esa

ECOSPHERE

Tests of the coupled expression of latex and cardenolide plant defense in common milkweed (*Asclepias syriaca*)

ANURAG A. AGRAWAL,[1,2,]† EAMONN T. PATRICK,[1] AND AMY P. HASTINGS[1]

[1]*Department of Ecology and Evolutionary Biology, Cornell University, Corson Hall, Ithaca, New York 14853-2701 USA*
[2]*Department of Entomology, Cornell University, Comstock Hall, Ithaca, New York 14853 USA*

Citation: Agrawal, A. A., E. T. Patrick, and A. P. Hastings. 2014. Tests of the coupled expression of latex and cardenolide plant defense in common milkweed (*Asclepias syriaca*). Ecosphere 5(10):126. http://dx.doi.org/10.1890/ES14-00161.1

Abstract. The coexpression of plant resistance traits suggests the hypothesis that they may have complementary functions in defense against herbivory. To address the extent to which defensive traits are necessarily coupled in plants grown under various conditions, we focused on latex and cardenolides, two potent defenses of milkweeds. We measured defenses across ontogenetic stages, different biotic and abiotic environments, and across genetic families of the common milkweed *Asclepias syriaca*. We first addressed the extent to which foliar cardenolides are derived from latex because latex actively flows through canals in leaves. We rinsed latex out of shredded leaves, which had no impact on foliar cardenolides, suggesting cardenolides are allocated to leaves independently of latex. Accordingly, there is potential for independent expression of the two traits. We next followed a cohort of plants from germination over three years; expression of both latex exudation and cardenolides increased annually, with the exception of a second year dip in cardenolides. Damage by monarch caterpillars induced ≈50% increases of both latex and cardenolides, with the former occurring rapidly within a day and the latter taking five days of herbivory; these responses were preceded by an earlier peak of the signaling hormones jasmonic acid and abscisic acid. Endogenous jasmonic acid showed an instantaneous positive correlation with latex exudation and foliar cardenolides. Under drought stress, latex and cardenolide expression were reversed, with water stress suppressing latex exudation, but nearly doubling cardenolide concentrations. These drought effects were not driven by phytohormones in the expected manner, as jasmonic acid was unaffected, salicylic acid was strongly suppressed, and abscisic acid tripled in response to drought. Finally, a meta-analysis of four previously published field studies representing 85 genetic families of *A. syriaca* revealed no evidence for a genetic correlation between latex exudation and foliar cardenolide concentrations. The same lack of a correlation was observed across 22 populations of *A. syriaca* when grown in a common environment. Thus, the two most important defensive traits of milkweeds, although often coexpressed, can become uncoupled during some ontogenetic stages, under some biotic and abiotic conditions, and there is no evidence that they evolve together.

Key words: cardiac glycosides; defense syndromes; drought stress; genetic correlations; jasmonic acid; monarch butterfly; plant-insect interactions; plant ontogeny.

Received 17 May 2014; revised 30 July 2014; accepted 5 August 2014; final version received 8 September 2014; **published** 21 October 2014. Corresponding Editor: T. Roulston.

† **E-mail:** aa337@cornell.edu

INTRODUCTION

Most plants have an incredible diversity of defensive traits (Duffey and Stout 1996, Agrawal and Fishbein 2006, Gaquerel et al. 2010). Hypotheses for the expression of multiple defensive

traits in a single species range from synergism or redundancy in their action, specificity of traits towards multiple attackers, to various non-adaptive explanations (Jones and Firn 1991, Berenbaum and Zangerl 1996, Rasmann and Agrawal 2009, Agrawal 2011, Moles et al. 2013). Yet, there are remarkably few tests of these hypotheses (e.g., Berenbaum et al. 1991, Müller et al. 2010). One approach to understanding plant defensive diversity is to unravel the phenotypic deployment of multiple traits. If specific defensive traits are co-expressed (i.e., increase or decrease in concert) across ontogeny, diverse environmental conditions, and genotypes, one would conclude that either the traits are severely constrained and must be co-expressed or that coexpression is beneficial. Alternatively, if the traits can easily become uncoupled, it begs the question of why is coexpression observed under some conditions.

The extent to which key defensive traits are coupled bears on potential constraints on trait expression, possible adaptive specificity in response to different attackers, and whether their expression can be independently shaped by natural selection. Our lab has been studying latex exudation and cardenolides, two strong defensive traits of *Asclepias* spp. with no known functions in primary plant metabolism (Agrawal and Konno 2009, Agrawal et al. 2012*b*). In particular, the common milkweed, *A. syriaca*, produces each trait constitutively and induces increases in both traits upon feeding by specialist monarch butterfly caterpillars. Latex is a physical defense, contains various secondary metabolites, and is also a carrier of cardenolides (Agrawal et al. 2008). Nonetheless, initial work suggested some specificity, differential induction, and a lack of a genetic correlation between the two traits (Van Zandt and Agrawal 2004, Bingham and Agrawal 2010).

In addition to studying induced responses to herbivory, here we consider plant ontogeny and an abiotic stress (drought) in order to address the coexpression of latex exudation and cardenolides under other conditions. In particular, we had no expectation that the two traits would be coexpressed along ontogeny or under drought stress. For example, as plant get larger, latex exudation is predicted to increase because of size dependent pressure and the growth of latex-delivering canals (Agrawal and Konno 2009); we have no such prediction about cardenolides. Under drought stress, we expected latex exudation to decline, as it is a water-intensive trait not involved in primary metabolism (Agrawal and Konno 2009, Barton 2014), but again we had no such expectation for cardenolides, which are carbon-based steroidal defenses (Agrawal et al. 2012*b*).

