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What doesn't kill you makes you stronger: The burdens and benefits of toxin sequestration in a milkweed aphid

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Abstract

1. Specialized insect herbivores commonly co-opt plant defences for protection against predators, but the costs, benefits and mechanisms of sequestration are poorly understood.

2. We quantified sequestration of toxic cardenolides by the specialist aphid *Aphis nerii* when reared on four closely related milkweed (*Asclepias*) species with >20-fold variation in cardenolide content, and in the presence or absence of generalist ladybug predators.

3. Increasing concentrations of apolar plant cardenolides increased sequestered amounts in aphids. High concentrations in plants impaired aphid population growth, but also reduced the top-down effects of predators. An in vitro enzymatic assay of total cardenolides in aphid bodies using the cardenolides' target (animal Na^+/K^+-ATPase) revealed that the subset of sequestered cardenolides is disproportionally more toxic than cardenolides in leaves.

4. All aphids accumulated two cardenolides not present in their host plant, even on plants with very low foliar cardenolide concentrations. Sequestration of potent cardenolides by *A. nerii* thus involves passive, concentration-dependent uptake from the host plant, as well as a presumably more active mechanism of modification and up-concentration of plant cardenolides.

5. The concentration of toxins in the host plant thus not only determines the negative impacts on growth and performance of an aphid, but also the ease and efficiency by which toxins are sequestered for the aphid's defence, making the costs and benefits of plant toxins highly context-dependent for both the plant and the herbivore. Therefore, variation in plant toxins is of central importance for co-evolutionary plant–insect interactions, particularly in environments with variable predator pressure.

KEYWORDS
Aphis nerii, *Asclepias*, cardenolides, multitrophic interactions, population growth, predator–prey, toxin tolerance

1 | INTRODUCTION

Plant secondary chemicals often provide an effective defence against generalist herbivores, but more specialized insects have evolved strategies to cope with these chemicals and may sequester them for their own defence (Ali & Agrawal, 2012; Zvereva & Kozlov, 2016). The benefit of gaining "free" protection by co-opting of plant defences has led to the evolution of a diversity of sequestration strategies (Opitz & Müller, 2009). Despite this apparent success as an evolutionary strategy, sequestration of plant chemicals often has

costs as well as benefits, and herbivores may be faced by a "dilemma of conflicting demands" (Björkman & Larsson, 1991). For example, pine sawfly larvae sequester defences after consuming resin acids from their host plant, and predation threat can increase feeding rate on high-resin plants, but the increased exposure to resin acids reduces larval growth rate (Björkman & Larsson, 1991). Similar to that, caterpillars of the buckeye butterfly, which sequester iridoid glycosides from their host plant, were consumed less often by predators than caterpillars reared on toxin-free artificial diet (Camara, 1997b), but high levels of some iridoid glycosides reduced growth rate, immune function and survival of caterpillars in the absence of predators (Adler, Schmitt, & Bowers, 1995; Camara, 1997a; Smilanich, Dyer, Chambers, & Bowers, 2009). While these well-studied systems clearly demonstrate both benefits and costs of sequestration, we frequently lack a mechanistic understanding of the metabolic and physiological processes involved in acquiring and accumulating these compounds.

To minimize costs of plant toxin accumulation in their bodies, most sequestering herbivores have specific mechanisms to tolerate toxins or to control their uptake and storage (Erb & Robert, 2016; Petschenka & Agrawal, 2016). As a key tolerance strategy, the primary molecular targets of plant toxins may mutate to a less sensitive form (e.g., target-site insensitivity, Dobler, Petschenka, & Pankoke, 2011; Heckel, 2014). Furthermore, several plant toxins require enzymatic activation, either by plant enzymes upon tissue damage or by insect enzymes upon digestion, enabling specialized insects to sequester these toxins by prevention or reversal of this activation (Dobler et al., 2011; Petschenka & Agrawal, 2016). Lastly, detoxification of plant compounds may be used as a strategy by sequestering herbivores to prevent accumulation of specific toxins from becoming detrimental (Cogni, Trigo, & Futuyma, 2012; Heckel, 2014; Kumar, Pandit, Steppuhn, & Baldwin, 2014).

