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RESEARCH

Potent Nitrogen-containing Milkweed Toxins are Differentially Regulated by Soil Nitrogen and Herbivore-induced Defense

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Abstract

Theories have been widely proposed and tested for impacts of soil nitrogen (*N*) on phytochemical defenses. Among the hundreds of distinct cardenolide toxins produced by milkweeds (*Asclepias* spp.), few contain *N*, yet these appear to be the most toxic against specialist herbivores. Because *N*- and non-*N*-cardenolides coexist in milkweed leaves and likely have distinct biosynthesis, they present an opportunity to address hypotheses about drivers of toxin expression. We tested effects of soil *N* and herbivore-damage on cardenolide profiles of two milkweed species differing in life-history strategies (*Asclepias syriaca* and *A. curassavica*), and the toxicity of their leaves. In particular leaf extracts were tested against the target enzymes (Na^+/K^+-ATPase extracted from neural tissue) from both monarch butterflies (*Danaus plexippus*) as well as less cardenolide-resistant queen butterflies, *D. gilippus*. Increasing soil *N* enhanced biomass of *Asclepias syriaca* but had weak effects on cardenolides, including causing a significant reduction in the *N*-cardenolide labriformin; feeding by monarch caterpillars strongly induced *N*-cardenolides (labriformin), its precursors, and total cardenolides. Conversely, soil *N* had little impact on *A. curassavica* biomass, but was the primary driver of increasing *N*-cardenolides (voruscharin, uscharin and their precursors); caterpillar induction was weak. Butterfly enzyme assays revealed damage-induced cardenolides substantially increased toxicity of both milkweeds to both butterflies, swamping out effects of soil N on cardenolide concentration and composition. Although these two milkweed species differentially responded to soil *N* with allocation to growth and specific cardenolides, leaf toxicity to butterfly Na^+/K^+-ATPases was primarily driven by herbivore-induced defense. Thus, both biotic and abiotic factors shape the composition of phytochemical defense expression, and their relative importance may be dictated by plant life-history differences.

Keywords Cardenolide · Cardiac glycoside · Chemical ecology · Coevolution · Phytochemical diversity · Sodium–potassium pump · Monarch butterfly · Na^+/K^+-ATPase

Introduction

Because nitrogen is widely known as limiting for plant growth in terrestrial ecosystems, it's availability in soil has played a major role in the conceptual development of the plant sciences (Mattson 1980; Lambers et al. 1998; Vidal and Gutiérrez 2008). And, to "grow or defend" has been the basis of prominent theories of plant allocation, with *N*-limitation typically being involved (Herms and Mattson 1992). In some systems, like solanaceous plants that produce nitrogen-hungry alkaloids for defense, classic work traced N-limitation and its differential allocation to producing seeds versus alkaloids (Baldwin et al. 1998). Indeed, when demand for alkaloids was high, as in the case following herbivore damage, tobacco plants traded off growth in favor of defense (Baldwin and Hamilton 2000). Nonetheless, under less threatening conditions, *N*-fertilization alone resulted in a down-regulation of alkaloid production (Lou and Baldwin 2004), with allocation favoring growth.

Despite the success of the *N*-limitation and defense paradigm, in part codified by the carbon-nutrient balance hypothesis (CNBH) (Bryant et al. 1983), the predictive power of

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CNBH across plant species has been dismal. In fact, following early reviews of hundreds of studies and meta-analyses, some prominent authors concluded that the hypothesis was hopeless, so much so that it should be completely abandoned (Haukioja et al. 1998; Hamilton et al. 2001). The diversity of plants, let alone the many biosynthetic classes of secondary metabolites present within and across species, perhaps makes a general theory of nitrogen fertilization elusive. Nonetheless, the notion that *N*-availability predictably impacts defense composition, at least in some systems, is abundantly true and certainly worthy of study.

Here we take a physiological approach, working with a remarkable system where a single class of highly toxic compounds, with a known mode of action, are themselves produced with and without nitrogen (Fig. 1). We have been working on the growth and defense of milkweeds (*Asclepias* spp.), a group of > 130 North American perennial plant species (Agrawal and Fishbein 2008; Agrawal et al. 2015a, b). Milkweeds are well-known for their relationship with monarch butterflies and other insect herbivores, with their interactions being heavily mediated by a group of steroidal glycosides, the cardenolides (Agrawal 2017). Although the lion's share of the hundreds of milkweed cardenolides that have been identified are composed of carbon, oxygen, and hydrogen, a few compounds have been found with nitrogen- and sulfur-containing rings (Seiber et al. 1983). We recently discovered that although *N*-containing cardenolides are not more toxic than non-*N*-cardenolides to generalist insects, they are among the most potent against highly adapted herbivores (Agrawal et al. 2021, 2022; Agrawal and Hastings 2023). Because these compounds coexist with non-*N*-containing cardenolides in the two most important host plants (*A. syriaca* and *A. curassavica*) of the monarch butterfly (*Danaus plexippus*), here we study cardenolide production in response to variation in available soil N and induction following damage by monarch caterpillars. We predicted that, all else being equal, plants should allocate more *N* to *N*-cardenolides when fertilized, and that this effect would be strongest when plants have had their defenses induced by specialist monarch caterpillar herbivory.

With regard to categorizing plant chemical defenses, Feeny (1976) had long ago suggested that those plant toxins that lack expensive nitrogen and occur in relatively high concentrations in plants may be considered generalized defenses against most consumers. Conversely, *N*-containing compounds were predicted to occur in lower concentrations, and with specific targets only tolerable by highly specialized herbivores. Thus, we test this notion among the milkweed cardenolides by assessing allocational responses of *N*- and non-*N*-cardenolides to soil *N* and herbivory; additionally, we test the effects of our treatments on the toxicity of leaves to the physiological target of cardenolides, the universal animal enzyme, Na^+/K^+-ATPase (i.e., the sodium pump). In particular, we test the leaf extracts *in vitro* on the sodium pump extracted from neural tissue of monarchs as well as queen butterflies (*D. gilippus*), both specialized feeders of *Asclepias*. Queens

