

Growth–defense tradeoffs for two major anti-herbivore traits of the common milkweed *Asclepias syriaca*

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Costs of plant defense are a key assumption in evolutionary ecology, yet their detection has remained challenging. Here we introduce a novel method for quantifying plant growth using the common milkweed *Asclepias syriaca* and repeated non-destructive size measurements to experimentally test for costs of defensive traits. We estimated mechanistic components of plant growth (relative growth rate, net assimilation rate, specific leaf area and leaf-mass ratio) at two levels of fertilization (high and low), and related them to production of toxic cardenolides and exudation of sticky latex. We found negative genetic correlations between cardenolides and growth (most strongly with net assimilation rate) at both nutrient levels. Additionally, plants varied in their cardenolide response to low nutrients, and genetic families maintaining higher cardenolide production at low nutrient availability suffered proportionally larger reductions in growth. In contrast, the amount of latex was positively correlated with plant growth. Because latex is instantly deployed from a plant-wide system of pressurized laticifers, larger plants may simply exude proportionally more latex when damaged and thus plant size is likely to mask potential costs of latex synthesis. Unbiased quantification of mechanistic growth processes, coupled with the manipulation of nutrient or stress levels, is thus an effective approach to demonstrate allocation to defense and tradeoffs with growth, especially in long-lived plant species.

The idea that defenses have a cost may well be one of the most central paradigms in evolutionary ecological studies of plant–herbivore interactions (Koricheva 2002, Cipollini et al. 2014), as the existence of these costs provides the most compelling explanation for variability of defensive traits in natural plant populations (Herms and Mattson 1992, Rausher 1996). Costs of defense prevent maximally defended plant genotypes from becoming fixed within populations, as less defended genotypes are favored by selection where herbivory is infrequent in space or time (Agrawal et al. 2012b, Züst et al. 2012). Although lifetime investment in seed reproduction is often considered the gold standard for measuring costs of defense, this view is biased by a focus on annual plants and neglects the large number of perennials, many of which may live for decades and may reproduce clonally. Especially for long-lived species, growth may represent an appropriate proxy for fitness, particularly during establishment. Therefore, plant growth measured at the seedling stage is an important substitute measure of plant fitness (Herms and Mattson 1992). Additionally, accurate measures of resource allocation to growth are critical for studying the eco-physiology of growth–defense tradeoffs in any species.

As a plant is growing, it continuously modifies its allocation strategy based on its current pool of resources in order to produce new vegetative tissue, reproductive tissue, defensive traits, etc. In most environments, some nutrients

are limiting and competition with other plants for resources is strong, therefore faster growth is often favored as a means to monopolize a larger fraction of the total available resources (Fakheran et al. 2010). However, plants need to protect their tissue against damage by herbivores using an array of defensive traits, and the expenditure in energy and nutrients that is used up by the production of these defenses can be considerable (Simms 1992, Gershenzon 1994). We therefore expect tradeoffs between fast growth and high defense levels ('allocation cost') to be virtually ubiquitous in plant species. Surprisingly, in a meta-analysis of a large body of correlational studies on costs of defense, Koricheva (2002) found little support for the existence of allocation costs, and thus concluded that other types of cost, such as those that arise from interactions with the biotic environment ('ecological costs'), could be more important. However, this conclusion contradicts findings of a number of recent studies that focused on annual plants and artificially manipulated defensive traits (Siemens and Mitchell-Olds 1998, Zavala et al. 2004, Züst et al. 2011).

Indeed, allocation costs of defenses are likely to be present in many plant species, but correlation-based tests might fail to detect tradeoffs between growth and defense, both due to biological and statistical reasons. For example, allocation costs might differ between different plant organs (e.g. shoots and roots, Parker et al. 2012), as resource limitation will

vary both between organs and throughout plant development. Detection of costs will thus depend on both the plant organs and the growth stage of the plant that is considered. Additionally, systematic bias in growth rate estimation can mask tradeoffs between growth and defense (Paul-Victor et al. 2010). Specifically, relative growth rate (RGR) is generally calculated as $\ln(M_{t+\Delta t} / M_t) / \Delta t$, where M indicates biomass at any two time points t and $t + \Delta t$ (Hoffmann and Poorter 2002). However, implicit in this calculation of RGR is that a plant grows exponentially, which is usually prevented by resource limitation and size-dependent processes such as self-shading and allocation to structural components (Evans 1972). In nature, RGR declines with size, and the effects of initial size differences can be strong enough to mask patterns of correlations between growth rate and other traits (Turnbull et al. 2008, Rose et al. 2009, Paul-Victor et al. 2010). Biases in estimating RGR can be resolved by recording plant growth using frequent biomass measures and a non-linear function that accurately captures the growth progression of the plant (Paine et al. 2012), and allows comparisons of plants at a common reference size (Paul-Victor et al. 2010).

Additionally, size-standardized RGR is a phenotypic measure that is commonly considered the product of three components (Hunt 1982), each of which relates to allocation and thus could be a target of selection: 1) the net assimilation rate (NAR), which represents the increase in total biomass per unit time and leaf area; 2) the specific leaf area (SLA), which is the average fresh leaf area per unit dry weight of leaves and is associated with leaf thickness, water content and leaf nitrogen; and 3) the leaf-mass ratio (LMR), which is the proportion of total aboveground biomass (including structural stem tissue) that is invested into leaves. All three components of RGR are highly inter-related, and plants in optimal environments might be able to compensate for negative effects of defense costs on one component by adjusting the other two, resulting in no detectable effect on RGR and a false rejection of defense costs. For example, NAR is less sensitive to environmental effects than RGR (McDonald 1990), thus tradeoffs with defense might be more consistently detectable for NAR across a range of different environments.

Interestingly, even though NAR can vary greatly between species this variation is often independent of RGR, while most of the differences in growth rates between species are linked to differences in LMR and SLA (Potter and Jones 1977, Poorter 1990, Poorter and Remkes 1990, Hunt and Cornelissen 1997, Rees et al. 2010). Costs of secondary metabolism and defense thus have mainly been attributed to differential allocation of photoassimilates into new leaf area, rather than to direct interference with photosynthesis (Hermis and Mattson 1992). However, this might only be true for across-species comparisons, where larger differences in SLA and LMR could easily mask any patterns between NAR and RGR or defense. In contrast, genotypes of the same species are likely to have more similar leaf characteristics. Thus partitioning of RGR into its functional components for a group of genotypes with different levels of defense has the potential to shed additional light on physiological tradeoffs within plants.

We use the common milkweed *Asclepias syriaca* (Apocynaceae) to demonstrate how such a growth-oriented approach to

plant allocation can provide a robust framework for studying costs of defense in perennial species. Milkweed uses an array of defensive traits, including sticky latex (Agrawal and Konno 2009) and a diversity of toxic cardiac glycosides (cardenolides) that vary both qualitatively and quantitatively among plants and are present in shoots and roots (Agrawal 2005, Agrawal et al. 2012c). Both traits can be induced upon herbivore damage (Agrawal and Konno 2009, Bingham and Agrawal 2010, Rasmann et al. 2011), which is already suggestive of some type of costs associated with these traits (Zangerl and Bazzaz 1992). Latex is a largely systemic trait that is deployed throughout aboveground tissues via pressurized canals (laticifers) and is exuded upon leaf damage (Agrawal and Konno 2009). Relatively little is known about the biosynthesis of cardenolides (Groeneveld et al. 1990), but evidence from *Digitalis* (Plantaginaceae) suggests that plant enzymes can modify the polarity of cardenolides to regulate cellular transport and storage in and out of vacuoles and cells (Agrawal et al. 2012c). Local production and systemic transport are therefore likely to interact to distribute cardenolides across plant tissues. Both latex and cardenolides are primarily carbon-based defenses of terpenoid origin, but their synthesis is likely to require nutrient-demanding enzymes (Gershenzon 1994).