Cardenolides are a class of potent defensive compounds found in the Apocynaceae and a few other plant families, and which act as highly specific inhibitors of animal Na^+/K^+-ATPase (Agrawal, Petschenka, Bingham, Weber, & Rasmann, 2012). Several specialist herbivores, including the oleander aphid *Aphis nerii*, have independently evolved the ability to sequester cardenolides from their host plants (Dobler, Dalla, Wagschal, & Agrawal, 2012; Malcolm, 1990; Rothschild, von Euw, & Reichstein, 1970; Züst & Agrawal, 2016b), with plant-derived cardenolides providing clear defensive benefits against generalist vertebrate and invertebrate predators (Brower, Edmunds, & Moffitt, 1975; Desneux, Barta, Hoelmer, Hopper, & Heimpel, 2009). Among these milkweed herbivores, sequestration of cardenolides is often linked to the gain of target-site insensitivity in the otherwise highly conserved Na^+/K^+-ATPase enzyme (Dobler et al., 2012; Zhen, Aardema, Medina, Schumer, & Andolfatto, 2012); in fact, the defensive benefit from cardenolide sequestration may have been a key evolutionary driver of target-site insensitivity (Petschenka & Agrawal, 2015). As a prominent exception among cardenolide-sequestering herbivores, *A. nerii* lacks a resistant Na^+/K^+-ATPase (Zhen et al., 2012), and instead may primarily rely on cardenolide detoxification (Birnbaum & Abbot,

2018; Birnbaum, Rinker, Gerardo, & Abbot, 2017). Furthermore, while sequestration of cardenolides in most herbivores involves at least partially active uptake (Dobler et al., 2011), sequestration by *A. nerii* appears to be primarily passive (Züst & Agrawal, 2016b).

Given its unique sequestration strategy, *A. nerii* may be trapped in a dilemma of conflicting demands. Here, we examined the effects of plant cardenolide content on sequestration by *A. nerii* and quantified the burden and benefits of sequestration in terms of population growth and susceptibility to predation. We grew populations of *A. nerii* on four closely related milkweed species with >20-fold differences in their cardenolide content and added generalist adult ladybug predators to half of all aphid populations to impose a predation threat. We predicted (a) that sequestration of cardenolides would be highest on cardenolide-rich plants; and (b) that increasing host plant cardenolide content and sequestration would negatively impact aphid population growth; but (c) that predation would be reduced for aphids with higher levels of sequestered cardenolides.

2 | MATERIALS AND METHODS

The oleander aphid *A. nerii* is broadly specialized to feed on many plants of the Apocynaceae (Blackman & Eastop, 2006). To evaluate the importance of host plant cardenolide concentrations, we selected the closely related milkweed species *Asclepias incarnata incarnata* (henceforth *A. incarnata*), *A. incarnata pulchra* (henceforth *A. pulchra*), *A. curassavica* and *A. perennis* for their large interspecific constitutive differences in cardenolide content (Fishbein, Chuba, Ellison, Mason-Gamer, & Lynch, 2011; Rasmann & Agrawal, 2011a). For each species, we grew 25–30 replicate plants. Three of the species were grown from seed, while *A. perennis* was grown from cuttings purchased from Shady Oak Butterfly Farm (Brooker, FL, USA). For germination, seeds were bleached, scarified and cold-stratified at 4°C for a week, after which they were germinated at 28°C. Seedlings and cuttings were planted in Lambert mix (Lambert Peat Moss Inc., QC, Canada). Cuttings of *A. perennis* were planted 2 weeks earlier than seedlings, to be cut at soil level when seedlings were planted. All plants were randomly arranged in a growth chamber (16-hr daylight, 26°C day/22°C night) and received one dose of fertilizer (N:P:K 21:5:20, 150 ppm N [μg/g]) 1 week after planting/cutting. Plants were grown for a total of 7 weeks, during which all plants were cut back to soil level twice to maintain plants at a compact size and to reduce effects of different origins. After the final cutting, regrowing plants were fertilized again and grown for three additional weeks before the aphid experiment was initiated.