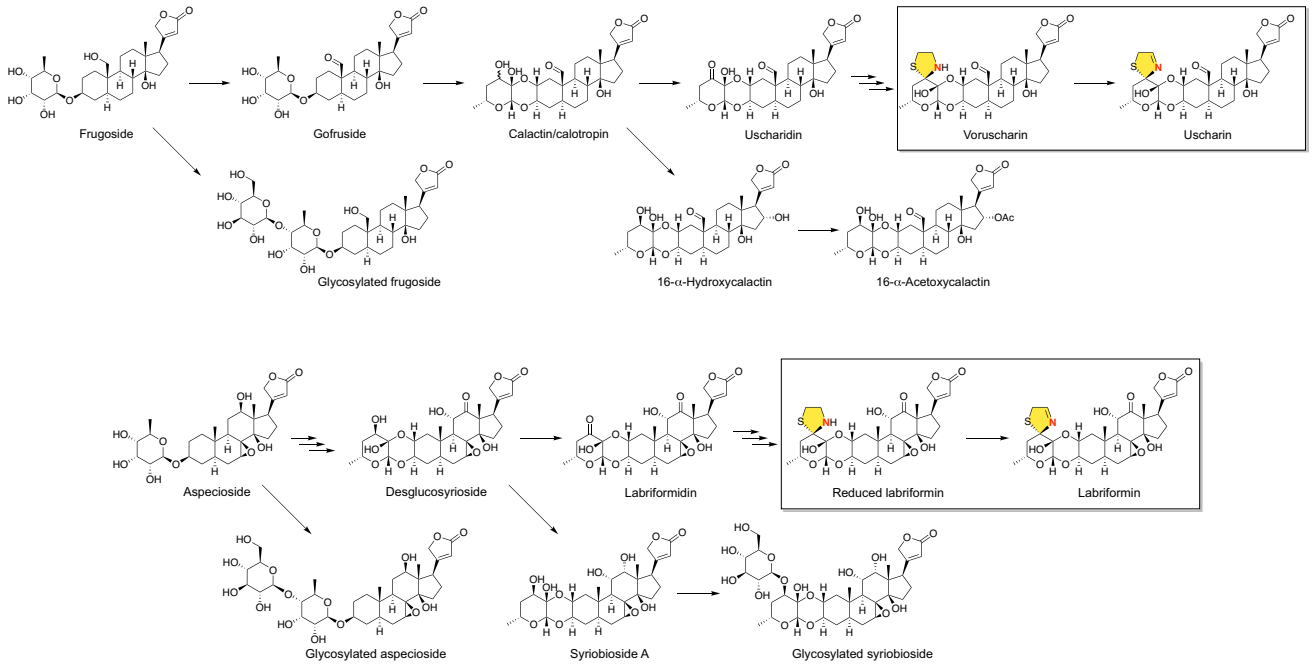


Fig. 1 Putative biosynthesis of nitrogen-containing and non-nitrogen-containing cardenolides in (**a**) *Asclepias syriaca* and (**b**) *A. curassavica*. The nitrogen is highlighted in red and the yellow ring shows the thiazolidine and thiazoline heterocycle resulting putatively from a cysteine enzymatic condensation reaction on cardenolides followed by enzymatic decarboxylation and reduction reactions (not shown)

are substantially less resistant to cardenolides than monarchs (Petschenka et al. 2013; Karageorgi et al. 2019), and thus we hypothesized that they may be more sensitive to changes in concentration and composition of milkweed toxins.

We take advantage of the well-characterized chemistry of *A. syriaca* and *A. curassavica*, and use liquid chromatography, coupling mass spectrometric and UV approaches, to assess plant responses in terms of cardenolide composition and concentration. Accordingly, in this study, we asked the following specific questions: 1) how does quantitatively altering soil N impact growth and leaf *N* content of two milkweed species, 2) what is the relative impact of soil N and monarch caterpillar induction on the concentration and composition of *N*- and non-*N*- cardenolides in leaves of these species, and 3) to what extent are the differentially adapted Na^+/K^+-ATPases of the two milkweed feeders impacted by N and herbivore-induced phytochemical changes?

Materials and Methods

Study System

A. syriaca and *A. curassavica* are the two most utilized host plants of monarch butterflies; they are distantly related among *Asclepias* spp., are temperate vs. tropical, respectively, and have dramatically different chemistries and life-histories. *A. syriaca* is a large, pubescent, low toxicity and aggressively clonal species, whereas *A. curassavica* is rather small, glabrous, high toxicity and typically single-stemmed (Woodson 1954; Seiber et al. 1983; Tao et al. 2014; Agrawal et al. 2021; Edwards et al. 2023). Monarchs (*D. plexippus*) and closely related queens (*D. gilippus*) are both native North American butterfly species that feed primarily on milkweeds in the genus *Asclepias*. The target enzyme (Na^+/K^+-ATPase) of monarchs is well-characterized and is substantially more resistant to cardenolides than that of queens (Petschenka et al. 2013; Karageorgi et al. 2019).

Plant Growth and Fertilization Treatments

Milkweed seeds (*A. curassavica* – Everwilde Farms, Fallbrook, CA; *A. syriaca* – collected locally in Ithaca, NY) were surface sterilized with 10% bleach for 10 min. They were rinsed, scarified, and kept moist at 4 °C for one week, then moved to an incubator at 30 °C for germination. Germinated seedlings were planted into 500 ml pots filled with pre-moistened LM-111 potting mix (Lambert, Quebec, Canada), and placed in a high-light growth chamber (photons of photosynthetically active radiation ≈350 µmol m^{-2} s^{-1}) with a 14-h daylength and 27 °C daytime, 23 °C nighttime temperature regime. Each pot was placed in a 90 mm × 15 mm petri dish to catch any overflow and ensure uptake of appropriate nutrient solutions.

