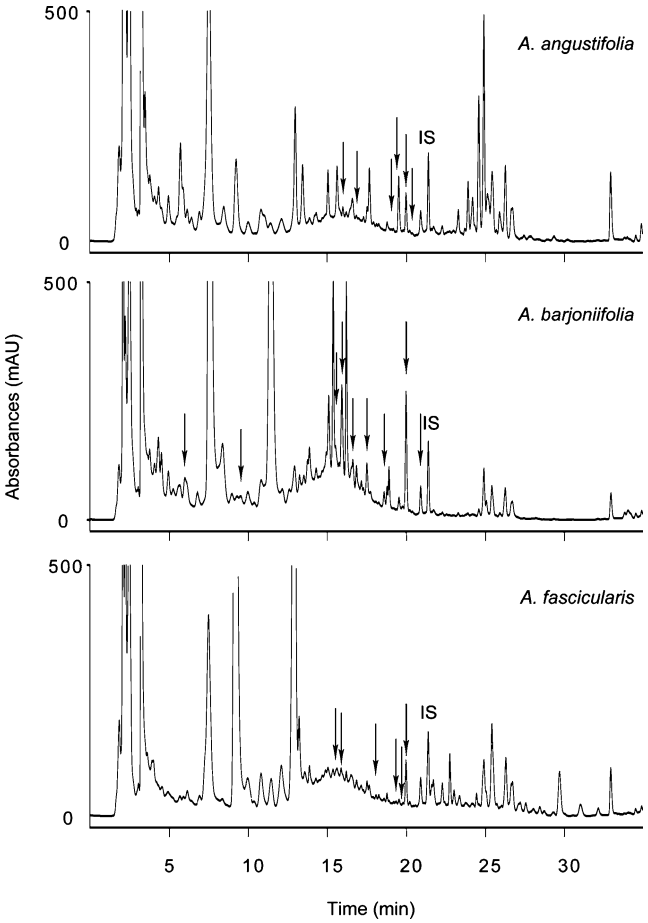

Fig. 2 Sample HPLC chromatograms of peak absorbance at 218 nm of leaf extracts for the three species of *Asclepias* studied. Scale is truncated at 500 mAU for better visualization of cardenolide peaks. Arrows mark all cardenolides found in undamaged plants. IS marks the internal standard (digitoxin)

photosynthesis. Consistent with the current findings, Agrawal and Fishbein (2006) showed that across 24 species of *Asclepias,* C/N correlated with leaf toughness, but not with qualitative defenses such as latex, cardenolides, or trichomes.

Cysteine proteases in latex drastically reduce herbivore growth (Konno et al. 2004). Across 36 species of *Asclepias,* Agrawal et al. (2008) showed that cysteine proteases in latex correlated positively with the amount of latex produced by the plant, but traded off with cardenolide concentration in latex. Here, the species with the highest leaf cardenolide level, *A. barjoniifolia*, also had the highest protease activity. We speculate that the consistently poor quality of *A. barjoniifolia,* irrespective of induced plant responses, was due to the high cardenolide, high latex, and high protease content of the species.

In the swamp milkweed *A. incarnata,* there was no influence of reduced latex flow on growth rates or survival of first-instar monarchs (Zalucki and Malcolm 1999). This is both a low latex and low cardenolide plant species, with smooth, soft, lanceolate leaves. The lack of a response to reduced latex flow in a low-latex and low-cardenolide milkweed lends credence to hypothesis that both latex and cardenolides are integrated defenses against early-stage monarch larvae. Similarly, Zalucki et al. (2001b) found that monarch larvae on nine *Asclepias* species generally grew faster and survived better on leaves when latex flow was reduced by partial severance of the leaf petiole. The outcome depended on milkweed species, and was related to the amount of latex and cardenolides produced, as well as to other plant characters, such as leaf hairs and microclimate. They concluded that several other parameters of plant quality/defense must play an important role in larval performance. We agree with this conclusion based on the three *Asclepias* species studied here.