Here we use novel methods to estimate whole-plant growth rate and to further partition RGR into its physiological components, thereby testing for allocation costs of the two main defensive traits of *A. syriaca* at different levels of organization and in above- and below-ground tissues. We grew a set of 24 plant genotypes at two nutrient levels to specifically address whether 1) cardenolides in roots and shoots and latex in shoots trade off with plant growth, 2) how traits and tradeoffs respond to nutrient limitation; and 3) whether costs are differentially apparent in tradeoffs with physiological components of RGR.

Material and methods

Plant material

The common milkweed is a native perennial plant that occurs in disturbed areas and early successional habitats across eastern North America, with individuals of *Asclepias syriaca* reproducing both asexually by underground rhizome-like stems and sexually by flowers. While any established plant typically persists for long periods of time through clonal propagation, sexual reproduction is important for the colonization of new habitats. During the first bouts of colonization, young seedlings must survive within a strong competitive environment, therefore suggesting that differences in growth rates may directly affect establishment success (Züst et al. 2011). Seeds of *A. syriaca* are sired by the insertion of a single pollinium into a flower; hence all seeds from a single fruit pod typically represent a full-sibling genetic family (Gold and Shore 1995), resulting in consistently significant heritabilities for defensive traits (Agrawal 2005, Bingham and Agrawal 2010). Whole seed pods from *A. syriaca* plants were collected in twelve natural populations in Tompkins County, New York (Supplementary material Appendix 1 Table A1). Several genetic families

per population were grown in a preliminary experiment to measure cardenolide concentrations and latex exudation. Based on these data we selected 24 genetic families, two from each population, ensuring representative sampling of the full natural range of defensive phenotypes.

Growth experiment

Accurate modelling of plant growth and growth rate components requires fine-scale data on the growth progression of leaf area, leaf biomass, and total biomass. Such data are ideally gathered using sequential destructive harvests (Rees et al. 2010), yet quite often the requirements in plant material and growth chamber space of such experiments are prohibitive. We therefore combined frequent non-destructive size measurements (stem height and approximate leaf area) with three sequential destructive harvests of subsets of plants. Given the different units of height and area, we transformed both measures from each time point into biomass, extrapolating from correlations of exact trait values in the three destructive harvests. Using these biomass estimates, we then modelled plant growth throughout the course of the experiment to compare genetic family means of RGR and its components at a common plant size.

Plants were grown in three completely nested temporal blocks with 10, 6 and 8 unique genetic families per block, respectively. For each genetic family we aimed for 18 individuals. Seeds were scarified, cold stratified at 4°C on moist filter paper for a week, and germinated in the dark at 26°C. Seedlings were planted in plastic pots (10 cm diameter) using a 1:1 mixture of perlite and potting soil that had been thoroughly rinsed to remove excess soluble nutrients. Plants in each genetic family were randomly assigned to one of two levels of nutrient availability: plants in the high-nutrient treatment received 200 ml solution of 0.01% fertilizer (N:P:K 21:5:20, 150 ppm N) in water once a week, while plants in the low-nutrient treatment received a solution of 0.001% fertilizer in water. The low-nutrient treatment resulted in a small phenotype not commonly observed in our milkweed experiments. Plants were additionally watered 2–3 times a week as needed and grown for a total of 44 days.

We repeatedly measured plant size using non-destructive measures of stem height and approximate leaf area on all plants on days 5, 9, 13, 18, 23, 28, 36 and 44. Stem height was measured as the distance from the lowest leaf node carrying the cotyledons to the apex of the plant, while approximate leaf area was calculated from measurements of leaf length and width on one leaf from each leaf pair of a plant. On days 13 and 28, one to two plants per genetic family were destructively harvested unless this would have reduced the final number of plants below three per nutrient level, and on day 44 all remaining plants were harvested. Before each destructive harvest, we measured latex exudation by cutting the tip (2–3 mm) off the second-youngest, fully expanded leaf, and collecting all exuding latex onto a pre-weighed 1 cm disc of filter paper until latex flow stopped naturally. Discs were then placed into a pre-weighed microcentrifuge tube and whole tubes were immediately weighed on a microbalance. We cut plants at soil level and removed all leaves from the stem. We scanned leaves and quantified

the leaf area from scanned pictures using ImageJ (Rasband 1997–2014). For plants in the final destructive harvest, roots were carefully separated from soil and washed, and the cleaned roots, stems and leaves were then oven-dried at 40°C for a week and weighed to the nearest microgram. After weighing, dried leaves and roots were ground for later cardenolide extraction and analysis.

Leaf area growth

We first modeled the growth of total leaf area using the statistical software R (<www.r-project.org/>). We adjusted the approximate (rectangular) total leaf area from all eight time points using genetic family means for the ratio of actual versus approximate leaf area measured in the subset of harvested plants to minimize systematic over-estimation of leaf area. There was a clear indication for leaf area growth to approach an asymptotic final size over the timescale of the experiment. We thus excluded zero values and fitted an asymptotic regression model (aka monomolecular) to log-transformed areas, as visual inspection of residuals indicated the most realistic fit to the data of all commonly used asymptotic models (Paine et al. 2012). The function of the asymptotic regression model approaches a maximal final size as time $\rightarrow \infty$, but in contrast to the logistic model it has no point of inflection and instead, its relative growth rate is fastest initially and continues to decrease exponentially thereafter (Paine et al. 2012). The asymptotic regression model is given by

$$\ln(A_t) = K_l + (A_0 - K_l)e^{-r_l t} \quad (1)$$

where the leaf area A at time t is a function of the final maximal leaf area K_p , the initial leaf area A_0 at $t=0$, and the logarithm of the rate constant r_l (the rate constant is log-transformed to ensure positive growth). Equation 1 is implemented in R as the *SSasympt* function in the *nlme* package (Pinheiro et al. 2013) and was fitted as a non-linear mixed effects model (function *nlme* in the *nlme* package) treating plant identity as a random effect to account for repeated measurements. We found strong evidence for temporal, non-linear heterogeneity in the variances throughout time and therefore allowed for independent spread in the variance for each measurement day (*varIdent* variance structure in *nlme* (Zuur et al. 2009)). We fitted fixed effects of nutrient level, genetic family, and an interaction term on the three model parameters A_0 , r_l and K_p , thereby estimating unique parameter values for each combination of genetic family and nutrient level. We then attempted to simplify models by stepwise removal of model terms and comparing nested models using Akaike's information criterion (AIC). Models with the lowest AIC were favored, but simpler models were favored when pairs of nested models were within two units of AIC.