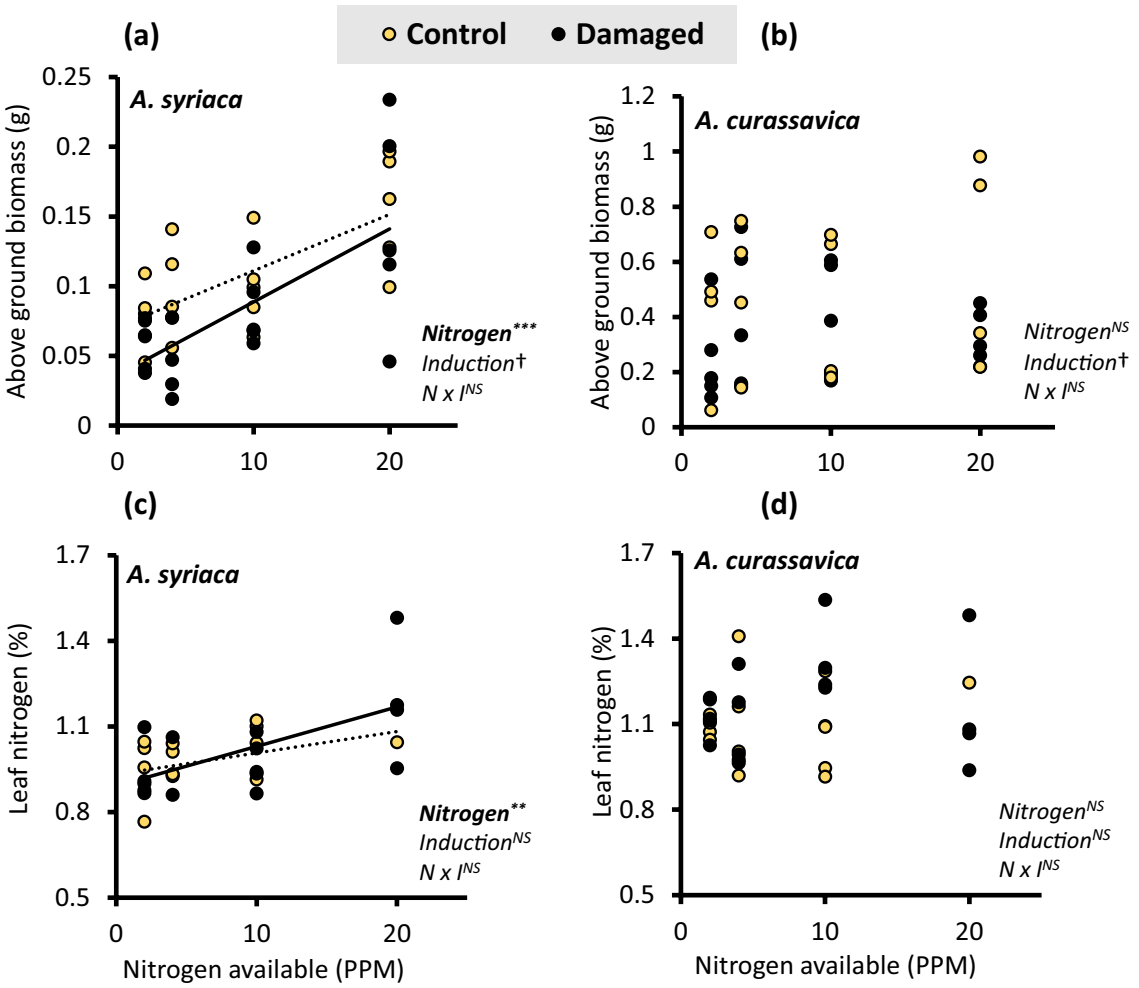
Plants were randomly assigned to five fertilization treatments: N2, N4, N10, N20, and a complete commercial fertilizer treatment (Jack's) ($n = 8$–10 plants per species in each treatment, total $n = 95$). Seedlings in the N2-N20 treatments were each given 150 mL of nitrogen-free nutrient solution (Broughton and Dilworth 1971; Table S1) once per week, as well as 100 mL of a ammonium nitrate (NH_4NO_3) solution at the appropriate concentration (N2 – 2 ppm *N*, N4 – 4 ppm *N*, N10 – 10 ppm *N*, N20 – 20 ppm *N*). Each seedling in the Jack's group was provided with 100 mL Jack's All-Purpose Fertilizer (20:20:20, JR Peters Inc,. Allentown, PA) at 1.25 ml per gallon, per week (equivalent to 60 ppm *N*). The Jack's treatment was primarily used as a visual control for growth of our experimental plants with varying levels of NH_4NO_3 provided through the Broughton and Dilworth solution (Fig. 2). As such, and because the Jack's fertilizer had different levels of phosphorus and potassium as well, we did not include it in the statistical analyses for effects of increasing N on cardenolides. All plants were provided with water ad libitum, with care taken to not have much water leaving the pots. Plants were grown for five weeks according to this regime; plant species and treatments were completely randomized in the growth chamber.

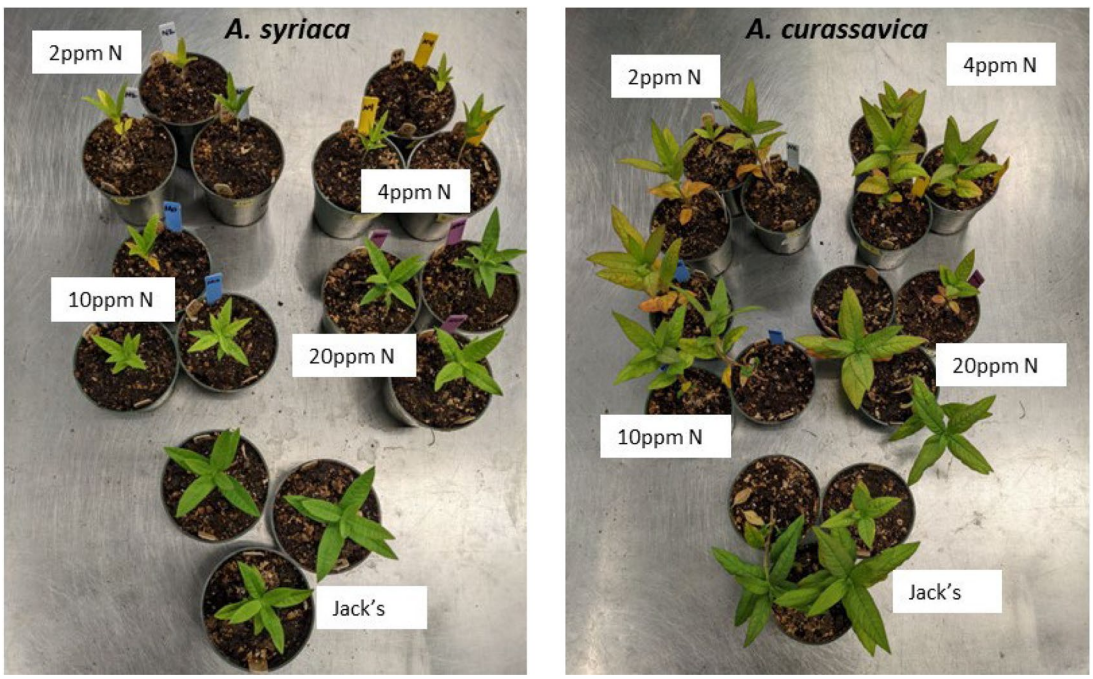
Herbivory Treatments

After five weeks of growth, half of the plants from each species and treatment combination were randomly selected for the herbivory treatment, while the other half served as controls. One neonate monarch caterpillar (eggs obtained from Monarch Watch, University of Kansas, Lawrence, KS) was applied to an upper leaf of each of these plants. Caterpillars were allowed to feed on their host plant for three (*A. syriaca*) or four (*A. curassavica*) days, then removed and weighed immediately on a microbalance. We allowed damage for different amounts of time on the two species but achieved a similar proportion of total leaf area damaged (10–20%). Plants received water ad libitum during the herbivory treatment. Above-ground tissues from herbivory and control plants were harvested two days after caterpillar removal, frozen at -80 °C and freeze-dried for subsequent analysis. After freeze-drying, total stem and leaf tissue from each plant was weighed, and then all leaf tissue was ground in a 2 mL tube with a 3 mm stainless steel bead using a shaker (Retsch Mixer Mill 300, Haan, Germany).