Specificity of Induction Jasmonic acid now is widely accepted to be a key intracellular signal in mediating responses to insect attack (de Bruxelles and Roberts 2001), eliciting both direct and indirect defenses resulting in lower herbivore performance (Thaler et al. 2002). Here, we showed that exogenous application of jasmonic acid resulted in species- and trait-specific responses, which were often different from the responses plants produced after caterpillar feeding (Fig. 1). For example, latex induction

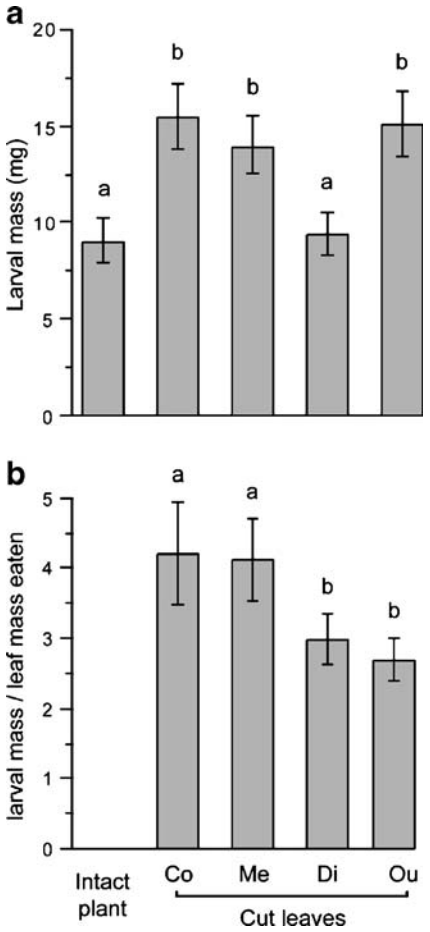

Fig. 3 **a**) Mean ± SE larval mass after 2 days feeding on *Asclepias angustifolia* intact plants, cut leaves (Co), cut leaves painted with methanol (Me), cut leaves painted with digitoxin (Di), or on cut leaves painted with ouabain (Ou). **b**) Mean ± SE conversion efficiency for each treatment other than the intact plant. Different letters above bars indicate significant differences among treatments ($P<0.05$)

was the same after jasmonic acid or monarch herbivory in *A. fascicularis*, but not for *A. barjoniifolia*.

One might think that the "sabotaging" behavior of monarch caterpillars, which notch veins and cut small moats through the leaves that reduce latex exudation at the feeding sites (Dussourd and Denno 1991; Zalucki and Brower 1992), was the cause of the reduced latex flow in *A. barjoniifolia.* However, in this species, latex was collected from healthy leaves, often opposite to the damaged one. Additionally, reduced latex production has been noted in other experiments with *A. barjoniifolia* when latex was collected several days after monarch feeding had ceased (S.C. Cook et al., unpublished data). Thus, the decrease in latex production in *A. barjoniifolia* following herbivory suggests that there may be other factors than mechanical sabotage that are involved in reducing latex production. Jasmonic acid, on the other hand, increased latex in the same species. This leads us to speculate that monarch larvae can impede or even disrupt some aspects of jasmonic acid induction in *A. barjoniifolia*.

Separating the Effects of Different Defense Components

Latex and cardenolides have detrimental effects on the growth rate of first-instar monarch larvae (Zalucki et al. 2001a, b; Agrawal 2005). Our results confirm this observation as we found that larvae fed on cut leaves perform better than larvae fed on cut leaves painted with digitoxin, or on intact plants. Based on field observations of naturally laid eggs, Zalucki et al. (1990) showed that survival in the first instars was weakly negatively correlated with plant cardenolide concentration in *A. humistrata*. Zalucki and Brower (1992) subsequently confirmed this observation experimentally and suggested that some of the high mortality in instars might be related to the cardenolide concentration in latex, or to the latex itself. This was confirmed by manipulative experiments in the field. By notching the basal side of the mid-vein of a leaf, Zalucki et al. (2001a) reduced latex flow and increased monarch larval growth rate. In our experiment, by removing the leaf from the plant, we similarly reduced latex exudation, thus also favoring larval growth. Here, however, we cannot completely exclude alterative explanations for the reduced resistance, such as the lack of responses that involves changes in systemic resource allocation.