We extracted model parameters from the best model to calculate the relative leaf growth rate (RLGR). RLGR at time t (Paine et al. 2012) is given by

$$RLGR = (K_l - A_0)e^{-r_l t} \quad (2)$$

thus allowing us to calculate mean RLGR values for all genetic families at both nutrient levels at any given time during the experiment.

Biomass growth and growth rate partitioning

In a second step we modeled total biomass growth of plants to partition the plant's relative growth rate (RGR) into its mechanistic components. We estimated biomass conversion functions for the non-destructive size measures based on the subset of plants that were destructively harvested throughout the experiment. The mass of plant stems M_S was modeled as a function of stem height H with a non-linear regression through the origin of the form $M_S = a_S H^b$, where a_S is the slope of the regression and b is a fitted parameter that accounts for the nonlinearity between mass and height (Supplementary material Appendix 1 Fig. A1a–b). Leaf biomass M_L was modeled as a function of leaf area A with a linear regression through the origin of the form $M_L = a_L A$, fitting a single parameter a_L for the slope of the regression as there was no evidence for nonlinearity (Supplementary material Appendix 1 Fig. A1c–d). Both conversion functions were fitted using generalized least-squares regression implemented as the function *gnls* in the *nlme* package for R. We specified effects of nutrient level, genetic family, and an interaction term on all model parameters, and then attempted stepwise model simplification, comparing nested models based on likelihood-ratio tests. Using the most parsimonious models for stem height and leaf area, we transformed all non-destructive size measurements into biomass measures and combined predicted stem and leaf biomass into total shoot biomass. Since this method artificially reduces variation in biomass estimates, we finally adjusted model predictions based on the known deviation of the actual mass of each plant on the day of harvest at the end of the experiment. Assuming plants deviated consistently and proportionally from genetic family means throughout the experiment, we adjusted all predicted values of a plant by the ratio of its final predicted versus actual biomass.

We modeled the adjusted total biomass growth M_t at time t using a four-parameter logistic model, as visual inspection of residuals indicated the most realistic fit for this model to the biomass data. The four-parameter logistic model is implemented in R as the *SSfpl* function in the *nlme* package and is given by

$$M_t = M_0 + \frac{K_m - M_0}{1 + e^{r_m(t_0 - t)}} \quad (3)$$

where the total shoot biomass M at time t of a plant is a function of the final maximal mass K_m , the initial mass M_0 , the time t_0 when the plant mass is midway between K_m and M_0 , and a growth rate parameter r_m . This model was fitted as a non-linear mixed effects model with identical random and variance structure as for leaf area, and fixed effects of nutrient level, genetic family, and an interaction term on all four model parameters. We simplified the full model based on AIC and extracted parameter values from the most parsimonious model. We calculated RGR at a common reference size to standardize for potential size differences between plants. As reference sizes we used the fortieth percentile from the distributions of plant masses at each nutrient levels (18.29 mg and 39.31 mg, corresponding to 15.7 and 16.4 days of growth), since all plants occurred at these sizes (unlike, e.g. initial mass) and size limitations should still be minimal at this point. RGR at reference size M_{ref} (Paine et al. 2012) is given by

$$RGR = (M_0 - M_{ref}) \frac{r_m (K_m - M_{ref})}{M_{ref} (M_0 - K_m)} \quad (4)$$

RGR can be factored into its physiological components (Rees et al. 2010) using

$$RGR = NAR \times SLA \times LMR \quad (5)$$

which is identical to

$$\ln\left(\frac{1}{M} \frac{dM}{dt}\right) = \ln\left(\frac{1}{A} \frac{dM}{dt}\right) + \ln\left(\frac{A}{M_L}\right) + \ln\left(\frac{M_L}{M}\right) \quad (6)$$

Note that all components are log-transformed to simplify partitioning of RGR (Rees et al. 2010). Solving Eq. 3 for t , we could then calculate the time at which each genetic family reached the reference size M_{ref} allowing us to calculate the corresponding values of leaf area A (Eq. 1), RLGR (Eq. 2), and NAR, SLA and LMR (Eq. 6) to directly compare all growth parameters at a common size. We then finally partitioned the variation in RGR among its three components using the methods outlined in Rees et al. (2010). Briefly, the variance in $\ln(RGR)$ is given by the sum of variances and covariances of the three log-transformed growth components. The importance of each component in explaining variation in RGR is then given by the ratio of the summed absolute values of the component's variance and covariances over the sum of absolute values for all variances and covariances (Rees et al. 2010). Importantly, this approach does not assign causality but simply allocates the covariance terms equally between components.

Cardenolide analysis

Leaf and root cardenolides were extracted and analyzed by HPLC following a protocol from Rasmann et al. (2009), modified for improved sample cleanup and high throughput in a 96-well microplate format. Specifically, phenolic compounds that otherwise interfere with UV-detection of cardenolides were removed by filtration through DEAE-Sephadex A-25. We found Sephadex to have a low but significant affinity for phenolic compounds but not cardenolides, thus allowing us to partition samples into a fast-eluting fraction containing cardenolides and a slower-eluting, discarded fraction containing mainly phenolic compounds.

To ensure representative sampling of tissue within a plant, a subset of leaf material, equally sampled from all leaf pairs, or all dried root material of each plant was crushed and ground to a fine powder in a Retsch mixer mill. Aliquots of fifty milligrams of this tissue, spiked with 20 μg of Digitoxin as internal standard, were extracted with 0.5 ml of 100% methanol. We added approximately 30 FastPrep beads to each sample and agitated samples twice for 45 s at a speed of 6.5 m s^{-1} on a FastPrep-24 homogenizer, followed by centrifugation at 14 000 rpm for 12 min. Filter tubes in a 96-well format were loaded with approximately 65 mg of dry Sephadex using a 80 μl column loader and primed with 0.5 ml of 100% methanol. The supernatant of each sample was then added to a filter tube with primed Sephadex and eluted with 0.5 ml of 100% methanol into 1.4 ml tubes in 96-well racks. The eluates were dried down at 35°C on a vacuum concentrator, re-suspended in 200 μl

of 100% methanol, and filtered using a MultiScreen filter plate with a 0.22 μm PTFE filter.

We analyzed 5 μl of each sample by HPLC using a C18 100×3 mm column with 2.3 μm particle size and a Agilent 1100 instrument with diode array detection. The injections were eluted at a constant flow of 0.5 ml min^{-1} with a fast gradient of acetonitrile and water as follows: 0–1.5 min at 15% acetonitrile; 1.5–6.5 min from 15% to 30%; 6.5–10 min from 30% to 45%; 10–14 min from 45% to 55%; 14–15 min from 55 to 100%; and 15–22 min at 100%, followed by a 8 min reconditioning step at 15%. Peaks were recorded at 218 nm and absorbance spectra were recorded between 200 nm to 300 nm. Peaks showing a characteristic single absorption maximum between 214 and 222 nm were considered cardenolides. Peaks in different samples with differences in retention time of less than 0.05 min were considered the same compound, while we rejected compounds that were present in less than 80% of all samples. Concentrations of 15 individual cardenolide compounds were calculated by relating peak areas to the area of the internal digitoxin standard and compounds were summed as an estimate of total cardenolide concentration. Additionally, we performed principal component analyses (PCA) on genetic family means of the ten most abundant compounds in shoots to identify potential compound-specific tradeoffs with growth. On average, these 10 cardenolides accounted for 89% of the total shoot and 87% of root cardenolide content of the plant samples.

