

## Toxicity of milkweed leaves and latex: chromatographic quantification versus biological activity of cardenolides in 16 *Asclepias* species

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### Abstract

Cardenolides are classically studied steroidal defenses in chemical ecology and plant-herbivore coevolution. Although milkweed plants (*Asclepias* spp.) produce up to 200 structurally different cardenolides, all compounds seemingly share the same well-characterized mode of action, inhibition of the ubiquitous Na<sup>+</sup>/K<sup>+</sup> ATPase in animal cells. Over their evolutionary radiation, milkweeds show a quantitative decline of cardenolide production and diversity. This reduction is contrary to coevolutionary predictions and could represent a cost-saving strategy, i.e. production of fewer but more toxic cardenolides. Here we test this hypothesis by tandem cardenolide quantification using HPLC (UV absorption of the unsaturated lactone) and a pharmacological assay (*in vitro* inhibition of a sensitive Na<sup>+</sup>/K<sup>+</sup> ATPase) in a comparative study of 16 species of *Asclepias*. We contrast cardenolide concentrations in leaf tissue to the subset of cardenolides present in exuding latex. Results from the two quantification methods were strongly correlated, but the enzymatic assay revealed that milkweed cardenolide mixtures often cause stronger inhibition than equal amounts of a non-milkweed reference cardenolide, ouabain. Cardenolide concentrations in latex and leaves were positively correlated across species, yet latex caused 27% stronger enzyme inhibition than equimolar amounts of leaf cardenolides. Using a novel multiple regression approach, we found three highly potent cardenolides (identified as calactin, calotropin, and voruscharin) to be

primarily responsible for the increased pharmacological activity of milkweed cardenolide mixtures. However, contrary to an expected trade-off between concentration and toxicity, later-diverging milkweeds had the lowest amounts of these potent cardenolides, perhaps indicating an evolutionary response to milkweed's diverse community of specialist cardenolide-sequestering insect herbivores.

**Key words:** cardiac glycoside, coevolution, macroevolutionary escalation, mode of action, monarch butterfly, Na<sup>+</sup>/K<sup>+</sup> ATPase, phylogenetic chemical ecology, plant-insect interactions, structure-activity relationships, target site insensitivity

## Introduction

The mode of action of plant defenses forms the mechanistic cornerstone of plant-insect coevolution (Berenbaum 1995; Berenbaum 1999; Fraenkel 1959). In some cases, herbivorous insects have evolved offensive strategies that not only disarm the plant, but also make use of the plant “defenses” for their own benefit. For example, insect herbivores have been shown to take advantage of defensive plant secondary metabolites for nutrition (Rosenthal et al. 1982), mating pheromones (Eisner and Meinwald 1995), and defense (Conner et al. 2000; Nishida 2002; Petschenka and Agrawal 2016). Because a phytochemical's mode of action dictates how an herbivore may overcome or make use of the compound, understanding such offense-defense interactions mechanistically is critical for advancing our knowledge of coevolution.

Plant defenses that have a specific target involved in essential animal physiology represent an effective evolutionary strategy for plants to tackle a broad range of herbivores and in consequence, such defenses have evolved repeatedly (Agrawal 2011). However, given the high specificity of such defenses, simple and convergent genetic responses in consumers can change the binding affinity of the target to these toxins, resulting in tolerance through so-called target site insensitivity (Despres et al. 2007; Dobler et al. 2012). Indeed, the steroidal cardenolides famously known from foxglove (*Digitalis*) and milkweeds (*Asclepias*) have a highly specific mode of action, binding to a key animal enzyme, the Na<sup>+</sup>/K<sup>+</sup> ATPase (Agrawal et al. 2012). Because many animal cells express Na<sup>+</sup>/K<sup>+</sup> ATPases, cardenolides are principally toxic to all animals. Nonetheless, a diverse community of specialized insect herbivores from at least six taxonomic orders have specialized and evolved target site insensitivity to cardenolides based only on a handful of amino acid substitutions in their Na<sup>+</sup>/K<sup>+</sup> ATPases (Dobler et al. 2012; Dobler et al. 2015; Petschenka et al. 2017).

Although all cardenolides share a highly specific mode of action, individual plants and closely related species frequently produce many different forms of this class of chemical compounds. Understanding the role of such phytochemical diversity has long been a central goal of chemical ecology, and several non-exclusive explanations for this widespread phenomenon have emerged (Firn and Jones 2003; Forbey et al. 2013; Petschenka et al. 2018; Rasmann and Agrawal 2009; Richards et al. 2016; Romeo et al. 1996); for example, different cardenolides could be targeted at distinct herbivores by optimizing herbivore-specific uptake or inhibition. Structural variation among individual cardenolides can influence the binding affinity of the compound to its Na<sup>+</sup>/K<sup>+</sup> ATPase target-site and thereby affect compound toxicity (Dzimiri et al. 1987; Petschenka et al. 2018). In a large comparative study using isolated cardenolides from a variety of source plants, Petschenka et al. (2018) demonstrated that such structural variation results in at least 4-fold differences in inhibition of Na<sup>+</sup>/K<sup>+</sup> ATPase from non-adapted herbivores, but in more than 400-fold differences in (the overall reduced) inhibition of Na<sup>+</sup>/K<sup>+</sup> ATPase from adapted, specialist herbivores, with some evidence for relative selectivity between Na<sup>+</sup>/K<sup>+</sup> ATPases from different species. However, it is unclear to what extent cardenolides within a single plant differ in activity.

While coevolutionary theory predicts escalation of plant defenses over evolutionary time (Ehrlich and Raven 1964; Farrell and Mitter 1998; Futuyma and Agrawal 2009), we previously demonstrated a phylogenetic decline of cardenolide concentrations and diversity in milkweeds (Agrawal et al. 2015; Agrawal and Fishbein 2008; Agrawal et al. 2009a). However, a decline in concentration could be compensated by increasing potency of individual compounds (Farrell and Mitter 1998), which may be a more efficient and less costly strategy to cope with increasingly resistant herbivores. Here we test for a potential evolutionary change in cardenolide toxicity by conducting a comparative analysis of 16 representative milkweed species in the genus *Asclepias*, covering a wide range of defensive strategies and a significant proportion of the evolutionary history of the genus (Fig. 1). We compare cardenolide content and profiles of latex and leaf tissue of all plants, as the former is a strong defense of the plant with concentrated cardenolides (Agrawal and Konno 2009) and the latter is the primary food of many herbivores of milkweed.