Various levels of several cardenolides are found in latex and all other parts of the plant (Malcolm 1991), Fig. 2). Malcolm (1991) suggested that different types of cardenolides, having different polarities, are absorbed at different rates from the insect gut (Frick and Wink 1995). We compared the impacts of two cardenolides with different polarities. Larvae feeding on cut leaves painted with digitoxin (less polar) grew more poorly then larvae growing on leaves painted with ouabain (more polar). Although our conclusions regarding cardenolide toxicity to monarchs based on digitoxin and ouabain are tentative (digitoxin is only found in *Digitalis* sp.), our results are consistent with the prediction that non-polar compounds are more readily absorbed than polar ones (Wright 1960; Duffey and Scudder 1974; Malcolm 1991). The species studied here have cardenolides that may have polarities similar to digitoxin, based on elution time on reversed phase HPLC. *Asclepias barjoniifolia* also had some cardenolides that may have been more polar (Fig. 2).

In summary, although it is convenient to consider plant defense as a single trait, plants typically utilize a broad arsenal of defensive traits against herbivores (Duffey and Stout 1996; Romeo et al. 1996). Even when a plant species apparently is defended by a single type or class of defense chemical (like latex or cardenolides in our case), typically there are many specific forms of those compounds (Berenbaum et al. 1986; Malcolm 1991; Bennett and

Wallsgrove 1994). Thus, it is more useful to think about plant defense as a suite of traits, which might include aspects of a plant's nutritional quality, physical characteristics, toxicity, phenology, regrowth capacity, and indirect defense (Agrawal and Fishbein 2006). Synergistic interactions between multiple traits are particularly important in providing a greater level of defense than would be possible if the traits were present independently (Broadway et al. 1986; Berenbaum et al. 1991; Rasmann and Agrawal 2009).

By studying closely related species, we have taken account of similarities due to shared ancestry, and have attempted to identify important differences in defensive features (Agrawal and Fishbein 2006). Interestingly, our closely related species were substantially different in defensive traits, suggesting that they may have evolved under dramatically different habitats and herbivory regimes. An additional dimension to the plant defense syndrome hypothesis would be to incorporate induction and repression of defensive traits following herbivore attack. We found that *A. angustifolia* is nearly defenseless, so we suggest that for this species induction would not be helpful because herbivores generally have high performance on this species. On the contrary, *A. barjoniifolia* is extremely toxic and induced responses seem to be redundant, not providing additional protection. Finally, *A. fascicularis* had intermediate traits, with inducible latex, which in coordination with the other components of the defensive system, promoted reduced larval growth.

Acknowledgments We thank Amy Hastings for laboratory assistance, Rayko Halitschke for help with chemical analysis, Jennifer Thaler for providing jasmonic acid, Susan Cook, Rayko Halitschke and Mike Stastny for comments on the manuscript, and Steve Malcolm for providing seeds of *A. barjoniifolia*. Chemical analyses were conducted in the Cornell Chemical Ecology Core Facility, with support from Paul Feeny, New Life Sciences Initiative, College of Agriculture and Life Sciences, Center for a Sustainable Future, Boyce Thompson Institute, and Departments of Ecology & Evolutionary Biology, Neurobiology & Behavior, Entomology, Plant Biology, and Horticulture. Our research was supported by NSF-DEB 0447550 to AAA, and postdoctoral fellowship from Swiss National Science Foundation PA0033-121483 to SR.

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