Trait correlations and use of graphical vector analysis

For analyses of the effect of the nutrient treatment on defense traits and biomass we used the full data and linear mixed effects models with a random effects structure of genetic family nested within block, thus accounting for interdependence between family means at the two nutrient levels and potential differences among temporal blocks of the experiment. In contrast, analyses involving growth rate estimates were based on family means ($n = 24$) for each nutrient level, since our best estimates of RGR and its components are given by the fixed effects of the non-linear mixed effects models of biomass and area growth (Eq. 3, 5). In all models we found strong evidence for heteroscedasticity between nutrient levels, thus we allowed for independent spread in the variance for each level (function *varIdent*). We report significance of fixed effects for all models as conditional F -tests, testing the marginal significance of each fixed coefficient when all other fixed effects are present in the model (Pinheiro and Bates 2000). If interactions with nutrient treatment were non-significant, we report estimates from the simplified, additive models. For models using the full data, we specifically tested for differences in the response of plant families to the nutrient treatment (i.e. interactions between fixed and random terms) using likelihood-ratio tests, while for models using mean data the reduced variation at the level of genetic family prevented us from performing such tests.

We used graphical vector analysis (GVA) to visualize the effect of nutrient limitation (i.e. the difference between the high- and low-nutrient treatment) on defense metabolism of both cardenolides and latex (Koricheva

1999). For cardenolides, the mean concentration (y-axis) for each genetic family at each nutrient level is plotted against the corresponding mean content (x-axis). Assuming that the tissue sampled for cardenolide analysis is representative for all leaves, content is simply the concentration multiplied by dry leaf biomass. Correspondingly, the amount of exuding latex (x-axis) is plotted against the amount of latex per total shoot biomass (= concentration, y-axis). In such a graph, diagonal lines through the origin represent the possible set of values for concentration and content of plants with identical biomass (Koricheva 1999). Movement along these diagonal lines in response to a treatment can be interpreted as changes in defense synthesis at constant biomass, while lateral movement indicates a change in both biomass and defense synthesis.

Results

Effects of nutrient limitation

Nutrient limitation had a strong negative effect on both above- and belowground plant biomass at final harvest, with plants in the low-nutrient treatment having on average 809% less shoot biomass ($F_{1,218} = 831.41$, $p < 0.001$) and 435% less root biomass ($F_{1,218} = 612.94$, $p < 0.001$) than plants in the high-nutrient treatment. Nutrient limitation decreased the shoot:root ratio by 85% (mean \pm SEM 0.77 ± 0.06 versus 1.42 ± 0.06 ; $F_{1,218} = 252.37$, $p < 0.001$). There was no support for an interaction between nutrient level and genetic family for shoot biomass (likelihood-ratio of nested models with and without random interaction term: LR = 1.75, $\Delta\text{DF} = 2$, $p = 0.416$), root biomass (LR < 0.1, $\Delta\text{DF} = 2$, $p = 0.999$), or shoot:root ratio (LR = 1.91, $\Delta\text{DF} = 2$, $p = 0.39$). Overall, shoot cardenolide concentrations were reduced by 27.7% ($F_{1,218} = 49.50$, $p < 0.001$) with marginal support of a nutrient level \times genetic family interaction (LR = 5.08, $\Delta\text{DF} = 2$, $p = 0.079$), while root cardenolides were reduced by 22.5% ($F_{1,218} = 63.89$, $p < 0.001$) independent of genetic family (LR = 0.90, $\Delta\text{DF} = 2$, $p = 0.638$). Similarly, mean latex exudation was decreased by 55.3% ($F_{1,216} = 16.78$, $p < 0.001$) in the low-nutrient treatment, but families strongly differed in their response to nutrient limitation (LR = 18.38, $\Delta\text{DF} = 2$, $p < 0.001$). Overall, nutrient limitation thus constrained both plant growth and allocation to cardenolides and latex.

Plant growth and growth rate components

The best model for biomass growth supported main effects of nutrient level and genetic family on all four terms of the logistic regression (Eq. 3), thereby estimating mean parameter values for each genetic family and overall additive effects of nutrient level on each parameter. There was support for a nutrient level \times genetic family interaction on M_p , K_m and t (Supplementary material Appendix 1 Table A2), allowing family-specific parameters to vary independently with nutrient level. Similarly, the best model for leaf area growth (Eq. 1) supported both main effects on all model terms and a nutrient level \times genetic family interaction on K_l (Supplementary material Appendix 1 Table A3). The family means