Here we use five approaches to understand the coexpression of latex exudation and cardenolides in the common milkweed (*A. syriaca*). (1) *Cardenolides in the latex*: We measured the relative concentration of cardenolides in latex and leaves. In addition, using a rinsing procedure to remove latex, we asked what contribution residual latex makes to the cardenolides measured in leaves. (2) *Ontogeny*: We followed a cohort of plants over 3 years and measured both traits each season to estimate their ontogenetic trajectories. (3) *Timing of induction*: using monarchs as herbivores, we assessed the timing of hormonal signal bursts (jasmonic acid, salicylic acid, abscisic acid) and the subsequent timing of induction of latex and cardenolides. (4) *Drought stress*: We assessed the impacts of this common abiotic stress on the expression of hormonal signals, cardenolides, and latex exudation. (5) *Genetic correlations*: Using a meta-analysis of four of our own previous field studies, we asked if there is a genetic correlation in the expression of latex exudation and cardenolides.

Methods

Cardenolides in latex.—Using two separate cohorts of plants, *A. syriaca* were germinated from seed collected in a natural population in Ithaca, NY, in petri dishes containing moist paper towels. Germinants were planted into 500-ml plastic pots with Pro-Mix BX soil and fertilized twice with a dilute fertilizer (NPK 21:5:20, 150 ppm N). We fully randomized the plants (one per pot) in a walk-in growth chamber (400 μmoles/m^2/sec light, 14h/10h light/dark cycle, 28°C/24°C temperature cycle). After growing for approximately 6 weeks, we clipped two of the youngest fully expanded leaves (opposing leaves in a pair) from each plant ($n = 19$). After excision, one leaf in each pair was kept intact, while the other leaf was quickly cut, with a razor blade, into small (1–2 mm) squares. We then followed the rinsing

protocol of Konno et al. (2004), washing all samples three times with tap water, which was reported as an effective method to clean residual latex out of leaves. Samples were then frozen at −80°C, dried and processed for cardenolide analysis (see below). Total cardenolide concentrations were analyzed with a paired analysis of variance.

To specifically address the cardenolide content of *A. syriaca* latex, we sampled 20 wild plants across field sites in Ithaca, NY and collected a single leaf from each as well as 20 μl of latex (from the cut petiole when the leaf was removed). Leaves were collected for cardenolide analysis as outlined below. The latex was pipetted into a pre-weighed microcentrifuge tube and weighed after drying overnight at 45°C; samples were subjected to the same protocol for cardenolide analysis given below. Again, a paired analysis of variance was used to determine variation in the cardenolide content of leaves and latex.

Ontogeny.—In this experiment, we followed a cohort of plants for three years following germination, and measured latex cardenolides once during each growing season. No treatments were imposed and all measures were taken from the youngest fully expanded leaves to standardize leaf age. In the summer of 2009, we germinated seeds of 10 full-sib families *A. syriaca* as above. After two months of growth in a growth chamber, a measure of latex and cardenolides was taken (n = 61; see below for methods). The plants were then moved outside (October 2009), hardened in a lath house, clipped, and mulched to overwinter. In May 2010, we uncovered the plants and transplanted them into 4-l pots containing a 1:1:1 mixture of topsoil, compost, and sand. These second year plants grew in full sun out of doors, were watered as needed, and were sampled for latex and cardenolides in July 2010. In this second year, because of minor overwintering mortality and the fact that some plants were utilized for a separate experiment, replication was reduced (n = 38). Plants were again clipped, mulched to overwinter, and were next sampled in July 2011 (n = 27). Herbivory was minimal and the plants did not flower by the end of 2011. To characterize latex and cardenolides in larger, mature, naturally occurring plants, in July 2009, we identified 24 *A. syriaca* plants across four field sites in Tompkins County, NY, USA, all occurring in full sun in open fields. All plants were flowering and were separated by at least 25 meters.

Timing of induction.—In this experiment we altered the amount of time monarch caterpillars fed on *A. syriaca* plants in order to assess the time-course of phytohormone (jasmonic acid, salicylic acid, and abscisic acid) expression and coupled latex and cardenolide induction. Plants from five full-sib families were grown from seed as above (n = 106) and half were randomly assigned to be infested with a single freshly hatched monarch caterpillar. We then proceeded to harvest an equal number of control and monarch-damaged plants at five time points: (1) after 24 hours of damage, (2) after 72 hours damage, (3) after 72 hours damage plus an additional 48 hours with the caterpillar removed, (4) after 72 hours damage plus an additional 96 hours the caterpillar removed, and (5) after 120 hours damage. After latex exudation was measured on each plant (see methods below), we divided the apical tissue for analyses of phytohormones and cardenolides.

We used a two-way factorial analysis of variance with treatment (monarch herbivory or not), harvest day, and their interaction as factors. For clarity and ease of interpretation, data for plant traits assessed two and four days after damaged ceased (time points 3 and 4 above) were not included in the same statistical model. We further assessed the instantaneous relationship between endogenous jasmonic acid and each of the two defenses (because tissue for all three analyses was simultaneously harvested) in a model with the treatment-by-harvest day combination included as a blocking factor.

Drought stress.—This experiment was replicated over two temporal blocks designed to assess the impact of an abiotic stress on the coexpression of latex exudation and foliar cardenolides. Plants from five full-sib families were germinated and grown in a growth chamber as described above. Plants were grown for three weeks with ad libitum water (i.e., to saturation). Plants were fertilized once (as above) 10 days after germination. At the three week mark, drought stress, achieved by differential watering, was randomly assigned and imposed on half of the plants for an additional 18 days prior to harvest. Control or

non-droughted plants were watered at first sign of dryness, every 2–3 days, keeping the soil noticeably moist, whereas drought treatment plants were not watered until the first sign of leaf wilt (often 7–10 days without water). Plants were checked for wilting daily. At first sign of wilting, droughted plants were watered with 50 mL water. Prior to watering plants in both treatments, the soil was gently punctured with a fork in a circular pattern approx. 2 cm from the stem of the plant to ensure permeability. Across the two blocks, a total of 103 plants were equally assigned to the two treatments, with half of the plants harvested for phytohormones and the other half harvested to measure cardenolides and latex; in both cases, apical leaves were harvested.