Our work integrates two methods of quantitation: 1) chromatographic separation of compounds in plant extracts by HPLC and quantification of individual cardenolides, using the UV light absorption by the unsaturated lactone that is present in each cardenolide molecule; and 2) quantification of the total cardenolide content using the pharmacological activity of plant extracts in an *in vitro* assay of Na<sup>+</sup>/K<sup>+</sup> ATPase inhibition relative to a reference

cardenolide (Petschenka et al. 2013). With these methods, we test the hypothesis that natural cardenolide mixtures derived from different milkweed species vary in their potency against a non-cardenolide-adapted  $\text{Na}^+/\text{K}^+$  ATPase (Malcom 1991; Seiber et al. 1983), and that more derived species compensate for lower total amounts of cardenolides by more potent toxins in their latex and leaf tissue (Farrell and Mitter 1998).

Specifically, we asked: 1) Is latex more potent in terms of cardenolide concentration than leaves? 2) Do (summed) cardenolide concentrations quantified by HPLC in latex and leaves mirror those estimated from the enzymatic inhibition by crude extracts, and is there evidence for differential toxicity of latex and leaf cardenolides? 3) Using the natural variation in cardenolide profiles among the 16 milkweed species, can we identify species and specific compounds with disproportionately strong inhibition of the  $\text{Na}^+/\text{K}^+$  ATPase? And 4), do macroevolutionarily derived (later-diverging) milkweed species have higher toxicity compared to earlier-diverging species, resulting in trade-offs between concentration and toxicity?

## **Materials and Methods**

### *Plant growth and tissue collection*

We grew five plants for each of *A. asperula*, *A. californica*, *A. cryptoceras*, *A. curassavica*, *A. exaltata*, *A. incarnata*, *A. labriformis*, *A. linaria*, *A. nivea*, *A. perennis*, *A. purpurascens*, *A. solanoana*, *A. syriaca*, *A. tuberosa*, *A. verticillata*, and *A. viridis* from seed. Seeds had been collected in the field or were provided by commercial suppliers. All seeds were surface-sterilized in 10% bleach and scarified with a razor blade, cold stratified at 4 °C on moist paper towels for one week, and then placed in the dark at 28 °C for four days to synchronize germination. Seedlings were planted in commercial potting soil (Metro-Mix, Sun Gro Horticulture, Canada CM Ltd.) in plastic pots (10 cm diameter) and grown in a growth chamber for 35 days (16 h daylight with ~400 microeinsteins of light, 26 °C day : 22 °C night). Plants were fertilized once after ten days [N:P:K 21:5:20, 150 ppm N (mg/g)] and watered as needed throughout the experiment. At harvest, we collected latex and leaf material from 5 plants per species for cardenolide analysis. For *A. linaria* and *A. solanoana*, not all plants survived and only 3 and 4 plants could be harvested, respectively. To collect leaves, the four youngest, fully expanded leaves of each plant were cut and frozen at -80 °C. Frozen leaves were later freeze-dried and ground to a fine powder on a mixer mill (Retsch, Haan, Germany) using 3 mm steel beads. Note that leaves contained residual latex at time of harvest, thus some overlap between leaf and latex cardenolide profiles is expected. Latex was collected from each plant in one of two ways. Plants were randomized by species, and for the first half of the

harvest, plants were cut at the base of the stem and exuding latex was collected onto pre-weighed discs of aluminum foil. As this protocol yielded small volumes of latex in some cases, for the second half of the harvest, we instead collected latex from the four petioles cut for leaf harvest. Laticifers of a plant are part of a connected system, thus latex exuding from stem and leaves should be identical in its composition (effect of exudation method on total latex cardenolide concentration:  $F_{1,58} = 0.292$ ,  $p = 0.591$ ). Exuded latex was immediately weighed to the nearest microgram. As rapid evaporation of the latex droplet interfered with stable balance readouts, weights were recorded at the latest 10 seconds after placing the aluminum disc with the droplet onto the microbalance. Discs with latex droplets were then dried and weighed again to measure latex dry weight.

### *Sample preparation*

We extracted 50 mg of dried, ground leaf material using 1.6 ml of methanol and approximately 30 FastPrep beads (MP Biomedicals, CA, USA) in plastic 2 ml screw-cap vials (Sarstedt, Nürmbrecht, Germany). Samples were agitated twice for 45 seconds at a speed of 6.5 m/sec on a FastPrep-24 homogenizer, followed by centrifugation at 14'000 rpm for 12 minutes. The supernatant was split into two aliquots, 1 ml to be used for HPLC and 0.2 ml to be used in the  $\text{Na}^+/\text{K}^+$ -ATPase assay (Fig. S1). The aliquot for HPLC was spiked with 20  $\mu\text{g}$  digitoxin (Sigma Aldrich, MO, USA), and both aliquots were evaporated at 35 °C on a vacuum concentrator (Labconco, MO, USA). Dried aliquots for HPLC were re-suspended in 300  $\mu\text{l}$  methanol and filtered through a 0.2  $\mu\text{m}$  syringe filter with a PTFE membrane (Kinesis, NJ, USA). Dried aliquots for the  $\text{Na}^+/\text{K}^+$ -ATPase assay were suspended in 100% dimethyl sulfoxide (DMSO, Sigma Aldrich, MO, USA) by adding 20  $\mu\text{l}$  of the solvent and sonicating samples twice for 5 minutes. Extracts were then brought to 10% DMSO by addition of 180  $\mu\text{l}$  water and vortexed to mix, followed by centrifugation to precipitate any undissolved particles.

We extracted dried latex from the aluminum disks using a similar protocol as for leaf tissue. Disks were placed into screw-cap vials containing 1.3 ml of methanol and approximately 30 FastPrep beads. Samples were agitated, centrifuged, and the supernatant was split into two aliquots as above (Fig. S1). The aliquot for HPLC was spiked with 5  $\mu\text{g}$  digitoxin, and both aliquots were evaporated at 35 °C on a vacuum concentrator. Dried aliquots for HPLC were prepared for analysis as above but re-suspended in 200  $\mu\text{l}$  methanol for analysis. Dried aliquots for the  $\text{Na}^+/\text{K}^+$ -ATPase assay were suspended in 5  $\mu\text{l}$  100% DMSO and brought to a 10% solution by addition of 45  $\mu\text{l}$  water. To account for the differences in collected latex amounts between plant species, we selected the sample with the lowest amount of dry latex as reference

concentration (86 ng latex  $\mu\text{l}^{-1}$  10% DMSO) and diluted all other samples to the same concentration by adding the required volumes of 10% DMSO in water.