Leaf Tissue Nutrient Analysis

In order to test how our nitrogen addition treatments affected plant elemental composition, we analyzed percent nitrogen in the leaf tissues. Three milligrams of ground, freeze dried


(a)
Control
Damaged
(b)
A. syriaca
A. curassavica
Above ground biomass (g)
Nitrogen***
Induction†
N x I^NS
Nitrogen^NS
Induction†
N x I^NS
(c)
(d)
Leaf nitrogen (%)
Nitrogen**
Induction^NS
N x I^NS
Nitrogen^NS
Induction^NS
N x I^NS
Nitrogen available (PPM)
Nitrogen available (PPM)


A. syriaca
2ppm N
4ppm N
10ppm N
20ppm N
Jack's
A. curassavica
2ppm N
4ppm N
20ppm N
10ppm N
Jack's

◂**Fig. 2** Effects of soil nitrogen availability on total above ground biomass (**a**, **b**) and leaf nitrogen content (**c**, **d**) for *Asclepias syriaca* and *A. curassavica*. Shown are the raw data and analyses from analysis of covariance with nitrogen availability and monarch caterpillar induction as the man effects. Yellow datapoints and dashed lines represent undamaged plants, while black-filled datapoints and solid lines represent herbivore-damaged plants. *** $p < 0.001$, † $p = 0.06$, NS = not significant. Shown below are representative replicates of each plant species grown under each of the soil nitrogen treatments

leaf tissue from each plant sample was subjected to combustion analysis at the Cornell University Stable Isotope Laboratory, for determination of %N and %C in each sample ($n = 2$–4 per combination of species x nitrogen treatment x herbivory treatment, total $n = 62$).

Cardenolide Extraction and Analysis

In order to address whether our nitrogen and herbivory treatments affected plant chemical defense, we extracted cardenolides from tissues as in Petschenka et al. (2022). Briefly, 25 mg of ground, freeze dried leaf tissue from each sample was extracted in 1 mL methanol, spiked with 20 µg hydrocortisone as an internal standard, with zirconia-silica beads in a FastPrep homogenizer (MP Biomedicals, Irvine, CA). Hydrocortisone does not inhibit the Na^+/K^+-ATPase and its inclusion allows for the subsequent use of these extracts in Na^+/K^+-ATPase inhibition assays, without interference. Extracts were then centrifuged for 12 min at 15,000 g and 750 µL of the supernatant was taken to dryness in a vacuum concentrator (Labconco, Kansas City, MO). Residues were then dissolved in 100 µL methanol and filtered through a 0.2 µm syringe filter (Restek, Bellefonte, PA). Extracts were stored at -20C and analyzed first by LC–MS (UPLC-HRMS) for determining relative concentrations of particular cardenolide compounds, and then analyzed by HPLC–DAD for estimating total cardenolide concentration.

UPLC-HRMS: Reversed-phase ultra-performant chromatography in a Dionex 3000 LC coupled to an Orbitrap Q-Exactive mass spectrometer controlled by Xcalibur software (ThermoFisher Scientific) were used for targeted metabolomics (Agrawal et al. 2021, 2022). Methanolic extracts (5 replicates per treatment per plant species) were separated on an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm, particle size 1.8 µm) maintained at 40 °C with a flow rate of 0.5 mL/min. Each sample (1 µL injected) was analyzed in positive electrospray ionization mode with *m/z* ranges 70–1000. MS2 spectra were obtained via Excalibur software (ThermoFisher Scientific). The acquired LC–MS data files were converted to mzXML files using the ProteoWizard MSconvert tool. LC–MS data was then pre-processed with the open-source MZmine 2 software (Pluskal et al. 2010) and consisted of peak detection, removal of isotopes, alignment, filtering, and peak filling. Data were normalized, log transformed and auto-scaled using with Metaboanalyst (Chong et al. 2018). We mined the generated feature table to retrieve cardenolide ion adducts of interest in *A. syriaca* and *A. curassavica* and confirmed their structure by comparing MS2 fragmentation spectra and retention time with pure isolated standards if available in our in-house library (see Table 1).

HPLC–UV: Fifteen µL of each sample were injected into an Agilent 1100 series HPLC and compounds were separated on a Gemini C18 reversed phase column (3 µm, 150 mm × 4.6 mm, Phenomenex, Torrance, CA). Cardenolides were eluted with a constant flow of 0.7 ml/min using the following gradient: 0–2 min 16% acetonitrile, 25 min 70% acetonitrile, 30–40 min 95% acetonitrile, and a 10 min post-run at 16% acetonitrile. UV absorbance spectra were recorded at 218 nm using a diode array detector (Agilent Technologies, Santa Clara, CA). Peaks with symmetrical absorption maxima between 218—220 nm were labeled as cardenolides and concentrations estimated using peak area, normalized to that of the internal hydrocortisone standard. We utilized a conversion factor to translate hydrocortisone peak area to the equivalent amount of digitoxin, our typical internal standard. Individual cardenolide concentrations were summed together to yield an estimate of total cardenolide concentration in each sample.

Na^+/K^+-ATPases Inhibition Assays

To measure the potency of the cardenolides in the leaf tissues, we measured the ability of a subset of the leaf extracts (see next paragraph) to inhibit the activity of Na^+/K^+-ATPase *in vitro*. In particular, we tested inhibition by leaf extracts of two cardenolide-adapted Na^+/K^+-ATPases, that of the monarch as well as the queen butterfly, a congener with an enzyme that is less resistant to cardenolides. Enzyme preparations were made as in Petschenka et al. 2022, by dissecting neural tissues of the butterflies that had been frozen alive at -80 °C, pooling and homogenizing brains in Millipore water using an all-glass grinder, and freeze-drying batches of single-use aliquots. Preps were stored at -80 until being brought up with cold Millipore water just prior to the assay.

To specifically contrast herbivory and nitrogen effects, we chose herbivory and control plants for each species from low (N4) and high (N20) nitrogen treatments ($n = 4$ each, and thus $n = 16$ per species). After LC analysis,

Table 1 Effects of Nitrogen treatment, Monarch caterpillar damage, and their interaction on specific cardenolides of *A. syriaca* (degrees of freedom = 1, 32) and *A. curassavica* (degrees of freedom = 1, 35). Shown are F-values; significance is as follows: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, † $p < 0.1$. For precursors of the nitrogen-containing cardenolides the proposed number of biosynthetic steps prior to production is given parenthetically); see biochemical pathway to production of the nitrogen-containing cardenolides in Fig. 1