Genetic correlations.—In four previously published experiments we had quantified latex and cardenolides across a set of genetic families (full siblings) grown in common field environments (Agrawal 2005, Mooney and Agrawal 2008, Bingham and Agrawal 2010, Agrawal et al. 2012*a*). Here we use these data to assess any general pattern in the coexpression of the two traits across families. All data come from field common gardens, unmanipulated plants (i.e., no treatment), sampled from the youngest fully expanded leaves, and where both latex exudation and cardenolides showed significant family-level variation (i.e., had heritabilities significantly greater than zero). Across the four experiments, 85 genetic families are represented from three populations in southern Ontario and central New York. Analysis of covariance was used to assess the association between latex and cardenolides while blocking by experiment.

An additional population level analysis was conducted based on cardenolide and latex data in Woods et al. (2012), which represents 22 populations of *A. syriaca* across the entire latitudinal range (11 degrees of latitude, from New Brunswick, Canada, to North Carolina, USA). Approximately five individuals of each population were grown in a field common garden in Ithaca, NY, USA and mean population values for cardenolides and latex exudation from the second year of growth were correlated here.

Measuring latex.—We measured latex exudation on all plants by cutting 2–3 mm off the tip of the youngest, fully expanded, undamaged leaf, and collecting the latex on a pre-weighed 1-cm filter paper disc. After absorbing all of the latex, we placed the disc into a pre-weighed microcentrifuge tube. These tubes were stored in a −80 freezer until they could be reweighed to estimate the mass of wet latex collected. This method is a repeatable measure of latex exudation and has been shown to predict resistance to herbivores (Van Zandt and Agrawal 2004, Agrawal 2005). Immediately following latex collection, two leaves (that from which latex was collected and the opposite leaf) were typically collected for cardenolide analysis.

HPLC methods for cardenolides.—Fully expanded leaves from each plant were placed in a coin envelope, frozen at −20°C overnight, dried at 45°C, ground to fine powder using a Retsch Mixer Mill 300, and weighed. Cardenolide concentrations were assessed by HPLC, following Bingham and Agrawal (2010). Briefly, 50 mg dried leaf tissue was extracted with 1.8 ml methanol (MeOH), spiked with 20 μg digitoxin as an internal standard, and samples were homogenized on a FastPrep instrument (MP Biomedicals, Solon, Ohio, USA) at 6 m/s for 45 s. After centrifugation, the supernatant was collected, dried, resuspended in 1 ml MeOH, and filtered through a 0.45-μm syringe driven filter unit. Fifteen microliters of extract was then injected into an Agilent 1100 series HPLC and compounds were separated on a Gemini C18 reversed phase column (3 μm, 150 × 4.6 mm, Phenomenex, Torrance, CA). Cardenolides were eluted on a constant flow of 0.7 ml/min with an acetronile–0.25% phosphoric acid in water gradient as follows: 0–5 min 20% acetonitrile, 20 min 70% acetonitrile; 20–25 min 70% acetonitrile, 30 min 95% acetonitrile, 30–35 min 95% acetonitrile. UV absorbance spectra were recorded from 200 to 400 nm by diode array detector. Peaks with absorption maxima between 217 and 222 nm were recorded as cardenolides and quantified at 218 nm. Concentrations were calculated and standardized by peak areas of the known digitoxin concentration.

LC/ms methods for plant hormones.—Hormones were quantified using an established liquid chromatography–mass spectrometry procedure, modified from Thaler et al. (2010). Briefly, flash frozen samples were transferred into 2-mL screw cap tubes containing 900 mg zirconia/silica beads

(BioSpec, Bartelsville, OK, USA) and 1 mL extraction buffer. d_4-SA, d_5-JA, d_6-ABA (CDN isotopes, Point-Claire, Canada) were added as internal standards and samples were homogenized in a FastPrep instrument at 6 m/s for 45 s. Samples were dissolved in 200 μL methanol after extraction with dichloromethane and solvent evaporation and 15 μL were analyzed on a triple-quadrupole LC-MS/MS system (Quantum Access; Thermo Scientific, Waltham, Massachusetts, USA). Analytes were separated on a C18 reversed-phase HPLC column (Gemini-NX, 3 μ, 150 × 2.00 mm; Phenomenex, Torrance, California, USA) using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 300 μL/min. The initial condition of 10% B was kept for 2 min and increased to 100% solvent B at 20 min. Phytohormones were analyzed by negative electrospray ionization (spray voltage: 3.5 kV; sheat gas: 15; auxiliary gas: 15; capillary temperature: 350°C), collision-induced dissociation (argon CID gas pressure 1.3 mTorr [1.3 micron Hg], CID energy 16 V) and selected reaction monitoring (SRM) of compound-specific parent/product ion transitions: SA 137→93; d_4-SA 141→97; JA 209→59; d_5-JA 214→62; ABA 263→153; d_6-ABA 269→159.

Statistical analyses.—All analyses were conducted in JMP version 9 using analysis of variance (ANOVA). Residuals were normally distributed unless otherwise noted. Blocking factors were treated as random effects and were tested with a one-tailed likelihood ratio test, which is based on a chi-squared distribution with one degree of freedom (Littell et al. 1996). Interaction terms among main fixed effects (but not blocking terms) were included in the models.

Results and Discussion

Cardenolides in latex.—Our analysis of 19 pairs of washed and unwashed *A. syriaca* leaves provided little evidence that residual latex is an important source of foliar cardenolides (controls, 0.819 ± 0.086 mg/g dry mass; washed leaves, 0.828 ± 0.086 mg/g dry mass [mean ± SE]; $F_{1,18} = 0.018$, $p = 0.895$). Nonetheless, our analysis was sensitive enough to reveal differences between our plants (paired leaves: $\chi^2 = 13.6$, $p < 0.001$). Overall, there is remarkably little data on the phytochemical contribution of latex to the defensive chemistry of leaves. Konno et al. (2004) demonstrated that cysteine proteases in papaya latex, and left residually in cut leaves, were an important inhibitor of caterpillar feeding. Although *Asclepias* latex is known to also have high concentrations of cysteine proteases, their role in defense is unclear (Agrawal et al. 2008). Several herbivore species (e.g., *Trichoplusia ni*) that cannot feed on intact milkweed plants can complete development on cut leaves (A. A. Agrawal, *personal observations*), suggesting that pressurized latex is crucial for plant defense. The relative contribution of the physical barrier, deterrents, and toxins has not been well studied.