2.1 | Growth and predation experiment

We initiated the experiment by placing five adult aphids on each plant and enclosing the whole plant using perforated plastic bags. Aphids came from a laboratory colony maintained on common milkweed *Asclepias syriaca*. The number of aphids on each plant was recorded

five times, every 2–3 days for 14 days. After the third census on day 8, we randomly allocated half of all plants to a predator treatment, to which we added an individual adult ladybug (*Coccinella septempunctata*) for 3 days. Adult *C. septempunctata* beetles came from a long-term rearing using a diet of the potato aphid *Macrosiphum euphorbiae*. Aphid populations were counted again upon removal of *C. septempunctata* on day 11. We then affixed preweighed aluminium discs to the stem of each plant below the second-youngest pair of fully extended leaves to capture honeydew and other excretion products (aphid excretions hereafter) dropped by aphids above it. On day 14, these discs were collected, freeze-dried and later reweighed. All plants were cut at soil level, placed in bags and freeze-dried with all aphids still on the plants. We separated dried aphids from plant material, counted all individuals and sorted them into winged and unwinged individuals for each plant. Three freeze-dried leaves from the top of the plant that had no visible sign of aphid excretions were ground in preparation for chemical analysis.

2.2 | Estimation of population growth and aphid consumption

Aphid populations grew at a near-exponential rate during the first few days of the experiment, but growth slowed with increasing population density, and many of the fastest-growing populations exhausted their host plant by the end of the experiment. Population growth before collapse was therefore best described by a power law function (Enquist, West, Charnov, & Brown, 1999; Paine et al., 2012), which assumes that absolute growth rate is proportional to current population size raised to some power. In a power law model of the form

$$\frac{dN}{dt} = rN^{\beta}, \tag{1}$$

the population size N is a function of the growth coefficient r and the scaling coefficient β. Using the first four aphid counts from control plants and the first three counts from plants of the predator treatment (before predator introduction), we modelled aphid population growth using the closed-form solution of Equation 1 when $\beta < 1$ (Paine et al., 2012), given by

$$\log(N_t) = \log\left(\left(N_0^{(1-\beta)} + rt\,(1-\beta)\right)^{1/_{1-\beta}}\right). \tag{2}$$

The population size at time t is thus a function of the initial estimated population size N_0 and the coefficients r and β. Note that both sides of Equation 2 are log-transformed, which does not alter the overall shape of the function but reduces the relative weight of very large aphid counts on the model fit. As both counting errors and the effects of stochasticity increase with population size, fitting population growth on the log-scale results in a more conservative growth rate estimate. We fitted Equation 2 as a custom nonlinear mixed-effects model within the NLME package for the statistical software R (version 2.15.1). Plant identity was treated as a random effect to account for repeated

aphid population counts, and plant species was fitted as a fixed effect on all three growth coefficients. Using the best species-specific estimates of these coefficients, we could then calculate the absolute population growth rate (AGR, the number of aphids gained per day) for any time point of the experiment (Paine et al., 2012) using

$$AGR = r\left(N_0^{(1-\beta)} + rt\,(1-\beta)\right)^{\beta/_{1-\beta}}. \tag{3}$$

To estimate a measure of uncertainty for AGR (a function of the three model parameters), we generated population prediction intervals (Bolker, 2008). This method assumes that the distribution of the model parameters is multivariate normal with a variance–covariance matrix given by the inverse of the information matrix. We used the function *mvrnom* in the MASS package for R, which selects multivariate normal random deviates, and extracted the variance–covariance matrix from the growth model using the function *vcov*. We generated 1,000 sets of parameters to calculate a distribution of AGRs for each species. The lower and upper 68% quantiles of these distributions are the boundaries of an approximate standard error.

While we did not quantify the number of aphids consumed by predators directly, we estimated daily aphid consumption from the difference between the actual population size after predation and a predicted population in the absence of predators. We extended Equation 1 to include a constant daily removal of aphids, giving

$$\frac{dN}{dt} = rN^{\beta} - k. \tag{4}$$

Daily consumption k was estimated using *lsoda*, a solver for ordinary differential equations in the DESOLVE package for R. For each plant, random deviates for r and β were extracted from the population growth model to generating plant-specific aphid growth parameters in the absence of predation, and the population size at time of predator introduction was calculated from these parameters. All other parameters being thus fixed, daily consumption k was estimated for each plant by minimizing the squared difference between the actual and the predicted aphid number after 3 days of predation. All plant-specific values of k were then analysed for effects of predation treatment and plant species using a generalized least squares model (function *gls* in R) in which a *varIdent* variance structure was included to account for large differences in variances among plant species (Zuur, Ieno, Walker, Saveliev, & Smith, 2009).