Cardenolide type	Cardenolide	Nitrogen	Damage	*N* xD
A. curassavica				
non-*N*	Desglucouzarin	0.223	0.679	0.002
non-*N*	16-α-Hydroxycalactin	0.809	1.529	0.013
non-*N*	16-α-Acetoxycalactin	7.173*	0	0.001
non-*N*	Glycosylated frugoside	0.019	7.690**	1.673
N-precursor (*n*-4)	Frugoside	0.229	3.094 +	0.3
N-precursor (*n*-3)	Gofruside	0.839	0.721	0.092
N-precursor (*n*-2)	Calotropin	0.168	0.559	0.017
N-precursor (*n*-2)	Calactin	2.503	4.310*	0.855
N-precursor (*n*-1)	Uscharidin	51.608***	1.089	2.954 +
N-cardenolide	Voruscharin	24.155***	0.144	0.04
N-cardenolide	Uscharin	98.883***	4.924*	1.561
mixure	Total cardenolides	0.165	6.099*	0.527
A. syriaca				
non-*N*	Glycosylated aspecioside	1.106	0.252	0.025
non-*N*	Glycosylated syriogenin	0.306	0.212	0.648
non-*N*	Diglycosylated syriogenin	0.476	0.001	1.037
non-*N*	Syriobioside A	0.001	0.106	0.415
non-*N*	Syriobioside B	0.509	4.134*	0.32
non-*N*	Glycosylated syriobioside	0.407	11.367**	1
N-precursor (*n*-?)	Aspecioside	0.265	4.736*	0.003
N-precursor (*n*-2)	Desglucosyrioside	0.194	21.082***	0.202
N-precursor (*n*-1)	Labriformidin	18.632***	201.825***	5.031*
N-cardenolide	Reduced labriformin	2.920 +	165.272***	9.778*
N-cardenolide	Labriformin	24.782***	88.199***	3.156 +
Mixture	Total cardenolides	2.354	19.810***	< 0.001

leaf extracts were dried down and reconstituted in 20% DMSO, at a concentration of 7.5 mg tissue per 200 uL. This stock solution was serially diluted fourfold to yield a series of 6 solutions (1, ¼, 1/16, 1/64, 1/256, 1/1024) which were used to create an inhibition curve for each extract. Individual background wells were included for all dilutions on each plate. Each sample was run with the monarch and queen Na^+/K^+-ATPase on the same plate, for a total of three technical replicates on each enzyme type.

Details of the assay can be found in Petschenka et al. 2022. Briefly, a master-mix containing the appropriate enzyme prep, ATP, and either a reaction (with potassium) or background (without potassium) buffer was added to leaf extract dilutions in a 96-well plate. The plate was incubated at 37 °C for 20 min to allow for enzyme activity, then activity was terminated using sodium dodecyl sulfate (SDS) and cleaved phosphate was stained (Taussky-Shorr reagent). The amount of phosphate was measured using a spectrophotometer (absorbance at 700 nm, BioTek, Agilent). For each dilution, the background absorbance was subtracted from that of the reaction to yield our measure of Na^+/K^+-ATPase activity.

For each plant species, we applied extracts from 16 individuals at five dilutions to enzymes of the monarch and the queen butterflies. We then used a 4-parameter logistic function to fit enzyme inhibition curves for each plant extract (Petschenka et al. 2022). Curves were fit using SSfpl with separate non-linear mixed effects models for each plant species (nlme, Pinheiro et al. 2023). For each model, we fit an identifier of 'plant extract by enzyme' as a random effect, while the average curve of all extracts was the only fixed effect; individual inhibition curves were thus estimated as random deviates from the average curve. We report the xmid parameter as dilution of each extract required to inhibit enzyme activity by 50% (i.e., IC50). These values were converted to units of mg tissue per reaction. Raw inhibition curves are provided in supplementary information (Figure S1).

Statistical analysis

For analyses of the effect of nitrogen (four levels) and herbivore damage (induction) on plant growth and specific cardenolides, we used analysis of covariance including both fixed main effects and their interaction term. Although the two plant species were grown intermixed at the same time, we conducted separate analyses by species for ease of interpretation (i.e., no three-way interaction terms).

For the analysis of butterfly enzyme inhibition, we used a similar statistical approach but also included butterfly species as term in the model, since assays were conducted simultaneously and randomized. Finally, we conducted an additional test of the predictors of enzyme inhibition including total cardenolides in the above model to assess whether treatment effects were driven only by cardenolide concentration (or other compositional changes). For ease of interpretation, statistical results are presented in the figures.

Results

Our two plants species, *A. syriaca* and *A. curassavica,* showed divergent responses to nitrogen fertilization, with the former species increasing in above ground biomass and leaf N content and the latter showing no detectable responses (Fig. 2). Although perhaps unexpected, the different life-histories of these two milkweeds and our results suggest that they use alternative N acquisition and allocation strategies. As expected, we did not detect any strong insect herbivory effect on growth or leaf N of either species, as damage was minimal (and used primarily as an induction treatment) (Fig. 2).

For *A. syriaca*, soil *N* significantly decreased concentrations of *N*-containing labriformin and its precursor labriformidin, but not other specific and abundant cardenolides (syriobioside and glycosylated aspecioside), or total cardenolide concentrations (Table 1). The *N*-containing intermediate biosynthetic product, reduced labriformin (Fig. 1), was enhanced by soil *N*, but only when plants were damaged (see interaction term between *N* and Damage in Fig. 3). Monarch induction increased concentrations of labriformin and its precursors, as well as total cardenolides, by 200–300%, in a largely consistent manner across the *N* treatments.