Many milkweed species also have concentrated cardenolides in their latex, with up to 50-fold more than leaves on a dry mass basis (e.g., *A. curassavica*) (Seiber et al. 1982, Agrawal et al. 2008). Nonetheless, we found slightly (but not significantly) lower cardenolide concentrations in latex compared to leaves (leaves, 1.123 ± 0.173 mg/g dry mass; latex, 0.821 ± 0.173 mg/g dry mass; $F_{1,19} = 1.524$, $p = 0.232$; pair: $\chi^2 = 13.6$, $p < 0.001$). For a subset of our samples ($n = 12$ pairs of leaves and latex), we weighed the leaf and latex masses wet and dry (the volume of latex collected was consistently 20 μl). Latex samples were largely consistent, with little variation in wet mass (37.37 ± 2.06 mg), dry mass (18.26 ± 2.03 mg), and percent water (an estimate of viscosity, 51.66 ± 3.65). In an analysis of cardenolides on a fresh mass basis, milkweed leaves had 43% higher cardenolides than the latex (leaves, 0.338 ± 0.042 mg/g wet mass; latex, 0.236 ± 0.042 mg/g wet mass; $F_{1,11} = 8.393$, $p = 0.015$). On average, *A. syriaca* had 0.43 (± 0.070) μg cardenolides per μl of latex.

Two previous studies reported tremendous variation in the cardenolide content of latex among *Asclepias* species (Seiber et al. 1982, Agrawal et al. 2008); for example, both studies found few to any cardenolides in latex of *A. speciosa* (a very close relative of *A. syriaca*), despite some moderate levels in leaves. Accordingly, it appears that although *A. syriaca* latex has modest cardenolides, cardenolides in leaves are not dependent on latex, and thus there is not an obvious constraint acting to force the joint expression of latex exudation and cardenolide concentrations in leaves.

Ontogeny.—Across three years of growth, our

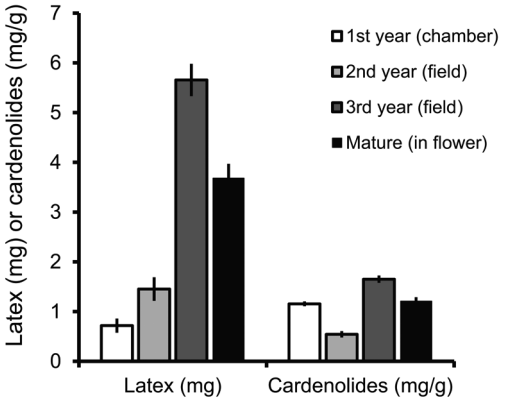

Fig. 1. Ontogenetic trajectories in the expression of latex exudation and foliar cardenolides. The first three time points follow the same cohort of plants while mature plants were assayed from a natural population. Note that the units for the Y axis are given next to the trait names. Shown are means ± SE.

cohort of common milkweed plants continuously increased the amount of latex exuded (Fig. 1). We previously reported a similar pattern, with latex exudation increasing nearly fourfold across two years in a field experiment (Agrawal 2005). In addition, latex exudation was genetically correlated across years, indicating that along-side this ontogenetic shift, the genetic component to latex exudation held constant (Agrawal 2005). This ontogenetic pattern is in contrast with cardenolides, which showed a 50% dip in year 2, followed by increases in the third year (Fig. 1). Although we do not have an explanation for the dip, it has been observed in another ontogenetic study of *A. syriaca* (M. D. Hunter, *personal communication*). Overall, this cohort of plants grew in the absence of competition in large pots and did not reach reproductive maturity. In contrast, mature, flowering plants (which were substantially larger in stature than our third year plants), naturally grew amongst old-field vegetation, and showed substantially lower values for latex and cardenolides (Fig. 1). Thus, across ontogeny, there appears to be a somewhat consistent co-deployment of latex and cardenolides; the main exception to this pattern is the dip in year 2 in the expression of cardenolides. Similarly, in Hawaiian prickly poppies, latex exudation and prickles on leaves are somewhat coexpressed, increasing across ontongeny within a growing season (Barton 2014).

Surprisingly few studies have followed ontogenetic trajectories of plant defense expression across multiple years. A recent meta-analysis found increasing investment in defensive chemistry across ontogenetic development for herbaceous plants, but this pattern was restricted to short term developmental changes, typically across the first year of growth (Barton and Koricheva 2010). A few other studies have followed insect attack on a cohort of plants across several years, but in these cases, the impact of changing plant phenotypes was unclear (e.g., Roininen et al. 1993). For common milkweed, which typically takes several years to mature, defense expression varies substantially during this process of maturity. Future work should evaluate the causes and consequences of this pattern, especially the second year dip in foliar cardenolide concentrations.

Timing of induction.—Across our previous work, we had begun to notice different patterns of cardenolide and latex induction associated with relatively minor differences in the extent of damage or timing of harvest (Mooney et al. 2008, Bingham and Agrawal 2010, Agrawal et al. 2012*a*). To address the kinetics of induction of the traits, we employed herbivory treatments of varying lengths and with harvests every two days. Results indicate that the jasmonic acid burst following herbivory peaks on day three (with relatively minor damage, $<3\%$ leaf tissue removal, caused by a neonate caterpillar), and declines thereafter, whether damage is continuous or not (Fig. 2). Although the pattern for abscisic acid was qualitatively the same, the decay to control levels was even more rapid (Fig. 2). Salicylic acid was not impacted by our treatments (data not shown). Results for latex exudation indicate a rapid and sustained response, whereas cardenolides did not induce until day five with continuous monarch damage (Fig. 2). In particular, for cardenolides, induction was neither evident on day 3, nor was it strong in subsequent assays if damage was ceased. Our interpretation of this result is that although the two defensive traits may be both regulated by jasmonic acid, the timing of their induction is distinct, suggesting independence of expression.