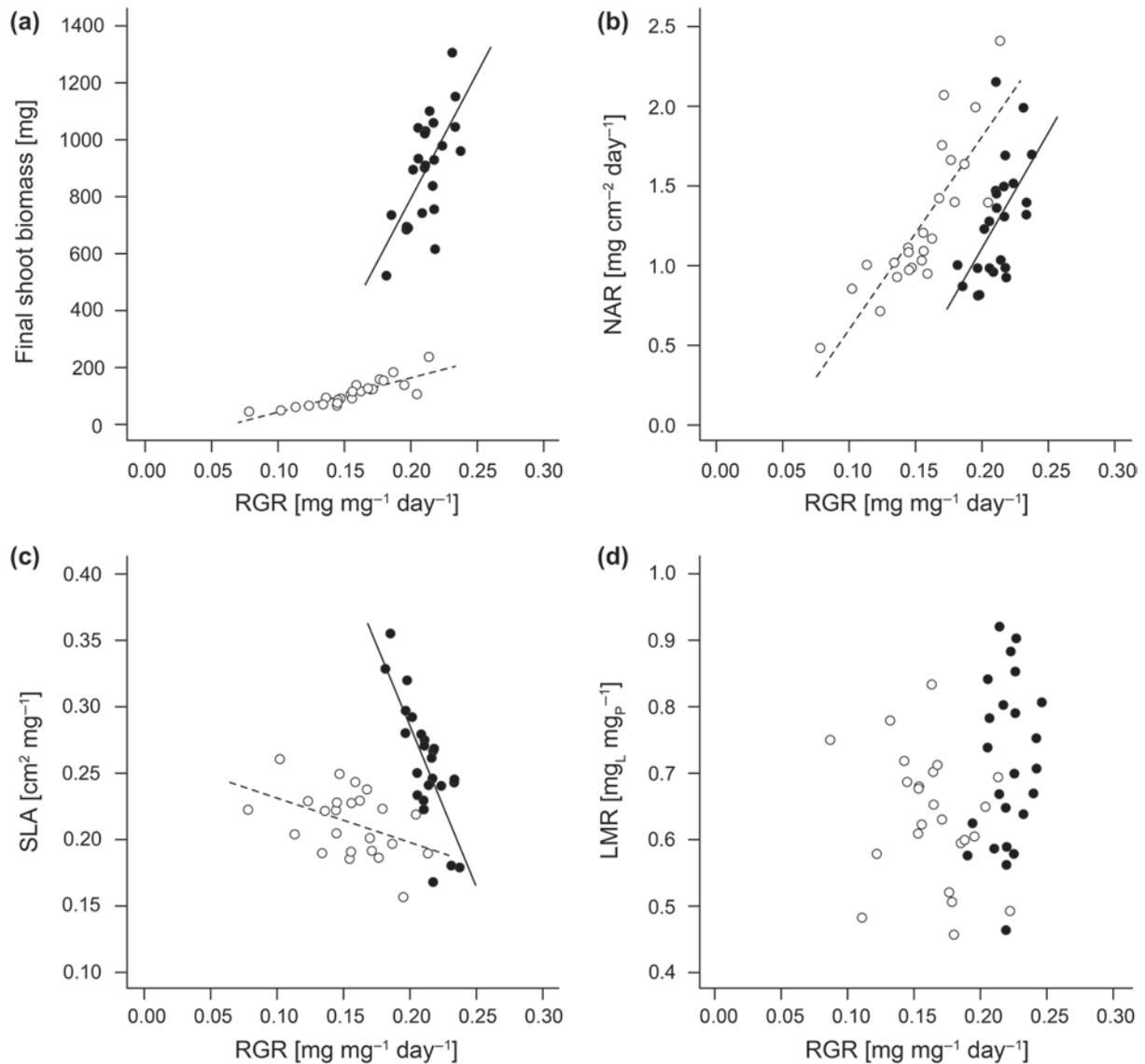


Figure 1. Genetic correlations for 24 plant families of common milkweed in the high- (filled circles, solid lines) and low-nutrient treatment (empty circles, dashed lines) between relative growth rate (RGR, assessed by repeated estimates of total shoot biomass) and (a) shoot biomass at final harvest, (b) net assimilative rate (NAR, absolute growth rate per unit leaf area), (c) specific leaf area (SLA, leaf area per unit weight), and (d) leaf mass ratio (LMR, proportion of shoot biomass invested in leaves). RGR and its components are estimated at a common reference size for each nutrient level, corresponding to the 40th percentile of the biomass distribution of plants within that level. Lines represent a significant correlation of two variables.

of relative leaf growth rate RLGR and RGR were correlated in the high- (Pearson's r , $r_p = 0.53$, $DF = 22$, $p = 0.008$) and low-nutrient treatment ($r_p = 0.84$, $DF = 22$, $p < 0.001$), justifying the use of RGR based on predicted biomass. RGR standardized to the fortieth percentile of biomass for both nutrient levels was positively correlated with final shoot biomass (Fig. 1a, high-nutrient: $r_p = 0.66$, $DF = 22$, $p < 0.001$, low-nutrient: $r_p = 0.85$, $DF = 22$, $p < 0.001$), root biomass (high-nutrient: $r_p = 0.50$, $DF = 22$, $p = 0.012$, low-nutrient: $r_p = 0.72$, $DF = 22$, $p < 0.001$), and with shoot:root ratio under nutrient limitation (high-nutrient: $r_p = 0.20$, $DF = 22$, $p = 0.338$, low-nutrient: $r_p = 0.44$, $DF = 22$, $p = 0.033$), even though RGR could only be measured on shoots due to lack of sequential data on root biomass growth.

RGR was positively correlated with the net assimilative rate NAR (Fig. 1b, high-nutrient: $r_p = 0.57$, $DF = 22$, $p = 0.004$, low-nutrient: $r_p = 0.83$, $DF = 22$, $p < 0.001$) and variance decomposition (Rees et al. 2010) revealed that NAR was by far the strongest driver of variation in RGR (high nutrient: importance value $I = 0.50$, low nutrient: $I = 0.65$). In contrast, SLA was negatively correlated with RGR but had a much weaker effect on its variation (Fig. 1c, high-nutrient: $r_p = -0.77$, $DF = 22$, $p < 0.001$, $I = 0.27$; low-nutrient: $r_p = -0.43$, $DF = 22$, $p = 0.036$, $I = 0.13$). LMR was not significantly correlated with RGR (Fig. 1d, high-nutrient: $r_p = 0.18$, $DF = 22$, $p = 0.393$, $I = 0.23$; low-nutrient: $r_p = -0.28$, $DF = 22$, $p = 0.181$, $I = 0.22$), even though it was significantly lower under nutrient limitation ($F_{1,22} = 5.78$, $p = 0.025$). Faster-growing