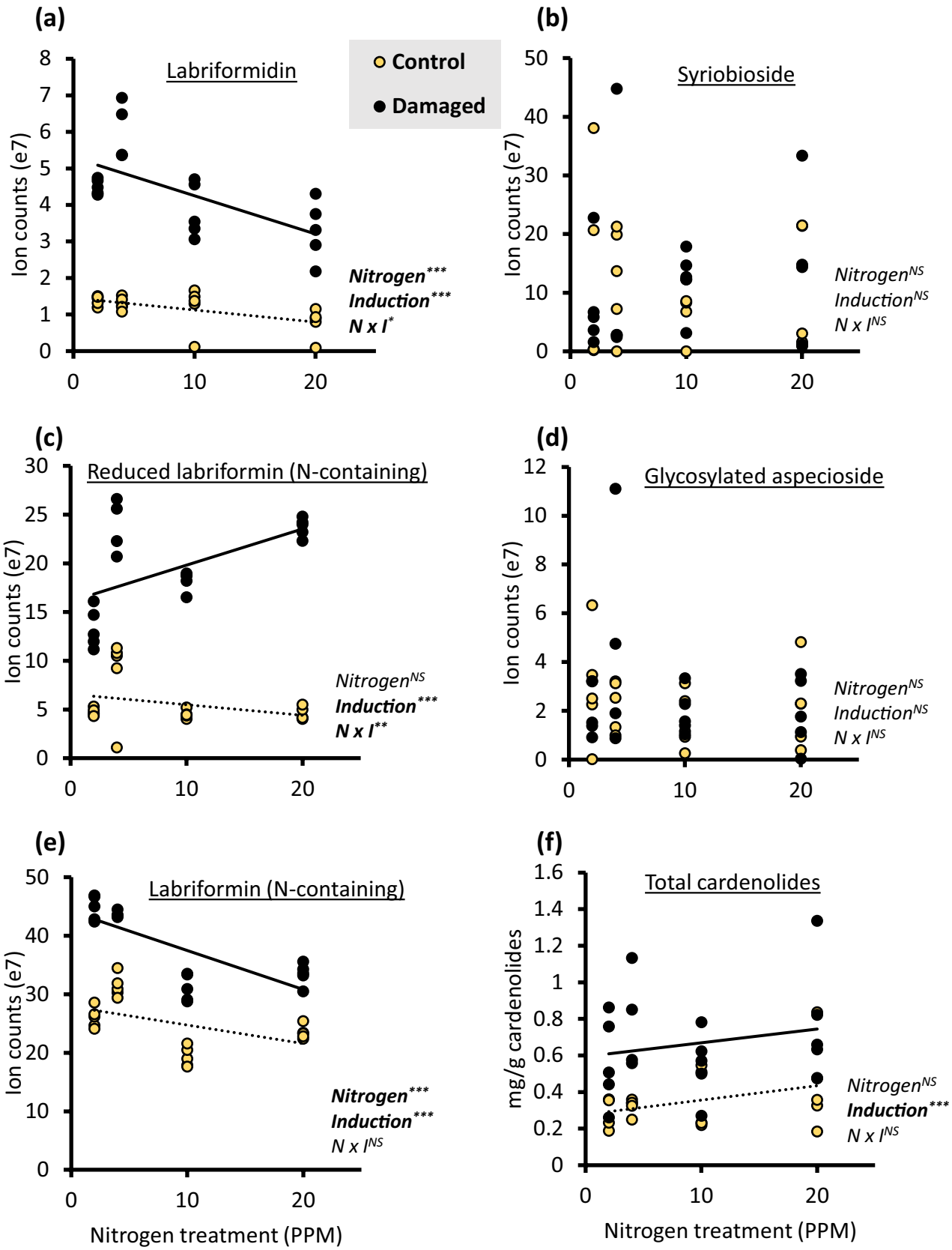
As expected, *A. curassavica* had nearly five times the total concentration of cardenolides, including N-containing cardenolides, compared to *A. syriaca*. For *A. curassavica*, soil *N* positively impacted production of uscharin and its precursors, but did not impact other abundant cardenolides or total concentrations (Table 1, Fig. 4). This is perhaps the main consistency we found between the two milkweed species: soil *N* availability had little impact the concentrations of non-*N*-containing cardenolides or the total concentrations of all cardenolides in leaves. We found modest effects of monarch induction on cardenolide concentration in *A. curassavica* (≈25% upregulation), although this was variable in direction depending on the specific compounds (Fig. 4). Overall, about half of the cardenolide compounds (as well as total cardenolides) were induced by monarch herbivory in across *A. syriaca* and *A. curassavica* (Table 1).

To understand the potential impacts of changes in cardenolide composition and concentrations on herbivores, we next conducted an *in vitro* assay of the extent to which the above leaf extracts inhibit the target of cardenolides, the Na^+/K^+-ATPase from monarch and queen butterflies. Although soil *N* availability did not impact this estimate of leaf toxicity, monarch induction consistently increased toxicity in all combinations of plant and insect species (Fig. 5). Indeed, across the board, about 30% less leaf tissue was needed to inhibit the insect Na^+/K^+-ATPases from induced plants compared to controls.

In a final set of analyses, we included total cardenolide concentrations as a covariate in the analysis of Na^+/K^+-ATPase inhibition to assess if the effects of plant induction were primarily through increasing total concentrations of cardenolides. We hypothesized that if including total cardenolides did not abolish the induction effect, then additional effects of cardenolide composition (or other compounds that modify their effects) were responsible for the enhanced inhibition. For *A. syriaca*, total cardenolides were not a significant predictor of enzyme inhibition ($F_{1,22} < 0.0001$, $p = 0.995$) and the induction effect remained significant ($F_{1,22} = 4.513$, $p = 0.045$). The same was true for *A. curassavica* (total cardenolides: $F_{1,22} = 0.360$, $p = 0.554$; induction: ($F_{1,22} = 6.382$, $p = 0.018$). Thus, for both milkweed species, monarch damage induced defense *compositional* changes in leaves, beyond the effect of higher cardenolide concentration, that increased foliar toxicity to the animal target enzyme.

Discussion

The availability of soil nitrogen has long been known to impact plant growth and defense strategies. Here we present data on the two most important host plants of the monarch butterfly, *A. syriaca* and *A. curassavica*, to test how N availability and herbivore damage change cardenolide profiles and shape toxicity using an *in vitro* assay. Importantly, these distantly related congeners had substantially different chemistries (i.e., no shared cardenolide genins) and distinct growth responses to *N*, yet both have *N*-containing cardenolides among many other non- *N*-containing compounds. While the highly clonal *A. syriaca* responded to *N*-fertilization


(a)
Labriformidin
Control
Damaged
Ion counts (e7)
Nitrogen***
Induction***
N x I*
(b)
Syriobioside
Ion counts (e7)
Nitrogen^NS
Induction^NS
N x I^NS
(c)
Reduced labriformin (N-containing)
Ion counts (e7)
Nitrogen^NS
Induction***
N x I**
(d)
Glycosylated aspecioside
Ion counts (e7)
Nitrogen^NS
Induction^NS
N x I^NS
(e)
Labriformin (N-containing)
Ion counts (e7)
Nitrogen***
Induction***
N x I^NS
Nitrogen treatment (PPM)
(f)
Total cardenolides
mg/g cardenolides
Nitrogen^NS
Induction***
N x I^NS
Nitrogen treatment (PPM)

◂**Fig. 3** Effects of soil nitrogen availability and caterpillar damage on foliar cardenolides of *Asclepias syriaca*. Nitrogen had a largely negative effect on concentrations of nitrogen-containing cardenolides (**c**, **e**) and their precursor (**a**) (see also Fig. 1); monarch herbivory substantially induced these compounds. Two abundant non-nitrogen-cardenolides (**b**, **d**) were unaffected by nitrogen availability or damage. Specific cardenolides were analyzed by LC–MS, while total cardenolides were estimated using UV-HPLC (**f**). Shown are the raw data and analyses from analysis of covariance with nitrogen availability and monarch caterpillar induction as the man effects. Yellow datapoints and dashed lines represent undamaged plants, while black-filled datapoints and solid lines represent herbivore-damaged plants.*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS = not significant