Given that the harvesting of tissue (for hormones, latex, and cardenolides) is destructive,

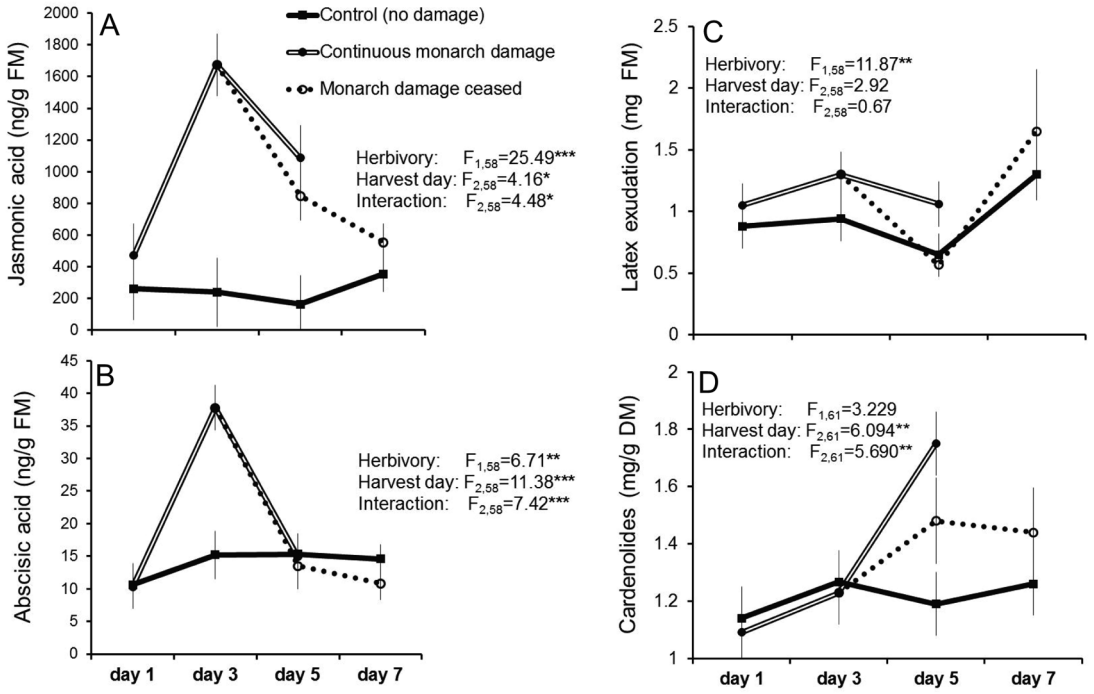

Fig. 2. Impacts of monarch damage on mean ± SE phytohormones (A, B) and defensive end-products (C, D) of common milkweed after 1, 3, and 5 days of continuous damage. Also shown are values for plants damaged for three days, but harvested 2 and 4 days after damaged ceased (not included in the statistical model). Data for salicylic acid is not shown as there were no significant treatment effects. FM = fresh mass; DM = dry mass.

we were only able to measure their simultaneous expression for individual plants. Endogenous jasmonic acid levels in leaves showed an instantaneous positive association with latex exudation ($F_{1,90.9} = 4.317$, $p = 0.041$) and cardenolides ($F_{1,65.6} = 4.830$, $p = 0.032$) (Fig. 3). Given the timing of hormonal induction and the difference in the timing of latex and cardenolide induction (i.e., a delay in cardenolide induction after the hormonal peak; Fig. 2), these relationships may be even stronger than appears in correlations based on simultaneous assessment of the traits.

Our patterns of the timing of cardenolide induction differ substantially from those reported by Malcolm and Zalucki (1996). There, mechanical damage (hole punches) were imposed on field plants of *A. syriaca*, and a time course of cardenolide concentrations was followed over six days and compared to the initial measure (at time zero). In the 24 hours following damage, cardenolides increased nearly threefold, but relaxed to control levels 5 days later (Malcolm and Zalucki 1996). However, this remarkably fast induction (evidenced within 10 minutes) could have been the effect of increased latex flow to the areas that were damaged, as cardenolide synthesis could not have occurred so quickly. In addition, our previous work indicates that mechanical damage induces a much weaker effect on cardenolides than real herbivore damage (Mooney et al. 2008). More generally, our findings on the timing of hormone and defense induction, although quantitatively different from some systems, follows the general pattern of hormonal signals showing an earlier peak than the defenses themselves (Baldwin et al. 1997). Previous work designed to address the relationship between jasmonic acid expression and the two defenses revealed that jasmonic acid typically correlates positively (and quantitatively) with latex exudation in five other milkweed species (Agrawal et al. 2014*b*). For cardenolides, however, although a jasmonic acid burst is associated with cardenolide induction, we have not previously observed a quantitative correlation between jasmonic acid levels and foliar

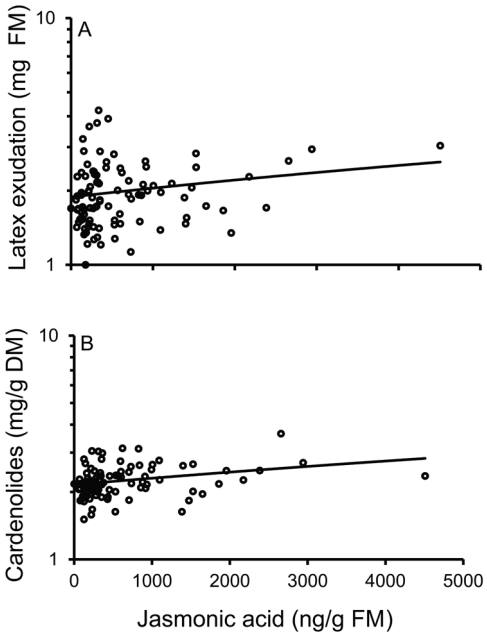

Fig. 3. Quantitative relationships between endogenous jasmonic acid levels and latex exudations (on a fresh mass basis, FM) (A) and cardenolide concentrations (on a dry mass basis, DM) (B). Shown are raw data +1 on a log scale. Analyses were conducted on log x+1 values and included treatment as a blocking factor in the statistical model.

cardenolide concentration (Agrawal et al. 2014*b*).