#### *HPLC analysis*

We analyzed 15  $\mu\text{l}$  of each sample by HPLC using a Gemini C18 reversed phase column (3  $\mu\text{m}$ , 150  $\times$  4.6 mm, Phenomenex, Torrance CA, USA) and an Agilent 1100 system with diode array detection. Cardenolides were eluted at a constant flow of 0.7 ml/min with a gradient of acetonitrile and water as follows: 0-2 min at 16% acetonitrile; 2-25 min from 16% to 70%; 25-30 min from 70% to 95%; 30-35 min at 95%; followed by 10 min reconditioning at 16% acetonitrile. 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, corresponding to an unsaturated lactone functional group, were considered cardenolides. All cardenolides contain a single unsaturated lactone and lack other light-absorbing features, thus equimolar amounts of different cardenolides have approximately equivalent UV absorption (Züst, unpublished data). Peaks in different samples with retention time differences of less than 0.05 min were considered the same compound. Concentrations of cardenolide compounds were calculated by relating peak areas to the area of the internal standard, digitoxin.

#### *Na<sup>+</sup>/K<sup>+</sup>-ATPase assay*

We measured the biological activity of leaf and latex extracts on the Na<sup>+</sup>/K<sup>+</sup>-ATPase from the porcine (pig, *Sus scrofa*) cerebral cortex (Sigma-Aldrich, MO, USA) using an *in vitro* assay introduced by Klauck and Luckner (1995); our methods were based on Petschenka et al. (2013). Briefly, we tested the inhibitory effect of each extract at three concentrations to estimate the sigmoid enzyme inhibition function from which we could determine the cardenolide content of the extract relative to a standard curve for ouabain (Sigma Aldrich, MO, USA). For leaves, we diluted extracts 1:5, 1:50, and 1:500 using 10% DMSO in water, while latex extracts (previously diluted to standardized concentrations of 86 ng  $\mu\text{l}^{-1}$ ) were used at full strength (= 1) and diluted 1:5 and 1:50.

In a 96-well plate, reactions were started by adding 80  $\mu\text{l}$  of a reaction mix containing 0.0015 units of porcine Na<sup>+</sup>/K<sup>+</sup>-ATPase to 20  $\mu\text{l}$  of leaf or latex extracts in 10% DMSO, to achieve final well concentrations (in 100  $\mu\text{l}$ ) of 100 mM NaCl, 20 mM KCl, 4 mM MgCl<sub>2</sub>, 50 mM imidazol, and 2.5 mM ATP at pH 7.4. To control for coloration of leaf extracts, we replicated each reaction on the same 96-well plate using a buffered background mix with

identical composition as the reaction mix but lacking KCl. As latex extracts were colorless, background correction was not necessary for these samples. In addition to extracts, we added two control wells to each plate, one with a reaction of fully active enzyme (active control), and one with a reaction lacking KCL and additionally inhibited by  $2 \times 10^{-3}$  M ouabain (inhibited control). Plates were incubated at 37 °C for 20 minutes, after which enzymatic reactions were stopped by addition of 100  $\mu$ l sodium dodecyl sulfate (SDS, 10% plus 0.05% Antifoam A) to each well. Inorganic phosphate released from enzymatically hydrolyzed ATP was quantified photometrically at 700 nm following the method described by Taussky and Shorr (1953).

Absorbance values of reactions were corrected by their respective backgrounds, and calculated as percent residual activity using the active and inhibited control wells in each plate as reference points for 100% activity and for 0% residual activity. Based on the residual enzymatic activity across the sample dilutions, we estimated the sigmoid dose-response curve using a logistic function with the upper and lower asymptotes fixed to 100% and 0% residual activity, respectively, using the statistical software R (function *gnls* in the *nlme* package). For each sample, we calculated the relative dilution at the inflection point (i.e., residual enzymatic activity of 50%). Using a calibration curve made with ouabain ranging from  $10^{-3}$  to  $10^{-8}$  M that was included on each 96-well plate, we then estimated the concentration of the undiluted sample in ouabain equivalents, i.e., the amount of ouabain required to achieve equivalent inhibition.

#### *Comparison of quantification methods*

We compared quantification by  $\text{Na}^+/\text{K}^+$ -ATPase and HPLC using a linear mixed effects model (function *lme* in the *nlme* package for R), treating  $\text{Na}^+/\text{K}^+$ -ATPase quantification as the response variable and sample type (leaf/latex) and HPLC quantification as fixed effects. Species identity was fitted as a random term. While the two types of quantification were overall highly correlated, several species clearly deviated from this relationship, with quantification based on  $\text{Na}^+/\text{K}^+$ -ATPase inhibition (relative to ouabain) revealing higher cardenolide concentrations than predicted by HPLC. If all plant cardenolide concentrations quantified by HPLC were equivalent in their inhibitory potency to the corresponding ouabain equivalents estimated by  $\text{Na}^+/\text{K}^+$ -ATPase inhibition, we would expect a 1:1 relationship between the two quantification methods; positive deviations therefore indicate that some species accumulate more potent cardenolide compounds against porcine  $\text{Na}^+/\text{K}^+$ -ATPase than ouabain.

To identify these compounds, we used a custom multiple regression approach to estimate compound multipliers (i.e., potency) that minimize the total deviation between the

two quantification methods for the combined leaf and latex samples. We restricted the data to the 43 most abundant cardenolide compounds (each constituting >5% of the total in at least one species/sample type combination). We then fit the total Na<sup>+</sup>/K<sup>+</sup>-ATPase-quantified cardenolide concentration  $N$  of the  $i$ th sample (both leaf and latex) as a function of the sum of HPLC-quantified concentrations  $H$  of the  $j$ th compound multiplied by a coefficient  $a_j$ . We constrained parameter space for  $a_j$  to values  $\geq 0$  and estimated compound-specific parameters by minimizing the total sums of squares ( $SS$ ) using equation 1 and function *optim* in R, with starting values for coefficients set to 1 (i.e., potency equal to ouabain).

$$SS = \sum_{i=1}^n \left\{ \left( \sum_{j=1}^m (a_j \times H_{i,j} + \dots + a_m \times H_{i,m}) - N_i \right)^2 \right\} \quad \text{eqn. 1}$$

Given their lower contribution to the total concentration, lower-concentrated compounds are more likely to have inflated parameter estimates. To test for significance of parameters, we therefore randomized concentration values within each compound (keeping each compounds' proportion of the total concentration constant) and re-calculated parameter estimates 1000 times. From these randomized estimates, we calculated the 2.5% or 97.5% quantiles, and only kept multipliers for compounds that lay outside these quantiles, while for all other compounds the multipliers were set to the default value of 1. Using this highly conservative list of potencies, we calculated an adjusted total cardenolide concentration, and correlated it again with the total Na<sup>+</sup>/K<sup>+</sup>-ATPase-quantified cardenolide concentration for leaves and latex. The R code and data to perform this analysis is provided as an online supplement.