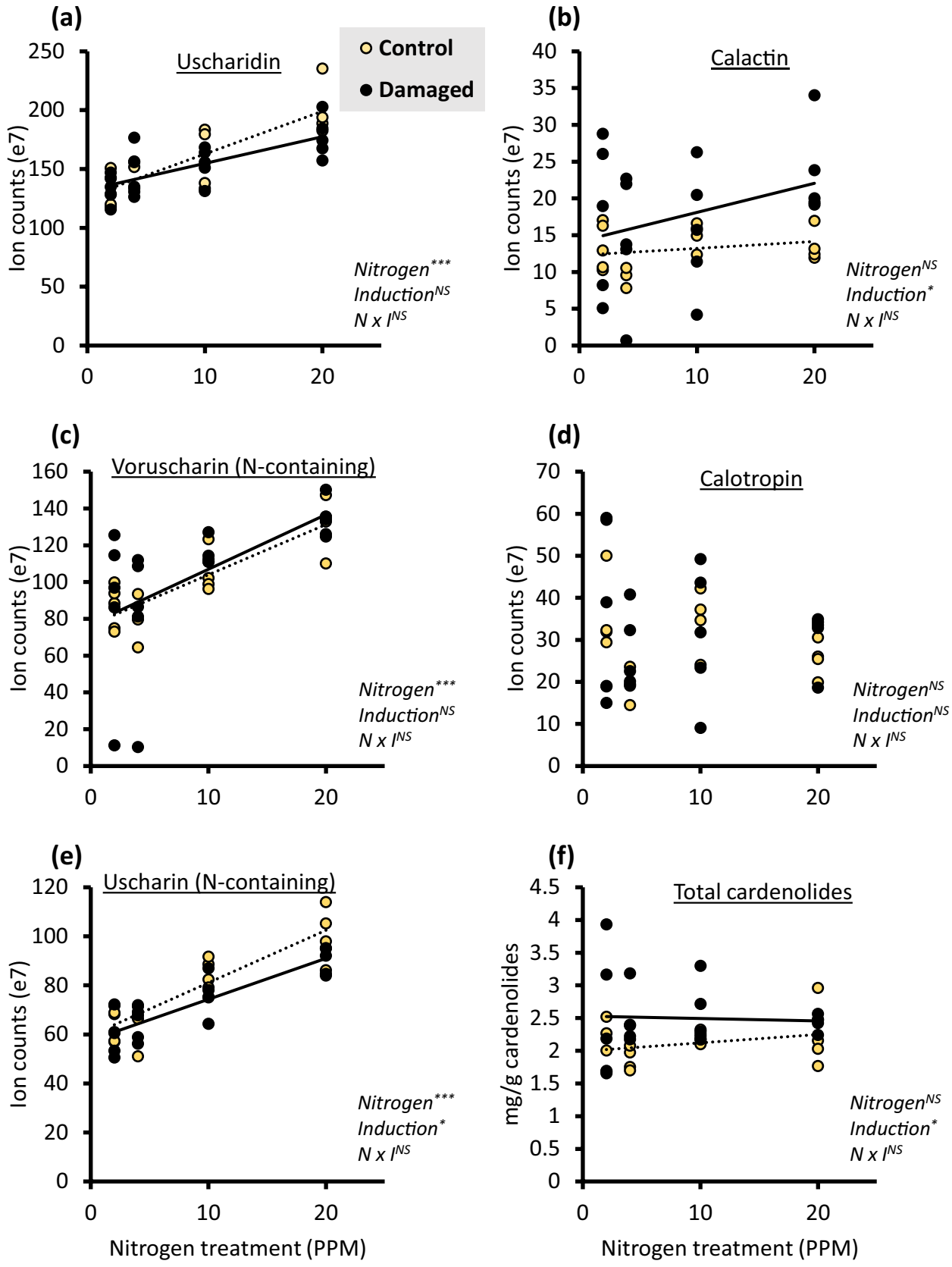
with increased above ground biomass and leaf *N* content, the same treatments did not impact *A. curassavica*.

Consistent with our findings on prioritizing growth, *A. syriaca* showed reduced abundance of its main *N*-cardenolide, labriformin, and the only effect on total cardenolides was via herbivore induction, not soil *N*. In other words, it appears that *A. syriaca* takes up excess *N* and allocates this primarily to growth; net cardenolide allocation was driven more so by a strong induced defensive response and this was also evident in our *in vitro* toxicity assays using both monarch and queen sodium pumps. Contrastingly, *A. curassavica* apparently allocated excess *N* to its two *N*-cardenolides, uscharin and voruscharin, but this did not affect total cardenolide concentrations, which were slightly enhanced by herbivore induction. *A. curassavica* is perhaps less nitrogen limited (Fig. 2), and perhaps other mineral nutrients were more limiting for growth and defense in this species.

For both plant species, given that total cardenolide concentrations were not impacted by soil *N*, it appears that *N*-mediated changes caused relatively modest shifts in the composition of specific cardenolides It is notable that the *N*-effects on cardenolides in both species occurred primarily through effects on the immediate precursors of the *N*-cardenolides (Table 1). Overall, the induction effect was also evident in the toxicity assays with *A. curassavica* extracts, suggesting the milieu of cardenolide changes with soil N balanced out to little net change. Enhanced cardenolide production in response to monarch herbivory has been widely reported in *A. syriaca* (Bingham and Agrawal 2010; Agrawal et al. 2012a, 2014a; Ali and Agrawal 2014), but less so for *A. curassavica* induction (Tan et al. 2018). For both species, relatively little work has been done on leaf position effects (but see Agrawal et al. 2014a) or local versus systemic induction; in the current study all leaves from each replicate were pooled and assayed, providing a whole plant perspective on defense chemistry, but leaving open the possibility of variation in responses among leaves.

For cardenolide-containing plants, there have been highly variable reports on the impact of fertilization on production of these secondary compounds. For example, a study of *A. curassavica* found that fertilization with ammonium nitrate increased leaf *N* and decreased total cardenolides (Couture et al. 2010). Similarly, Tao and Hunter (2015) applied ammonium nitrate to *A. curassavica* and found that leaf *N* increased (mostly at high soil *P*) and total cardenolides were variably affected, but declined at low *P*. Agrawal et al. (2012b) showed that NPK fertilization substantially reduced cardenolide concentrations across several species of *Asclepias*, including *A. curassavica*. One study examined the effects of NPK fertilization on *A. syriaca* growth and defense and found that both were increased with fertilization (Züst et al. 2015). Finally, in natural populations of *Digitalis obscura* (Plantaginaceae), soil nitrogen did not correlate with leaf cardenolides (Roca-Pérez et al. 2005). Although plant ontogeny and other aspects of the growth conditions (e.g., background levels of *P* and K) are certainly likely to impact cardenolide production, it appears that detailed studies of the specific cardenolides will be needed to further unravel the impacts of soil N dynamics for allocation to defense in *Asclepias*.

Although induced defense effects on monarch growth have been shown repeatedly for caterpillars on *A. syriaca* (Agrawal et al. 2012a, 2014a), no such data has been reported for *A. curassavica*. For *A. syriaca*, some fraction of this effect is due to latex, which increases substantially following herbivory (Agrawal et al. 2012a, 2014b). *A. curassavica* typically does not show inducible latex. Thus, the demonstration here of an induced defense effect on Na^+/K^+-ATPase activity in both species is a new finding. Furthermore, although we detected increases in cardenolide concentration of leaves from insect-damaged plants, this effect appears to be compositional in nature. In particular, after controlling for cardenolide concentrations, we still find a strong induced effect in both species (and on both butterfly enzymes). At this stage it is unclear whether these effects are due to cardenolide diversity, relative abundance, or some other modifying factor. Interestingly, *Asclepias* species are known to produce pregnane glycosides, which are based on a steroidal genin with an oligosaccharide chain. Such compounds have been proposed to increase the efficacy of cardenolides in inhibiting the sodium pump by disrupting cell membrane permeability and enhancing cardenolide uptake (Malcolm 1991; Wink 2008). This possibility is certainly worthy of study.