In other systems, the reported jasmonic acid burst is typically much faster than we typically observe for milkweed. For example, in corn there is typically a peak in jasmonic acid after 4–12 hours of herbivory (around 40–80 ng/g fresh mass), although these studies stopped after 24 hours (Schmelz et al. 2003, Dafoe et al. 2011); thus, it is unknown whether there are further increases in jasmonic acid as herbivory proceeds. Nonetheless, during these initial hours of herbivory, there is a tight quantitative correspondence between jasmonic acid levels and volatile terpene emissions from corn leaves (Schmelz et al. 2003). For wild tobaccos, wound-induced rapid bursts of jasmonic acid (peaking within 30–90 minutes) correlate with later nicotine and protease inhibitor expression, but these correlations degrade over time, with increasing bouts of simulated damage (and application of caterpillar oral secretions) (Baldwin et al. 1997, Stork et al. 2009). Importantly, we measured the hormones systemically in the apical tissues, whereas most other studies specifically measure the hormones only in the damaged tissues.

Drought stress.—We had predicted that drought stress would have divergent effects of latex exudation and cardenolides. Our treatment clearly had an impact on plants, as droughted plants had 23% fewer leaves than controls (controls, 14.97 ± 1.72; drought stressed, 11.58 ± 1.73; $F_{1,51} = 21.800$, $p < 0.001$). Analyses of plant hormones showed that jasmonic acid was unaffected by drought, salicylic acid was suppressed by 62%, and abscisic acid induced more than threefold (Fig. 4). The effect on defensive end-products was not concordant; we saw a >30% reduction in latex exudation and doubling of foliar cardenolides under drought compared to controlled conditions (Fig. 4). Nonetheless, the results of our phytohormonal analysis are consistent with other systems, whereby abscisic acid is induced by water stress and is antagonistic with salicylic acid (Thaler and Bostock 2004). Still, the opposing results of this drought treatment on latex exudation and cardenolides suggest an uncoupling of their expression, at least under some abiotic conditions.

The effects of latex and foliar cardenolides under drought may be due both to induced physiological processes as well as the reduced biomass of drought-stressed plants; the latter being relevant to the fact that we measure cardenolides as a concentration on a dry mass basis, and that larger plants typically exude more latex. Across other systems, plant defense compounds often increase in response to drought, but water content often declines (Briske and Camp 1982, English-Loeb et al. 1997). Accordingly, our results with milkweed may reflect the general pattern of increased secondary metabolite concentrations under drought, but the reduced latex may simply depend on water availability.

Genetic correlations.—We found no evidence of a genetic correlation between latex exudation and foliar cardenolide concentrations in our meta-analysis of four previously published field experiments. ($F_{1,76.3} = 1.205$, $p = 0.276$; Fig. 5). As would be expected, our experiments differed substantially in their mean trait values ($\chi^2 = 14.2$, $p < 0.001$). Similarly, when plants from across *A. syriaca*'s entire latitudinal distribution were

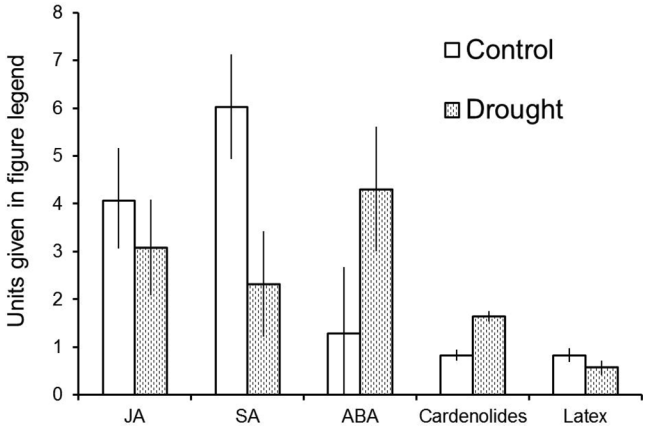

Fig. 4. Impacts of drought treatment on common milkweed on endogenous jasmonic acid (JA, μg/0.1g fresh mass), salicylic acid (SA, μg/0.1g fresh mass), abscisic acid (ABA, ng/g fresh mass), cardenolides (mg/g dry mass), and latex exudation (mg dry mass). Shown are means ± SE.

grown in a common environment, we also found no evidence for correlated expression of latex and cardenolides ($n = 22$ population means, $r = 0.151$, $p = 0.504$; data from Woods et al. 2012). This finding is consistent with Koricheva et al.'s (2004) analysis, which reported very little evidence for trade-offs between defense traits in plants. The robustness of our results and its consistency with the rest of the literature suggests that these two traits can evolve independently in common milkweed.

Conclusion and speculation

Latex and cardenolides can each negatively impact specialist milkweed herbivores, including monarch butterflies (Zalucki and Malcolm 1999, Zalucki et al. 2001, Agrawal 2004, Van Zandt and Agrawal 2004, Agrawal 2005, Agrawal et al. 2012*a*, Agrawal et al. 2014*a*), although we do not have information on their independent versus joint effects. Here we have shown that there are cardenolides in latex, but they are not concentrated compared to leaves (as is the case in other species; Seiber et al. 1982, Agrawal et al. 2008), and residual latex may not contribute substantially to what we measure as "foliar cardenolides". Because latex is rapidly transported following damage (Agrawal and Konno 2009), it is still unclear what impact latex flow can have on the rapid delivery of toxic cardenolides after damage occurs (Malcolm and Zalucki 1996).