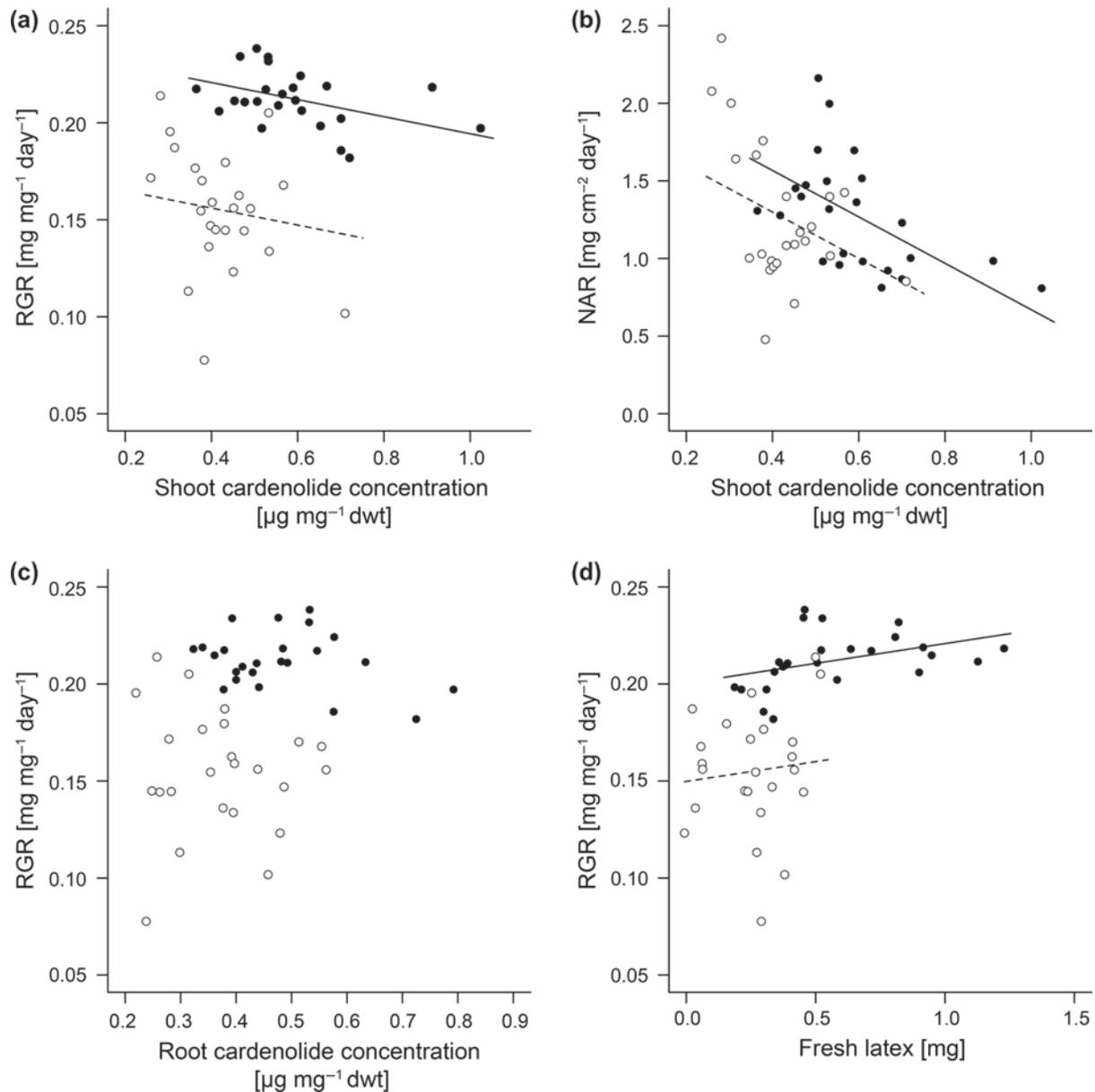


Figure 2. Genetic correlations between defensive traits and growth measures in 24 families of common milkweed. Symbols are mean values for the families in the high (filled circles, solid lines) and low-nutrient treatment (empty circles, dashed lines). Lines represent the linear model fits between cardenolide concentration and plant growth measures. (a) Shoot cardenolides were correlated negatively with relative growth rate (RGR) at both nutrient levels. (b) This correlation was even stronger between cardenolides and the most important component of RGR, the net assimilate rate (NAR). (c) For root cardenolide concentrations, no correlation was found with any plant growth measure. (d) Latex exudation was positively and correlated with plant growth, most strongly with the relative growth rate (RGR). Milkweed does not produce latex in the roots.

plants thus had thicker or denser leaves, while allocation to leaves and stems varied independently of growth. See Supplementary material Appendix 1 Table A4 for a complete list of pairwise trait correlations.

Costs of cardenolides and latex

We quantified 15 cardenolide compounds in leaf tissue, nine of which were also present in roots. Roots contained no tissue-specific cardenolides but lacked several shoot compounds, including the most abundant cardenolide in shoots (no. 6.2, Supplementary material Appendix 1 Fig. A2). There

was a significant negative genetic correlation between total shoot cardenolide concentration and RGR, independent of nutrient level (Fig. 2a, main effect: $F_{1,22} = 8.54$, $p = 0.008$, interaction term: $F_{1,21} = 2.06$, $p = 0.166$). Concordant with the positive correlation among RGR, final shoot biomass, and NAR, shoot cardenolides were also negatively correlated with shoot biomass (main effect: $F_{1,22} = 12.79$, $p = 0.002$), and most strongly with NAR (Fig. 2b, main effect: $F_{1,22} = 14.73$, $p < 0.001$). Shoot cardenolides correlated positively with SLA independent of nutrient level (Supplementary material Appendix 1 Table A4, main effect: $F_{1,22} = 19.04$, $p < 0.001$, interaction term: $F_{1,21} = 0.26$, $p = 0.615$), while LMR and

cardenolides varied independently (main effect: $F_{1,22} = 1.91$, $p = 0.181$).

Shoot and root cardenolides were positively correlated among genotypes (high-nutrient: $r_p = 0.43$, $DF = 22$, $p = 0.036$, low-nutrient: $r_p = 0.48$, $DF = 22$, $p = 0.017$), but despite this (and the positive correlation between RGR and root biomass), root cardenolide concentrations did not significantly predict RGR (Fig. 2c, main effect: $F_{1,22} = 1.86$, $p = 0.187$, interaction term: $F_{1,21} = 0.07$, $p = 0.789$), NAR (main effect: $F_{1,22} = 0.43$, $p = 0.519$), or root biomass (main effect: $F_{1,22} = 0.88$, $p = 0.358$). Latex exudation was positively predicted by both RGR (Fig. 2d, main effect: $F_{1,22} = 4.69$, $p = 0.041$, interaction term: $F_{1,21} = 0.01$, $p = 0.915$) and shoot biomass (main effect: $F_{1,22} = 5.19$, $p = 0.033$, interaction term: $F_{1,21} = 2.36$, $p = 0.139$), but not by NAR (main effect: $F_{1,22} = 1.81$, $p = 0.192$, interaction term: $F_{1,21} = 0.62$, $p = 0.437$). Latex exudation and shoot cardenolide concentration were not correlated with each other (high-nutrient: $r_p = 0.11$, $DF = 22$, $p = 0.608$, low-nutrient: $r_p = 0.11$, $DF = 22$, $p = 0.560$). Thus, not only were latex and cardenolides independently deployed as defensive strategies, but they also show divergent relationships with components of plant growth.

We next used PCA on the genetic family means for the ten most abundant cardenolide compounds in shoots to reduce the dimensionality of the dataset using an Eigenvalue > 1 as cut-off. At high nutrient availability, three principle components (PCs) accounted for 69% variation, two of which were not correlated with NAR, while PC2 was strongly correlated with NAR (Supplementary material Appendix 1 Table A5). Interestingly, only three cardenolide compounds had strong loadings on PC2 (> 0.5 , and more than double the loading of any of the other compounds), indicating that the overall negative correlation with growth at high nutrient availability might have been driven mainly by these specific cardenolides. Under nutrient limitation, four PCs explained 79% of the variation in cardenolides. Two PCs were correlated with NAR (Supplementary material Appendix 1 Table A6), with PC1 showing substantial loadings for all but one compound. Accordingly, it appears that most compounds contributed to the negative correlation with growth under nutrient limitation.

Costs of defense in response to nutrient limitation

As additional tests of allocation costs associated with defense traits, GVA revealed divergent responses to low nutrient availability by different families for both cardenolides and latex. For cardenolides, the majority of plant families responded with a proportional decrease in concentration (μg cardenolides per mg dry leaf mass) and content (concentration \times total dry leaf mass), indicating reduced cardenolide synthesis in shoots (Fig. 3a). However, six genetic families deviated from this pattern and either maintained or increased their relative allocation to cardenolide synthesis under nutrient limitation. Overall, the change in shoot cardenolides in response to nutrient limitation significantly predicted the change in NAR (Fig. 3b, $F_{1,22} = 11.09$, $p = 0.003$). In other words, not only did we detect costs of cardenolides as a negative genetic correlation with growth, most strongly with NAR, but the family-specific relative change in allocation to

cardenolides in response to our nutrient manipulation predicted their growth response, further supporting the notion of a direct allocational relationship between cardenolide metabolism and plant growth.

Cardenolides in roots largely mirrored this result, with an overall reduction in synthesis in response to low nutrient availability, and four families deviating from this pattern (Supplementary material Appendix 1 Fig. A3a). The family-specific magnitude of cardenolide reduction was weakly correlated between roots and shoots (Supplementary material Appendix 1 Fig. A3b, $r_p = 0.39$, $p = 0.060$), and correspondingly the relative change in root cardenolides only weakly predicted the change in NAR (Supplementary material Appendix 1 Fig. A3c, $F_{1,22} = 3.32$, $p = 0.082$). The reduction in latex exudation in response to low nutrient availability was less extreme than the reduction in biomass, resulting in higher latex-to-biomass ratios in the low nutrient treatment (Fig. 3c). Nonetheless, the relative change in plant growth rate in response to low nutrient availability predicted the reduction in latex exudation (Fig. 3d, $F_{1,22} = 9.66$, $p = 0.005$). Thus, the positive relationship between RGR and latex exudation is further supported by the responses of the genetic families to our nutrient manipulation.