### *Macroevolutionary analyses*

We previously published evidence for macroevolutionary declines of leaf cardenolide concentrations among 49 *Asclepias* species using two different quantification methods (spectrophotometry, Agrawal and Fishbein 2008; and HPLC, Agrawal et al. 2009a) and with a variety of phylogenetic models. Here we used the simplest of these tests which provided equivalent results to more complex models in our previous work: we regressed the log-transformed concentration of cardenolides in the 16 species (HPLC and Na<sup>+</sup>/K<sup>+</sup>-ATPase quantification) for leaves and latex against the number of hypothesized speciation events (phylogenetic nodes) from a comprehensive molecular phylogeny (Fishbein et al. 2011). In this



analysis, we thus consider the association between speciation and cardenolide concentration and activity.

## Results

The 16 evaluated milkweed species differed in cardenolide concentrations by several orders of magnitude, from *A. exaltata*, *A. tuberosa*, *A. purpurascens* and *A. incarnata* with virtually no detectable cardenolide levels in leaves or latex, to *A. curassavica*, *A. linaria*, and *A. perennis* with the highest levels in latex. Standardized by unit dry mass, latex had, on average, 100-fold higher cardenolide concentrations than leaf tissue (Fig. 2), and cardenolide concentrations of leaves and latex were highly correlated ( $r_{\text{Pearson}} = 0.882$ ,  $p < 0.001$ ). However, total latex production was largely independent of cardenolide concentration in latex, with *A. verticillata*, *A. tuberosa*, *A. incarnata* and *A. perennis* producing little to no latex in our experiment but spanning the full range of latex cardenolide concentrations. Latex water content was relatively constant across species (Table S1), with most species ranging from 1.1 to 3.1 units water per unit dry mass, but up to six times as much for *A. curassavica* with a water ratio of 6.3. Therefore, while the ranking of latex toxicity was unchanged for most species between wet and dry latex, the strong dilution of latex in *A. curassavica* resulted in an intermediate toxicity per unit wet latex, despite it being one of the most toxic species per unit dry latex.

Overall, there was a strong linear correlation between cardenolide quantification of the same samples by HPLC and  $\text{Na}^+/\text{K}^+$ -ATPase, yet quantification by  $\text{Na}^+/\text{K}^+$ -ATPase consistently yielded higher concentrations, indicating stronger inhibition by at least some *Asclepias* cardenolides than by the reference compound ouabain (Fig. 2, Table S1). For leaves, quantification by  $\text{Na}^+/\text{K}^+$ -ATPase yielded  $1.77 \pm 0.06$  (mean  $\pm$  1 SE) units cardenolides per unit quantified by HPLC. For latex, quantification by  $\text{Na}^+/\text{K}^+$ -ATPase was even higher, yielding  $2.25 \pm 0.08$  units cardenolide per unit quantified by HPLC. Cardenolide mixtures in latex were therefore on average 27 % more potent than foliar cardenolides across all species ( $F_{1,125} = 31.75$ ,  $p < 0.001$ ). The deviation between quantification methods was most pronounced for species with higher cardenolide content; the exception to this was *A. labriformis*, which despite accumulating the highest foliar cardenolide concentrations of all species did not inhibit  $\text{Na}^+/\text{K}^+$ -ATPase more than an equivalent amount of ouabain (Fig. 3a-b).

Using a constrained multiple regression approach to adjust the relative contribution of individual cardenolide compounds to the HPLC-quantified total, we identified three compounds as the main drivers of the deviation of  $\text{Na}^+/\text{K}^+$ -ATPase relative to HPLC

quantification. Two of these compounds were identified as calactin and calotropin in a separate study (Petschenka et al. 2018), with estimated multipliers of 5.50 and 4.45, respectively. Compound #19.9, here tentatively identified as voruscharin based on its exact mass (Fig. S2), is one of the less common nitrogen-containing cardenolides (Seiber et al. 1982) and had a multiplier of 5.28. The most abundant compound of *A. labriformis* (#15.9) was identified as labriformin based on exact mass and NMR (Fig. S2 & Table S2), and another abundant compound present in several species (#19.3) was tentatively identified as uscharin (Fig. S2). However, neither of these two compounds appeared to contribute to a stronger inhibition by plant cardenolide mixtures than by ouabain. With the default multiplier thus set to 1 for all compounds except calactin, calotropin, and voruscharin, almost all deviations between the two quantification methods could be accounted for, resulting in correlation coefficients of  $>0.93$  between HPLC- and  $\text{Na}^+/\text{K}^+$ -ATPase quantification for both latex and leaves (Fig. 3c-d).

Further comparison of cardenolide profiles of leaves and latex revealed that the increased toxicity of cardenolide mixtures in latex relative to leaves are primarily driven by higher accumulation of the three potent cardenolide compounds in latex (Fig. 3e-l). Cardenolides present in the latex of a species were consistently present in its leaves, but we cannot distinguish whether these accumulated in leaf cells or were present in residual latex. In contrast, several predominantly polar cardenolide compounds were detected exclusively in leaves, but none of these compounds were identified as being more (or less) potent than ouabain. Note, however, that the constrained multiple regression approach used here is less sensitive to identify potencies lower than ouabain, particularly for compounds only occurring at low concentrations.

Earlier-diverging species had the highest cardenolide concentrations in both leaves and latex (Fig. 4), and there was a significant decrease in cardenolide concentrations with increased phylogenetic branching (Latex:  $F_{1,30} = 8.16$ ,  $p = 0.008$ ; Leaves:  $F_{1,30} = 12.84$ ,  $p = 0.001$ ). Nonetheless, species with low concentrations of cardenolides were scattered across the phylogeny of *Asclepias* (Figs. 1, 4), resulting in large amounts of unexplained variation. Even though quantification by  $\text{Na}^+/\text{K}^+$ -ATPase inhibition yielded on average higher cardenolide concentrations than HPLC, the decrease in cardenolide concentration with increased phylogenetic branching was independent of quantification method (nodes-by-method interactions for Latex:  $F_{1,28} = 0.01$ ,  $p = 0.918$ ; Leaves:  $F_{1,28} = 0.02$ ,  $p = 0.902$ ). In further support for the lack of a trade-off between concentration and toxicity, the three highly potent cardenolides were mostly, but not exclusively, present in early-divergent species (Fig. 1).