Across macroevolutionary time, latex exudation and cardenolide concentrations have both declined in the most recently diverging *Asclepias* species (Agrawal and Fishbein 2008, Agrawal et al. 2009). Nonetheless, the two traits show no genetic correlations within *A. syriaca*, suggesting that they are free to evolve independently. Indeed, there are milkweed species possessing all combinations of high and low values for foliar cardenolide concentrations and latex exudation

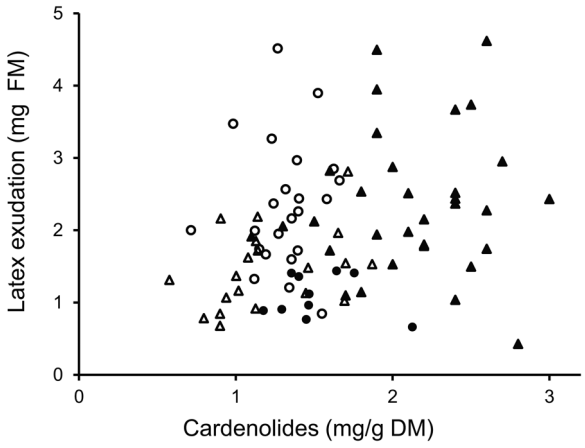

Fig. 5. Lack of genetic correlations between foliar cardenolides (on a dry mass basis, DM) and latex exudation (on a fresh mass basis, FM) across full-sib families of *Asclepias syriaca*. Open circles = Agrawal (2005), closed circles = Agrawal et al. (2012*a*), open diamonds = Bingham and Agrawal (2010), and closed diamonds = Mooney and Agrawal (2008).

(Agrawal and Fishbein 2008, Agrawal et al. 2009). Although the two traits are largely (although not completely) coupled ontogenetically and both are quantitatively regulated by endogenous jasmonic acid levels, they show different patterns of induction and have divergent responses to an abiotic stress (drought). Interestingly, different chewing herbivores cause differential induction of latex and cardenolides (Van Zandt and Agrawal 2004, Mooney et al. 2008, Bingham and Agrawal 2010, Agrawal et al. 2014*b*). Also, latex has an immediate effect upon leaf damage, while cardenolides are delayed, both in induction and their action upon feeding. Thus, given that coexpression of the two major defenses of milkweed is not inevitable, their joint induction following monarch herbivory suggests that they have complementary functions in defense against this species.

Acknowledgments

All chemical analyses were conducted in the Cornell Chemical Ecology Group's Core Facility with help from Rayko Halitschke. This research was supported by NSF-DEB 1118783 (AAA) (http://www.herbivory.com), NSF REU supplements that supported ETP, and the Templeton Foundation. We thank Alexis Erwin for help with data collection for analysis of plant ontogeny, and Lina Arcila Hernandez, Georg Petschenka, T'ai Roulston, Tobias Züst, and anonymous reviewers for helpful comments on the study.

Literature Cited

Agrawal, A. A. 2004. Plant defense and density dependence in the population growth of herbivores. American Naturalist 164:113–120.

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

Agrawal, A. A. 2011. Current trends in the evolutionary ecology of plant defence. Functional Ecology 25:420–432.

Agrawal, A. A., J. G. Ali, S. Rasmann, and M. Fishbein. 2014*a*. Macroevolutionary trends in the defense of milkweeds against monarchs: latex, cardenolides, and tolerance of herbivory. *In* K. Oberhauser, S. Altizer, and K. Nail, editors. Monarchs in a changing world: biology and conservation of an iconic insect. Cornell University Press, Ithaca, New York, USA.

Agrawal, A. A., and M. Fishbein. 2006. Plant defense syndromes. Ecology 87:S132–S149.

Agrawal, A. A., and M. Fishbein. 2008. Phylogenetic escalation and decline of plant defense strategies. Proceedings of the National Academy of Sciences USA 105:10057–10060.

Agrawal, A. A., M. Fishbein, R. Halitschke, A. P. Hastings, D. L. Rabosky, and S. Rasmann. 2009. Evidence for adaptive radiation from a phylogenetic study of plant defenses. Proceedings of the National Academy of Sciences USA 106:18067–18072.

Agrawal, A. A., A. P. Hastings, A. C. Knight, and E. T. Patrick. 2014*b*. Specificity of herbivore-induced hormonal signaling and defensive traits in five closely related milkweeds (*Asclepias* spp.). Journal of Chemical Ecology 40:717–729.

Agrawal, A. A., E. E. Kearney, A. P. Hastings, and T. E. Ramsey. 2012*a*. Attenuation of the jasmonate burst, plant defensive traits, and resistance to specialist monarch caterpillars on shaded common milkweed (*Asclepias syriaca*). Journal of Chemical Ecology 38:893–901.

Agrawal, A. A., and K. Konno. 2009. Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. Annual Review of Ecology, Evolution and Systematics 40:311–331.

Agrawal, A. A., M. J. Lajeunesse, and M. Fishbein. 2008. Evolution of latex and its constituent defensive chemistry in milkweeds (*Asclepias*): a test of phylogenetic escalation. Entomologia Experimentalis et Applicata 128:126–138.

Agrawal, A. A., G. Petschenka, R. A. Bingham, M. G. Weber, and S. Rasmann. 2012*b*. Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. New Phytologist 194:28–45.

Baldwin, I. T., Z. P. Zhang, N. Diab, T. E. Ohnmeiss, E. S. McCloud, G. Y. Lynds, and E. A. Schmelz. 1997. Quantification, correlations and manipulation of wound-induced changes in jasmonic acid and nicotine in *Nicotiana sylvestris*. Planta 201:397–404.

Barton, K. E., and J. Koricheva. 2010. The ontogeny of plant defense and herbivory: Characterizing general patterns using meta-analysis. American Naturalist 175:481–493.