Discussion

We present two lines of evidence that cardenolide synthesis is costly and trades off with plant growth. First, we found negative correlations between cardenolide concentrations and plant growth within each nutrient treatment. Variance partitioning of RGR revealed that differences in growth rate were mainly associated with variation in NAR, and in fact this component of growth had the strongest negative correlation with foliar cardenolide levels. NAR is the amount of new biomass produced by a plant per leaf area per day, thus its negative correlation with cardenolide levels is a strong indication for allocation costs. Plants with high allocation to defense divert a larger fraction of photoassimilates into secondary metabolites, while plants with low defense allocation can immediately re-invest these photoassimilates into primary metabolism where they contribute further to growth.

Second, our nutrient-limitation treatment provided additional support for a causal interpretation for allocation costs. Plants in the low-nutrient treatment were severely impaired in their physiology and growth, and generally responded to nutrient limitation by a proportional decrease of cardenolide synthesis in shoots and roots. However, the degree to which a genetic family reduced cardenolide synthesis in response to the experimentally diminished pool of resources was correlated with the reduction in growth it experienced. Indeed, families that maintained or increased shoot cardenolide production in the face of nutrient limitation suffered the greatest reduction in shoot growth and NAR.

Compared to NAR, SLA was only of minor importance in explaining variation in RGR. Nonetheless, SLA traded off negatively with growth in both nutrient treatments. Plants were therefore either fast-growing with thicker leaves and low defense, or slower-growing with thinner leaves and high defense. Interestingly, low nutrient availability significantly decreased SLA, which was mostly due to low-nutrient

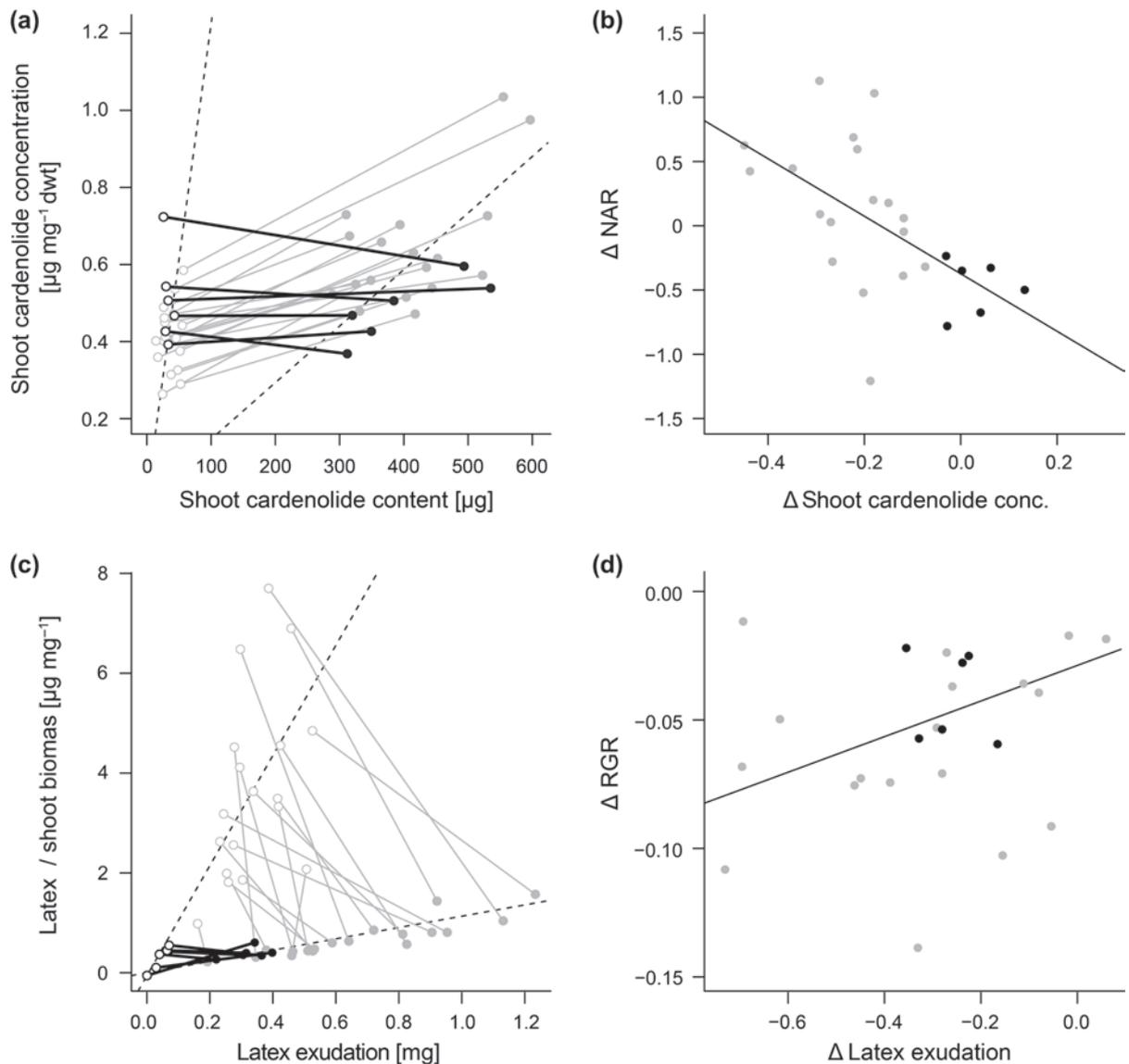


Figure 3. (a) Graphical vector analysis (GVA) for the effect of nutrient limitation on genetic family means of cardenolide concentration and content (concentration \times dry mass) in shoots. Lines link family means in the high (filled circles) and low-nutrient treatment (empty circles). The majority of plants decreased cardenolide concentration in response to nutrient limitation (grey symbols), while six genetic families showed no change or an increase in concentration (black symbols, arbitrary cut-off at -0.05). Dashed lines represent the mean plant biomass at each nutrient level (given that content = concentration \times biomass); they serve to visualize how content and concentration can change while biomass is held constant. (b) Genetic correlation between the absolute change in shoot cardenolide concentration and change in net assimilation rate for the 24 genetic families in the low- relative to the high-nutrient treatment. (c) GVA for the effect of nutrient limitation on genetic family means of latex and the latex-to-biomass ratio. Different treatments are indicated as above, while six families with horizontal slopes are highlighted (black symbols). (d) Genetic correlation between the absolute change in latex exudation and change in relative growth rate for the 24 genetic families in the low- relative to the high-nutrient treatment.

plants having smaller but tougher leaves. In cross-species comparisons, low values of SLA are commonly associated with tough, unpalatable leaves of slow-growing plants (Schädler et al. 2003, Agrawal and Fishbein 2006), resulting in positive correlations between SLA and growth. In contrast, shading experiments using a single plant species often find faster-growing plants to have thicker mesophyll layers and thus a lower SLA (Agrawal et al. 2012a), resulting in a negative correlation between SLA and growth. The diverging patterns with SLA within and between nutrient treatments could thus have been caused by different underlying mechanisms. In general, large differences in leaf toughness

could easily mask more subtle effects of mesophyll thickness, unless plants are matched for physiological state.