## Discussion

A key issue in plant-herbivore coevolution is not only understanding mechanisms of defense-offense interactions, but also deciphering how natural selection could operate on these mechanisms. Because the wide array of defensive plant traits have diffuse or unknown modes of action, progress has been slow in defining structural attributes that affect biological activity, which could directly impact defense-offense coevolution. Nonetheless, some classic work stands out. For example, studies of furanocoumarins revealed mechanisms of how adaptive structural changes of phytochemicals can influence toxicity and detoxification (Berenbaum 1978; Berenbaum and Zangerl 1993). Furthermore, despite the fact that the precise mode of action of glucosinolate defenses (and their hydrolysis products) in the Brassicaceae is not well-defined (Jeschke et al. 2016), numerous offensive traits of insects to deactivate the reactive derivatives have been identified and are manifold, including the prevention or diversion of hydrolysis (Jeschke et al. 2015). The variety of specific offense traits suggests multiple trajectories of toxin specific interactions with pest insects.

For cardenolide defenses acting against insect herbivores, it has been suggested that apolar, lipophilic cardenolides should have greater toxicity due to better absorption into the herbivore's body (Malcom 1991). Moreover, specific structural features of a cardenolide compound should increase biological activity on the level of the organism (Farrell and Mitter 1998; Seiber et al. 1983) and the target site  $\text{Na}^+/\text{K}^+$ -ATPase (Dzimiri et al. 1987; Petschenka et al. 2018). Even though highly convergent genetic substitutions of  $\text{Na}^+/\text{K}^+$ -ATPase that mediate cardenolide resistance are widespread in the community of cardenolide-exposed insects (Dobler et al. 2012; Dobler et al. 2015; Petschenka et al. 2017), there is still some evidence of sensitivity to the toxins in adapted herbivores (Rasmann and Agrawal 2011; Rasmann et al. 2009b; Zalucki et al. 2001), and in fact structure-activity effects appear to be exacerbated for adapted  $\text{Na}^+/\text{K}^+$ -ATPases (Petschenka et al. 2018). In the current study, we thus took a first step towards understanding variation in levels of cardenolide concentrations and the corresponding biological activity of cardenolide mixtures by comparing leaf extracts and latex of 16 *Asclepias* species.

Here we confirmed that chromatographic quantification versus biological activity (inhibition of a sensitive vertebrate  $\text{Na}^+/\text{K}^+$ -ATPase) of plant extracts reveals highly correlated estimates of total cardenolide concentrations. Among the dozens of cardenolides in the 16 milkweed species examined, three stood out as having strong support for highly divergent activity compared to their quantification by HPLC (Figure 3). Calactin and calotropin are two moderately apolar cardenolides, while voruscharin is highly apolar (and nitrogen-containing),

and all three are common in *A. curassavica* and *Calotropis procera* (Seiber et al. 1983). These compounds are linked to their sugar moieties via cyclic bridges and share an identical genin (calotropagenin). Interestingly, calactin and calotropin are preferentially sequestered by the monarch butterfly as well as other milkweed insects (Reichstein et al. 1968; Roeske et al. 1976; Von Euw et al. 1967), while other compounds in this group are metabolically converted to calactin and calotropin by monarchs (Marty and Krieger 1984; Seiber et al. 1980). Remarkably, calactin and calotropin were not only independently identified as two of most active inhibitors of sensitive  $\text{Na}^+/\text{K}^+$ -ATPase, but they were also among the strongest inhibitors of the adapted monarch  $\text{Na}^+/\text{K}^+$ -ATPase, which is on average at least 100-fold more resistant to cardenolides (Petschenka et al. 2018). Despite similar patterns of inhibition for these two highly active compounds on  $\text{Na}^+/\text{K}^+$ -ATPases from two organisms, other cardenolide compounds can exhibit high selectivity of inhibition of  $\text{Na}^+/\text{K}^+$ -ATPases from different species (Petschenka et al. 2018). While we only focused on porcine  $\text{Na}^+/\text{K}^+$ -ATPase as a proof of principle here, our study sets the foundation for future studies that should involve  $\text{Na}^+/\text{K}^+$ -ATPases from additional herbivorous species to elucidate such selectivity in the effects of specific cardenolide toxins.

The defensive system of laticiferous plants such as milkweed is complex because toxins are often concentrated in latex, but can also occur in plant tissues lacking latex (Agrawal and Konno 2009). For example, despite the fact that neither roots nor floral nectar of *Asclepias* spp. contain latex, these tissues contain correlated levels of cardenolides to leaves (Manson et al. 2012; Rasmann et al. 2009a). Although, as we show here, cardenolides are indeed concentrated in latex, residual latex in leaf tissue does not seem to be the (exclusive) driver of foliar cardenolide levels in milkweeds (Agrawal et al. 2014). Additionally, although leaves and latex generally show correlated patterns of cardenolide concentration and composition, some *Asclepias* species such as *A. californica* show very little cardenolides in latex, despite producing copious quantities of latex and having concentrated cardenolides in leaves (Agrawal et al. 2008; Seiber et al. 1982). Thus, milkweed defense is likely to be independently determined by latex amount as well as cardenolides in latex and other tissues (e.g., Zalucki et al. 2001). Although we found a high overlap regarding cardenolide types in leaves and latex of a given species in the present study, on average, latex was a more potent inhibitor of the  $\text{Na}^+/\text{K}^+$ -ATPase when standardized on cardenolide concentration. We attribute this difference, at least in part, to the greater relative proportion of calactin, calotropin, and voruscharin in latex compared to other cardenolides (Figure 3). Interestingly, proportionally higher accumulation

of less polar cardenolides (including voruscharin) in latex was previously documented but not yet linked to higher toxicity of latex (Seiber et al. 1982).

Cardenolides found exclusively in leaves were predominantly among the most polar compounds and accumulated to comparably low levels, and none were identified as more potent than our standard, ouabain. It is currently unclear whether these compounds accumulate as tissue-specific defenses, or perhaps are intermediate products of cardenolide synthesis. While the three most toxic cardenolides also were among the highly concentrated compounds of the species producing them, a few species accumulated high levels of additional cardenolides with low activity (against porcine  $\text{Na}^+/\text{K}^+$ -ATPase): several species accumulated compound #19.3 (tentatively identified as uscharin), and *A. labriformis* accumulated high levels of compound #15.9 (identified as labriformin). Voruscharin, uscharin, and labriformin are structurally similar nitrogen-containing cardenolides, yet we only found voruscharin to be a highly potent inhibitor of porcine  $\text{Na}^+/\text{K}^+$ -ATPase. Nonetheless, labriformin was reported to be toxic to livestock (Benson et al. 1979) and highly emetic to bird predators, but is metabolically converted and not stored by monarchs (Brower et al. 1982); thus, perhaps the defensive function of these compounds goes beyond inhibition of (porcine)  $\text{Na}^+/\text{K}^+$ -ATPase.