Barton, K. E. 2014. Prickles, latex, and tolerance in the endemic Hawaiian prickly poppy (*Argemone glauca*): variation between populations, across ontogeny, and in response to abiotic factors. Oecologia 174:1273–1281.

Berenbaum, M. R., J. K. Nitao, and A. R. Zangerl. 1991. Adaptive significance of furanocoumarin diversity in *Pastinaca sativa* (Apiaceae). Journal of Chemical Ecology 17:207–215.

Berenbaum, M. R., and A. R. Zangerl. 1996. Phytochemical diversity: Adaptation or random variation?. Pages 1–24 *in* J. T. Romeo, J. A. Saunders, and P. Barbosa, editors. Phytochemical diversity and

redundancy in ecological interactions. Plenum Press, New York, New York, USA.

Bingham, R. A., and A. A. Agrawal. 2010. Ecological genetics of herbivore-specific induced defenses in common milkweed. Journal of Ecology 98:1014–1022.

Briske, D. D., and B. J. Camp. 1982. Water-stress increases alkaloid concentrations in threadleaf groundsel (*Senecio longilobus*). Weed Science 30:106–108.

Dafoe, N. J., A. Huffaker, M. M. Vaughan, A. J. Duehl, P. E. Teal, and E. A. Schmelz. 2011. Rapidly induced chemical defenses in maize stems and their effects on short-term growth of *Ostrinia nubilalis*. Journal of Chemical Ecology 37:984–991.

Duffey, S. S., and M. J. Stout. 1996. Antinutritive and toxic components of plant defense against insects. Archives of Insect Biochemistry and Physiology 32:3–37.

English-Loeb, G., M. J. Stout, and S. S. Duffey. 1997. Drought stress in tomatoes: Changes in plant chemistry and potential nonlinear consequences for insect herbivores. Oikos 79:456–468.

Gaquerel, E., S. Heiling, M. Schoettner, G. Zurek, and I. T. Baldwin. 2010. Development and validation of a liquid chromatography-electrospray ionization-time-of-flight mass spectrometry method for induced changes in *Nicotiana attenuata* leaves during simulated herbivory. Journal of Agricultural and Food Chemistry 58:9418–9427.

Jones, C. G., and R. D. Firn. 1991. On the evolution of plant secondary chemical diversity. Philosophical Transactions of the Royal Society B 333:273–280.

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

Koricheva, J., H. Nykanen, and E. Gianoli. 2004. Meta-analysis of trade-offs among plant antiherbivore defenses: Are plants jacks-of-all-trades, masters of all? American Naturalist 163:E64–E75.

Littell, R. C., G. A. Milliken, W. W. Stroup, and R. D. Wolfinger. 1996. SAS system for mixed models. SAS Institute, Cary, North Carolina, USA.

Malcolm, S. B., and M. P. Zalucki. 1996. Milkweed latex and cardenolide induction may resolve the lethal plant defence paradox. Entomologia Experimentalis Et Applicata 80:193–196.

Moles, A. T., B. Peco, I. R. Wallis, W. J. Foley, A. G. Poore, E. W. Seabloom, et al. 2013. Correlations between physical and chemical defences in plants: tradeoffs, syndromes, or just many different ways to skin a herbivorous cat? New Phytologist 198:252–263.

Mooney, K. A., and A. A. Agrawal. 2008. Plant genotype shapes ant-aphid interactions: Implications for community structure and indirect plant defense. American Naturalist 171:E195–E205.

Mooney, K. A., P. Jones, and A. A. Agrawal. 2008. Coexisting congeners: demography, competition, and interactions with cardenolides for two milkweed-feeding aphids. Oikos 117:450–458.

Müller, R., M. Vos, J. Y. Sun, I. E. Sønderby, B. A. Halkier, U. Wittstock, and G. Jander. 2010. Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. Journal of Chemical Ecology 36:905–913.

Rasmann, S., and A. A. Agrawal. 2009. Plant defense against herbivory: progress in identifying synergism, redundancy, and antagonism between resistance traits. Current Opinion in Plant Biology 12:473–478.

Roininen, H., P. W. Price, and J. Tahvanainen. 1993. Colonization and extinction in a population of the shoot-galling sawfly, *Euura amerinae*. Oikos 68:448–454.

Schmelz, E. A., H. T. Alborn, E. Banchio, and J. H. Tumlinson. 2003. Quantitative relationships between induced jasmonic acid levels and volatile emission in *Zea mays* during *Spodoptera exigua* herbivory. Planta 216:665–673.

Seiber, J. N., C. J. Nelson, and S. M. Lee. 1982. Cardenolides in the latex and leaves of 7 *Asclepias* species and *Calotropis procera*. Phytochemistry 21:2343–2348.

Stork, W., C. Diezel, R. Halitschke, I. Galis, and I. T. Baldwin. 2009. An ecological analysis of the herbivory-elicited JA burst and its metabolism: plant memory processes and predictions of the moving target model. Plos One 4.

Thaler, J. S., A. A. Agrawal, and R. Halitschke. 2010. Salicylate-mediated interactions between pathogens and herbivores. Ecology 91:1075–1082.

Thaler, J. S., and R. M. Bostock. 2004. Interactions between abscisic-acid-mediated responses and plant resistance to pathogens and insects. Ecology 85:48–58.

Van Zandt, P. A., and A. A. Agrawal. 2004. Specificity of induced plant responses to specialist herbivores of the common milkweed, *Asclepias syriaca*. Oikos 104:401–409.

Woods, E. C., A. P. Hastings, N. E. Turley, S. B. Heard, and A. A. Agrawal. 2012. Adaptive geographical clines in the growth and defense of a native plant. Ecological Monographs 82:149–168.

Zalucki, M. P., L. P. Brower, and A. Alonso. 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.

Zalucki, M. P., and S. B. Malcolm. 1999. Plant latex and first-instar monarch larval growth and survival on three North American milkweed species. Journal of Chemical Ecology 25:1827–1842.