Even though the variance partitioning approach by Rees et al. (2010) does not assign causality, our data are indicative of allocation-related costs of cardenolides that are most apparent with NAR. In turn, it appears that NAR drives the variation in RGR, while SLA simply reflects the allocation to primary metabolism (i.e. mesophyll thickness) and in consequence is inversely related with cardenolide concentration. These results are in contrast to results from across-species comparisons (Potter and Jones 1977, Poorter 1990, Poorter and Remkes 1990, Hunt and Cornelissen 1997, Rees et al.

2010), where NAR was found to vary independently of RGR. However, negative covariances between components of RGR can mask important effects of individual components on growth rate (Rees et al. 2010). Both SLA and LMR are likely to be more variable between- than within-species (differences in leaf toughness, architecture, etc.), hence any potential relation between NAR and RGR will be more difficult to detect.

Although our results are perhaps the clearest, most mechanistic example to date for demonstrating allocation costs of cardenolide production, the findings are supported by previous work that indirectly point to such costs. For example, both Bingham and Agrawal (2010) and Rasmann et al. (2011) found a strong genetic trade-off between constitutive and induced levels of cardenolides in common milkweed. Such a tradeoff would only be expected if cardenolides are indeed costly (Zangerl and Bazzaz 1992), yet it might also be the result of other types of costs, such as differential selection by generalist versus specialist herbivores. In another study, Vannette et al. (2013) demonstrated a phenotypic tradeoff between cardenolide concentrations and biomass in the absence of mutualistic arbuscular mycorrhizal fungi using eight species of *Asclepias*, which supports the notion that cardenolide synthesis is indeed dependent on plant nutrient status.

We only found evidence for costs of cardenolides in shoots, while root cardenolides appeared to be uncorrelated with any measures of shoot growth. Although shoot and root cardenolide levels are typically correlated (Rasmann and Agrawal 2011), evidence from grafting experiments suggests that there is not substantial transport of cardenolides between the above and belowground organs (Agrawal et al. unpubl.). Shoot and root biomass responded differently to nutrient limitation, resulting in a marked decrease of the shoot:root ratio and highlighting the functional difference between the two tissue types. The differential prevalence of allocation costs in shoots versus roots is therefore perhaps unsurprising, yet we currently lack a mechanistic understanding of cardenolide synthesis to fully explain this pattern. Similarly, while we found strong patterns with total cardenolide concentrations in shoots, PCA analyses indicated that not all cardenolide compounds followed the same pattern. At high nutrient availability, the tradeoff with growth was largely driven by three compounds, while most other compounds varied independently of growth. In contrast, under nutrient limitation all but one compound correlated negatively with growth.

In contrast to cardenolides, latex exudation was most strongly and positively correlated with RGR, while it was uncorrelated with NAR and cardenolide concentration. This highlights differences in allocation strategies between the two defensive traits: cardenolide synthesis appears to be a local process that is directly related to the availability of resources, while latex is a systemic trait whose expression is largely determined by plant size. Latex therefore showed no evidence for allocation costs in our study, which corroborates the lack of a tradeoff between constitutive and induced latex levels reported by Bingham and Agrawal (2010). Indeed, latex is a complex trait and for a number of reasons could be expected to positively, rather than negatively, co-vary with plant size. First, latex has been shown to travel large distances within and across tissues of at least some plant

species (Buttery and Boatman 1976), and thus the majority of latex within a plant might be available for deployment constantly, and larger plants simply contain higher amounts of latex. Second, latex is delivered via pressurized laticifers, i.e. canals on the inside of milkweed leaves formed by specialized multi-nucleate cells. The size of laticifers likely scales with plant size; hence larger, more vigorous plants should have larger laticifers, exuding more latex upon damage. Thus even though the amount of latex exuding upon damage is clearly relevant for herbivores feeding on plants, this simple measure may be insufficient to detect costs of latex production. Standardizing exuding latex by plant size might be a potential solution, although strong correlations between final biomass and growth rate such as in our study could introduce spurious negative correlations between a size-standardized latex metric and plant growth (Jasienski and Bazzaz 1999).

Nutrient limitation had a decreasing effect on both cardenolide concentration and latex in plants. Both traits are predominantly carbon-based defenses which are assumed to be less affected by nutrient availability than nitrogen-based defenses. Indeed, increased nitrogen fertilization over a more moderate range of nutrient levels decreased total cardenolide concentration by 45% across five *Asclepias* species (Agrawal et al. 2012c), while shading (and thus a reduced carbon-to-nitrogen ratio) caused a decrease in cardenolide concentration in *A. syriaca* (Agrawal et al. 2012a, c). Both of these responses are in accordance with predictions for carbon-based defense compounds in response to relative carbon availability in the plant (Herms and Mattson 1992). However, both primary and secondary metabolic pathways in the plant depend on nutrient-rich enzymes (Gershenzon 1994), while nutrient status changes the physiology and allocation patterns in plants (Herms and Mattson 1992) and influences the ability to acquire other types of resources (Hamilton et al. 2001). In our experiment the low nutrient treatment resulted in a small, stunted phenotype, thus plants were likely impaired both in their pool of photosynthetic carbon as well as in their cellular machinery for secondary metabolism.

We estimated RGR and its components at a common reference size that most plants reached within the first two weeks of growth. At this point, effects of nutrient limitation can be expected to be detectable while effects of size-limitation such as self-shading should still be minimal, and differences in growth estimates are most likely to be caused by differences in allocation. These early estimates of RGR and NAR were highly correlated with the single biomass measure at final harvest, demonstrating that small differences during early growth stages can accumulate over time to cause large differences later on. Even though final biomass in this experiment was almost as good a predictor of cardenolides as RGR, we believe that mechanistic growth measures are superior estimates of plant growth for at least two reasons. First, growth rate and carrying capacity (as approximated by final biomass) of an organism can often vary independently, depending on experimental conditions (Underwood 2007). The relatively short growth period in our experiment allowed the two growth parameters to co-vary, but other environmental factors such as pot size could easily affect only one parameter, causing the positive correlation to disappear. Second, while we minimized asynchrony

in germination by scarification and cold stratification, and even further standardized the beginning of growth by planting all seedlings within a block on the same day, asynchrony can otherwise mask growth rate differences (Turnbull et al. 2008, Rose et al. 2009, Paul-Victor et al. 2010). Since it is difficult to predict when biomass and growth rate will be concordant, size-standardized estimates of growth promise to be more consistently accurate predictors of plant growth processes than simple biomass measures.

Conclusion

Using a combination of mechanistic plant growth modeling and comparing allocation of defense traits to shoots and roots at different levels of nutrient availability, we have found strong indications for costs of cardenolides in common milkweed *A. syriaca*. Genetic differences in the allocation of resources to cardenolide synthesis were correlated with differences in growth rate that accumulated over time to cause large differences in plant size. In contrast, we were unable to detect costs of latex due to underlying positive correlation with plant size. Wider screening of natural genotypes for variation in latex exudation while controlling for plant growth and size could reveal a cost for this trait as well. Allocation costs have sometimes proven difficult to detect, especially in long-lived species. Therefore, the accurate estimation of mechanistic growth at crucial, biologically relevant life-stages such as seedling establishment provides an invaluable tool to pave the way for further understanding constraints on the evolution of defense.

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Supplementary material (available online as Appendix oik.02075 at <www.oikosjournal.org/readers/appendix>). Appendix 1.