#### *Defense escalation and conclusion*

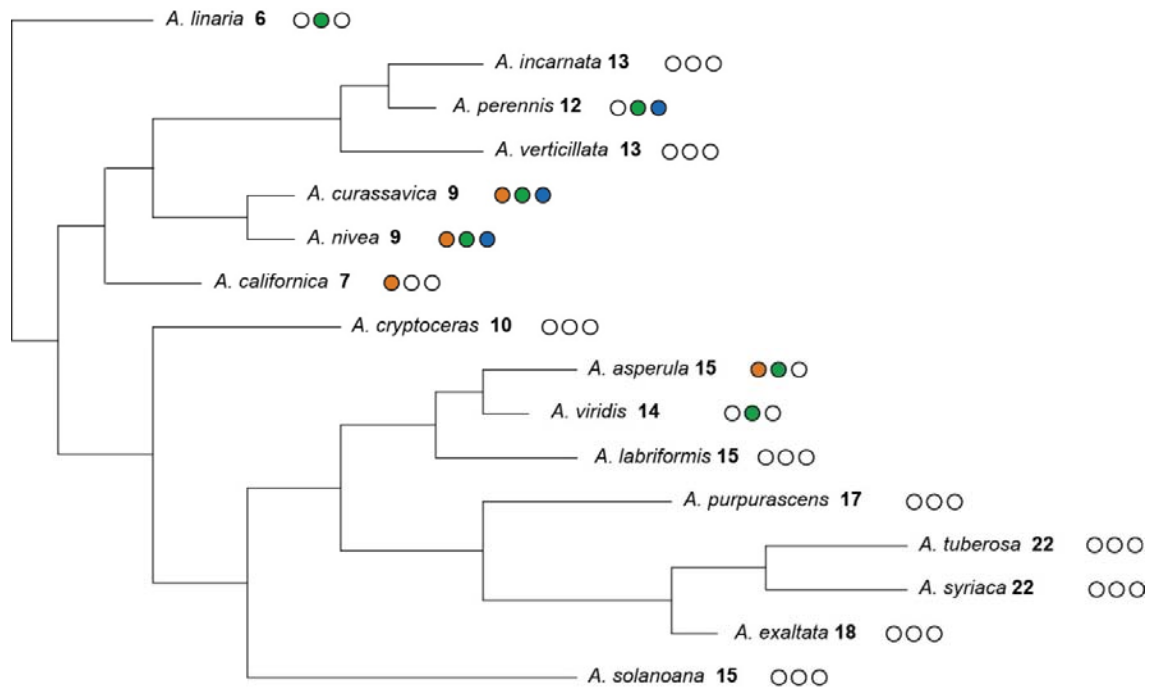
Farrell and Mitter (1998) proposed a very attractive hypothesis of cardenolide escalation that was based on Ehrlich and Raven's (1964) coevolutionary model. Although not fully resolved, the weight of the evidence suggests that milkweeds have not escalated their cardenolide defenses over macroevolutionary time. Indeed, both latex amounts and cardenolide concentrations have phylogenetically declined (Agrawal and Fishbein 2008; Agrawal et al. 2009a), and monarchs grow faster on later-diverging milkweeds (Agrawal et al. 2015). Nonetheless, monarch sequestration of cardenolides also decreased on these same later-diverging species (Agrawal et al. 2015), thus the goal to make monarchs more vulnerable to predators could have been a driver of macroevolutionary defense declines. Here we have shown that the cardenolides produced by later-diverging milkweed species have not only declined in concentration, but also do not produce compounds most effective at inhibition of a sensitive  $\text{Na}^+/\text{K}^+$ -ATPase. Importantly, the two cardenolides calactin and calotropin, which are preferentially sequestered by monarchs, are absent from later-derived milkweeds, perhaps making monarchs more susceptible to predation. In contrast, the concentration of phenolic compounds and the tolerance ability following defoliation has phylogenetically increased in

milkweed (Agrawal et al. 2015; Agrawal and Fishbein 2008; Agrawal et al. 2009b), which may represent an evolutionary response to these co-evolved herbivores.

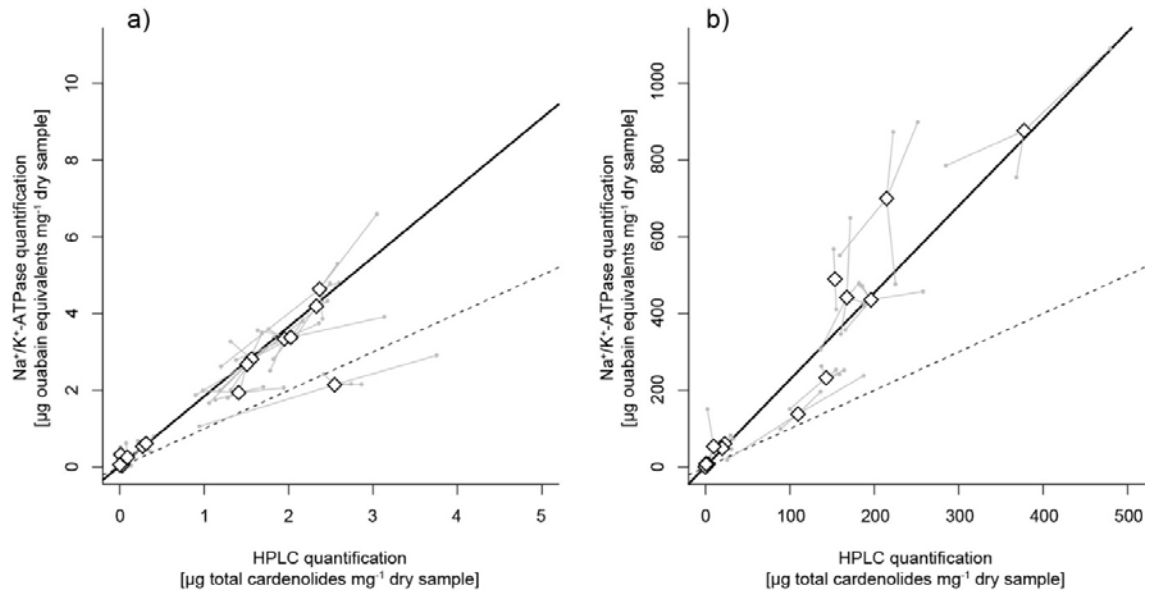
Although we found a high degree of correspondence between total cardenolide concentration in milkweed species and total *in vitro* inhibition of a sensitive vertebrate Na<sup>+</sup>/K<sup>+</sup>-ATPase, our results are in support of a central role of phytochemical diversity for plant-herbivore coevolution. First, we did detect three compounds with disproportionately high biological activity. Second, because our assays were conducted *in vitro* with an isolated enzyme, we bypassed intermediary physiological steps such as detoxification, excretion, or absorption of cardenolides into the body before reaching the target Na<sup>+</sup>/K<sup>+</sup>-ATPase. Structural attributes of cardenolides are likely to contribute to membrane permeability, where they travel in the herbivore, and their level of toxicity (Agrawal et al. 2012). Third, we present evidence for some specificity in the expression of cardenolides in leaves versus latex, and other work indicates similar selectivity in roots and floral nectar, allowing for the potential for tissue-specific cardenolides to defend against particular attackers (Manson et al. 2012; Nelson et al. 1981; Rasmann and Agrawal 2011). And finally, we predict that assays of the Na<sup>+</sup>/K<sup>+</sup>-ATPases from specialized milkweed herbivores will reveal surprises for some of the compounds, indicating stronger or weaker than predicted levels of toxicity. Given that milkweeds are primarily attacked by a community of highly specialized and sequestering insect herbivores, it is certainly possible that the route this plant lineage has followed is one of defensive de-escalation (Agrawal 2017). Both hypotheses and evidence are increasingly beginning to reveal this pattern in other systems, suggesting novel directions in the study of chemically-mediated coevolution (Cacho et al. 2015; Livshultz et al. 2018).