(a)
Uscharidin
Control
Damaged
Ion counts (e7)
Nitrogen***
Induction^NS
N x I^NS
(b)
Calactin
Ion counts (e7)
Nitrogen^NS
Induction*
N x I^NS
(c)
Voruscharin (N-containing)
Ion counts (e7)
Nitrogen***
Induction^NS
N x I^NS
(d)
Calotropin
Ion counts (e7)
Nitrogen^NS
Induction^NS
N x I^NS
(e)
Uscharin (N-containing)
Ion counts (e7)
Nitrogen***
Induction*
N x I^NS
Nitrogen treatment (PPM)
(f)
Total cardenolides
mg/g cardenolides
Nitrogen^NS
Induction*
N x I^NS
Nitrogen treatment (PPM)

◂**Fig. 4** Effects of soil nitrogen availability and caterpillar damage on foliar cardenolides of *Asclepias curassavica*. Nitrogen had a strongly positive effect on nitrogen-containing cardenolides (**c**, **e**) and their precursor (**a**) (see also Fig. 2). Two non-nitrogen-cardenolides (**b**, **d**) were unaffected by nitrogen availability. There were weak and variable effects of damage on cardenolides. Specific cardenolides were analyzed by LC–MS, while total cardenolides were estimated using UV-HPLC (**f**). Shown are the raw data and analyses from analysis of covariance with nitrogen availability and monarch caterpillar induction as the man effects. Yellow datapoints and dashed lines represent undamaged plants, while black-filled datapoints and solid lines represent herbivore-damaged plants. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS = not significant

Biochemistry

As outlined previously (Agrawal et al. 2021), we assume that the first stage of cysteine condensation is the key step for introducing *N* into cardenolides (Fig. 1), and it is clear that *N*-cardenolide regulation follows different trajectories depending on biotic and abiotic stresses. Nonetheless, we note that the most abundant *N*-cardenolide is the first compound produced after introduction of the cysteine (voruscharin) in *A. curassavica*, whereas in *A. syriaca* labriformin is the most abundant *N*-cardenolide and requires an additional imine formation step with supposedly a desaturase (Fig. 1). This successive diversification step is also a source of regulatory contrast. The regulation of *N*-cardenolides is different in *A. syriaca* and *A. curassavica*, and they follow their own pattern and differ from non-*N*-cardenolides within each species. The different trajectories of *N*- and non-*N*-cardenolide regulation in *A. syriaca* and *A. curassavica* suggest unique responses to abiotic and biotic stresses within milkweed necessitating detailed investigations into the specific biosynthetic pathways.

Conclusion

Continued interest in the impacts of abiotic factors on plant defense has been fueled by global change and greater understanding of specific plant metabolic pathways (Sampedro et al. 2011; Jamieson et al. 2017; Sun et al. 2020; Wang et al. 2022). Here we have shown that specific, yet uncommon, nitrogen-containing milkweed toxins have distinct regulation in two common *Asclepias* species. Despite these differences between plant species, and the somewhat independent regulation of non-*N*-cardenolides, the effects of *N*-fertilization were minimal compared to the effects of herbivore-induction in our enzymatic assays. Given the altered feeding, growth, and sequestration of monarchs on fertilized milkweeds (Lavoie and Oberhauser 2004; Tao and Hunter 2015) or those treated with other abiotic stresses (Agrawal et al. 2012a, 2014b; Faldyn et al. 2018), several plant and herbivore factors appear to be at play. As our knowledge of cardenolide biosynthesis and specificity grows (Agrawal et al. 2021), progress is likely in terms of deciphering the role of abiotic factors in the mechanisms of plant resistance.

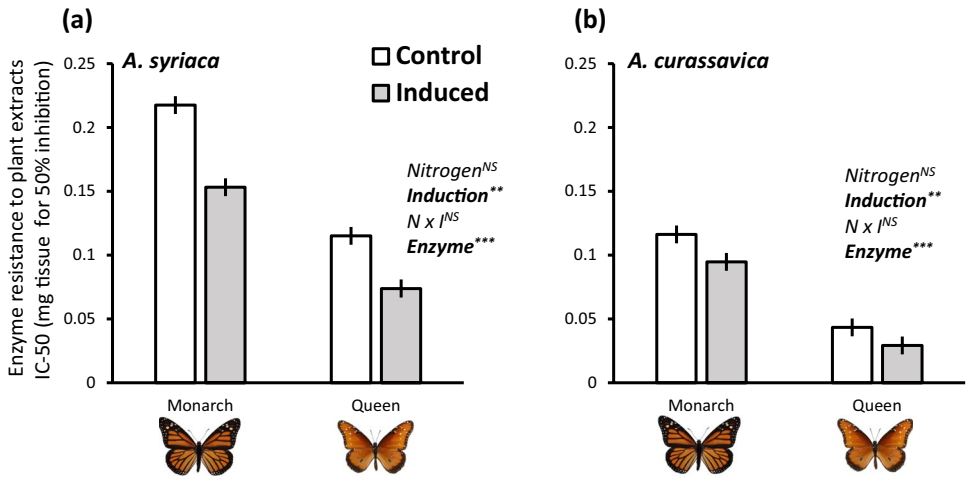


Fig. 5 Effects of caterpillar damage on foliar toxicity of *A. syriaca* (**a**) and *A. curassavica* (**b**) measured as inhibition of the monarch and queen butterfly sodium pump enzymes (Na^+/K^+-ATPase). Shown is a measure of enzyme resistance, the IC-50, or amount of leaf tissued extract needed to inhibit the Na^+/K^+-ATPase by 50%, with higher values indicating greater resistance of the pump (or lower toxicity). Soil nitrogen availability did not impact toxicity and so least squares means (±SE) for the other factors is what is shown. Note that, as expected, *A. curassavica* is substantially more toxic than *A. syriaca* and the queen enzyme is more susceptible than the monarch enzyme. Error degrees of freedom are 23 for *A. syriaca* and 27 for *A. curassavica*. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS = not significant

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Author Contributions All authors contributed to the conceptualization of the study, design of the experiments, and writing of the manuscript. CD conducted chemical analysis, APH led all other empirical aspects, and AAA conducted statistical analyses and led writing of the first draft of the manuscript.

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Data Availability The raw data for this study is available at https://doi.org/https://doi.org/10.6084/m9.figshare.26860192.v1, except the sodium pump inhibition data which is given in the supplementary information.

Declarations

Competing Interests The authors declare no competing interests.

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