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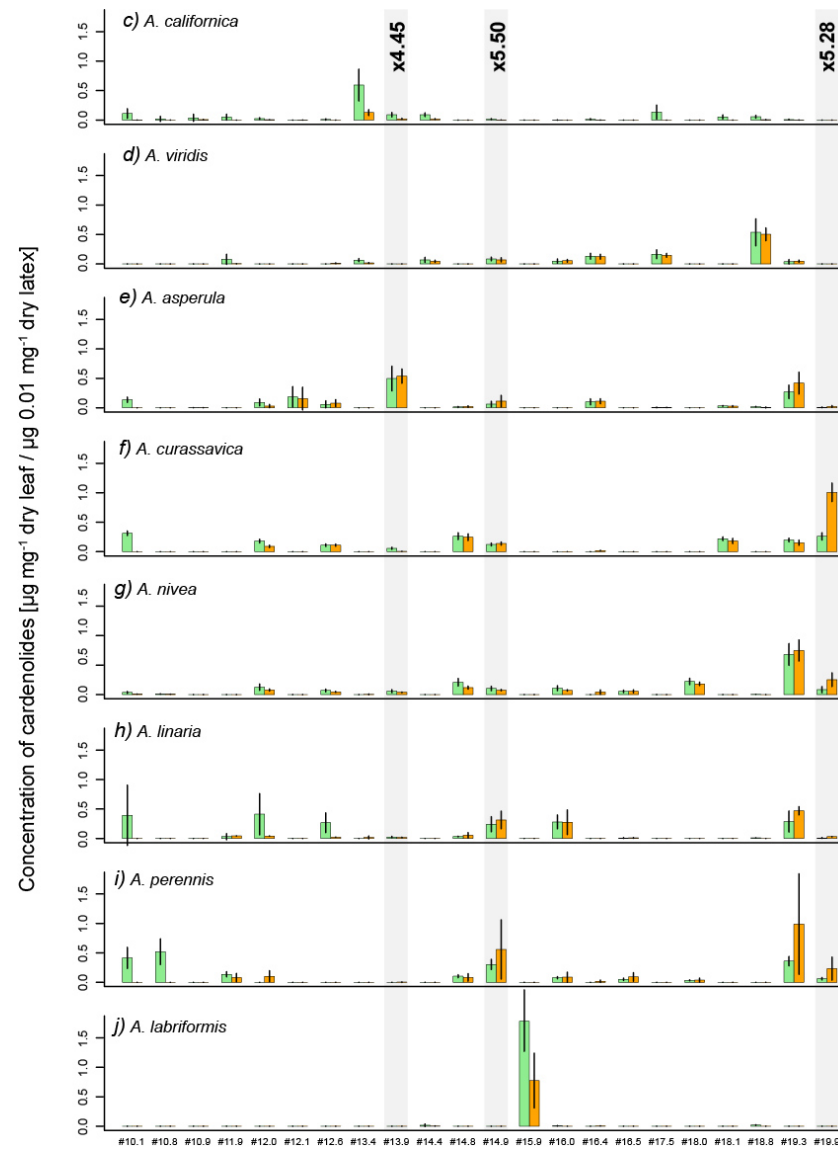
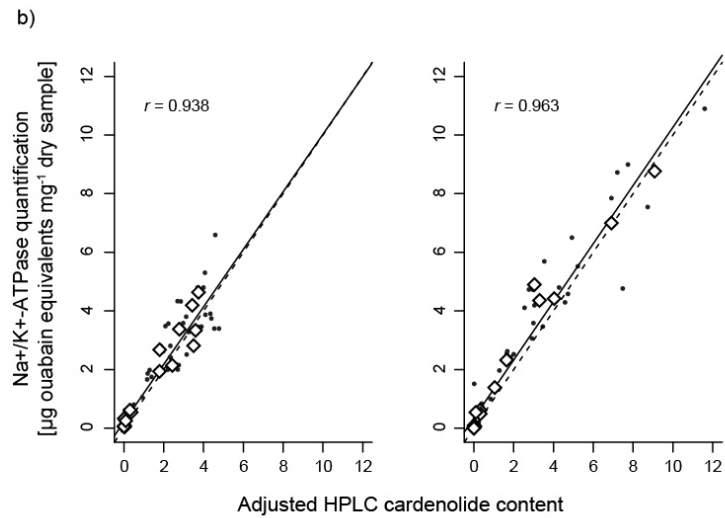
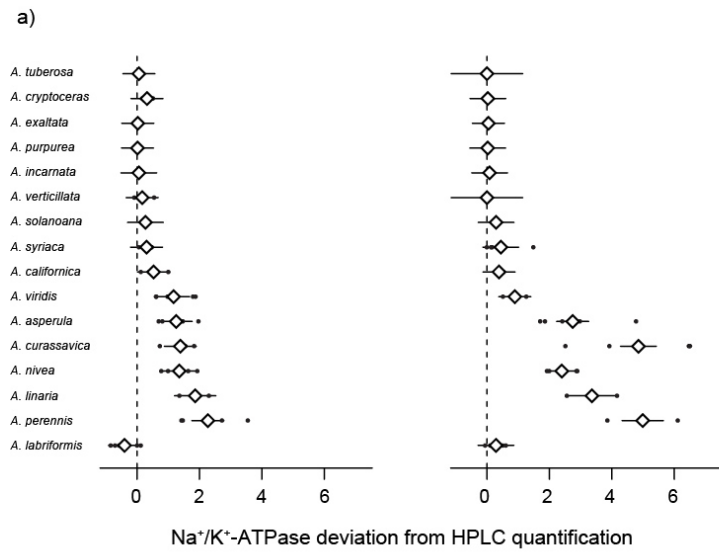
**Figure 1.** Phylogeny of *Asclepias* species based on three non-coding loci from their plastid genomes (Fishbein et al. 2011). This tree is the all-compatible consensus of phylogenies sampled in a Bayesian analysis of a comprehensive sample including 155 taxa, pruned to the 16 species studied here. Branch lengths are proportional to the number of hypothesized speciation events between nodes (shown after the species name); branch lengths are not equivalent because multiple speciation events in the complete phylogeny may occur on a single branch of this pruned phylogeny. The three circles after each tip represent the three highly potent cardenolides: left to right: calotropin (orange), calactin (green), and voruscharin (blue), respectively. Filled circles indicate concentrations significantly different from zero (at  $p < 0.05$ ).

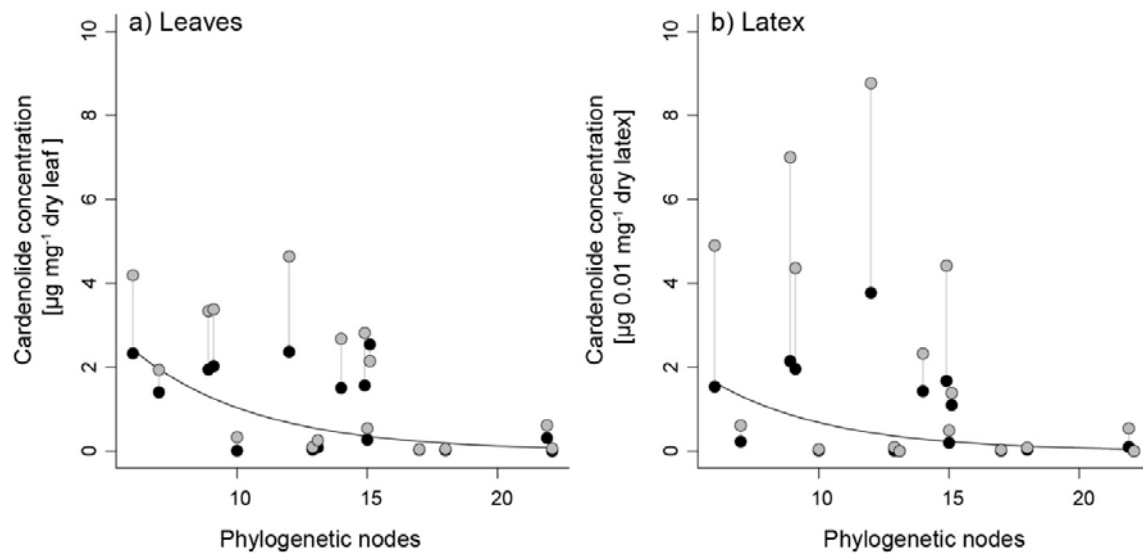


**Figure 2.** Comparison of cardenolide quantification methods for a) leaves and b) latex. Grey points are individual samples, with grey lines linking them to their respective species average (diamonds). Dashed lines represent the 1:1 ratio of equal cardenolide estimation, while solid lines are regression lines on the species means. All concentrations are expressed on a dry mass basis. Na<sup>+</sup>/K<sup>+</sup>-ATPase quantification corresponds to the concentration of the reference cardenolide compound ouabain required to achieve an equivalent enzymatic inhibition as by the leaf or latex extract.



**Figure 3 (below).** a) Species deviations of Na<sup>+</sup>/K<sup>+</sup>-ATPase quantification from HPLC quantification for leaves (left panel) and latex (right panel). Species are listed in order of increasing leaf cardenolide content as determined by HPLC. The deviation is zero (dashed line) when plants accumulate cardenolides with equal inhibitory potency as the reference cardenolide ouabain, while positive deviations indicate the accumulation of more potent cardenolide compounds. Black dots are individual plant samples, diamonds are species means, and horizontal lines are the 95% confidence interval. b) Deviations in Na<sup>+</sup>/K<sup>+</sup>-ATPase quantification are primarily caused by three compounds which are exceptionally strong inhibitors of porcine Na<sup>+</sup>/K<sup>+</sup>-ATPase. If the HPLC-determined concentrations of these three compounds are adjusted by compound-specific multipliers, almost all deviation of Na<sup>+</sup>/K<sup>+</sup>-ATPase quantifications from HPLC predictions can be explained for both leaves (left panel) and latex (right panel). c-j) Concentrations of cardenolide compounds for leaves (green bars) and latex (orange bars) of the eight species with intermediate to high cardenolide content. Only compounds that constitute at least 10% of the total in any species and sample type are shown for simplicity. The three positively weighted compounds are highlighted in light grey and the compound-specific multiplication factor is given on top. Note that for comparability across sample types, concentrations of latex are presented per 0.01 mg dry weight. Cardenolide compounds are named and ordered according to their HPLC retention time. For reference, digitoxin elutes at 19.6 minutes in this HPLC method, and ouabain elutes at 4.7 minutes. Compounds #13.9, #14.9, and #19.9 are calotropin, calactin, and voruscharin, respectively.





**Figure 4.** Change in cardenolide concentration in relation to distance from the hypothesized common ancestor of *Asclepias* (expressed as the number of branching points on the phylogeny) for leaves and latex. Black points are species means of cardenolide concentrations quantified by HPLC, and grey filled points are concentrations quantified by  $\text{Na}^+/\text{K}^+$ -ATPase inhibition. Species with the same number of phylogenetic nodes are shifted sideways to minimize overlap. Grey vertical lines connect mean trait values belonging to the same species, and solid black lines are the back-transformed linear regressions of log-transformed concentrations averaged for the two quantification methods. Note that latex has 100-fold more concentrated cardenolides than leaves.

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