2.3 | Cardenolide extraction and quantification

We extracted cardenolides from a subset of 10 samples per species (five from predator treatments and five from controls) for ground-up leaf material (50 mg), unwinged aphid bodies (20 mg, approximately 300–400 aphids) and aphid excretions (10 mg dry weight). Note that aphids feed on phloem, which may have a different cardenolide composition than leaves. However, we previously demonstrated that aphids either accumulate or excrete cardenolides from the full

range of foliar cardenolides (Züst & Agrawal, 2016b), making leaves a convenient approximate measure. For all three sample types, cardenolides were quantified by HPLC, and for plant and aphid samples, cardenolides were additionally quantified by an in vitro enzymatic assay utilizing the compound's specific biological activity on animal Na^+/K^+-ATPase (Petschenka et al., 2013). Plant and aphid samples were extracted using approximately 20 FastPrep beads (MP Biomedicals, CA, USA) and two washes of 1 ml 100% methanol. For each wash, samples were agitated on a FastPrep-24 homogenizer for 45 s at 6.5 m/s, followed by centrifugation at 20,800 g for 12 min. Supernatants of the two extraction steps were pooled, split 1:9 for the two quantification methods and dried down separately in a vacuum concentrator. Before drying, the larger fraction of each sample was spiked with 20 µg of digitoxin (Sigma-Aldrich, MO, USA) as an internal standard for HPLC analysis.

Dried plant extracts for HPLC were resuspended in 0.5 ml 100% methanol and filtered through a column of DEAE-Sephadex (Sigma-Aldrich) to eliminate phenolic compounds that could otherwise mask cardenolide peaks (Züst, Rasmann, & Agrawal, 2015). An additional 0.5 ml of 100% methanol was used to elute the samples from the Sephadex column. The filtered samples were dried and resuspended with 200 µl of 100% methanol and filtered through a 0.22-µm PTFE filter (Durapore®; EMD Millipore, MA, USA). Dried aphid extracts for HPCL were dissolved in 2 ml water with the help of a sonicator. Fats and oils were removed by washing the water phase with 3 × 1 ml petroleum ether. Cardenolides were then extracted from the water phase with three washes of 1 ml chloroform. Chloroform fractions were pooled and evaporated. The residue was resuspended with 200 µl of 100% methanol and filtered through a 0.22-µm PTFE filter. The absence of any cardenolides in the petroleum ether washes and the water phase were confirmed by HPLC.

Dried plant and aphid extracts for the Na^+/K^+-ATPase assay were dissolved in 10 µl of dimethylsulphoxide (DMSO) and diluted to 10% DMSO by the addition of 90 µl water. This extract was then diluted 1:10 and 1:100 using 10% DMSO to generate a concentration series for each sample. We quantified the biological activity of cardenolides in each dilution series using purified Na^+/K^+-ATPase from the porcine cerebral cortex (Sigma-Aldrich). Reactions were performed in 96-well microplates on a BioShake Iq microplate shaker (Quantifoil Instruments, Jena, Germany) set to 37°C. Each reaction was carried out in a total volume of 100 µl and consisted of 20 µl sample (1, 0.1 or 0.01 strength), 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 50 mM imidazole, 0.0015 units Na^+/K^+-ATPase and 25 µl ATP (ditrissalt, pH adjusted to 6.5 with Tris, 2.5 mM final well concentration) added as the final step to initiate the reaction. After 20 min, reactions were stopped by the addition of 100 µl of 10% SDS/0.05% Antifoam A. Inorganic phosphate released from enzymatically hydrolysed ATP was photometrically quantified at 700 nm following the method of Taussky and Shorr (1953). To account for coloration of plant extracts, we included a background well for each reaction well with identical content but lacking KCl. On each microplate, we also included wells with an uninhibited control, a completely inhibited reaction by 10^{-2} M ouabain, and a calibration curve made with ouabain ranging

from 10^{-3} to 10^{-8} M. Absorbance values of reaction wells were corrected by their respective backgrounds and calculated as per cent residual activity using reference points of the uninhibited reaction as 100% and the completely inhibited reaction as 0% residual activity. Based on the residual enzymatic activity inhibited by an extract at three concentrations, 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 (function gnls in the NLME package for R). For each extract, we calculated the relative dilution at the inflection point (i.e., residual enzymatic activity of 50%) and estimated the cardenolide concentration of the undiluted plant or aphid sample in ouabain equivalents based on the calibration curve.

Aphid excretions were solubilized by placing the dried aluminium discs in glass test tubes and adding 1 ml of 10% methanol in water. After extraction, the aluminium discs were dried and reweighed to estimate the efficiency of extraction. After adjusting weights of aphid excretions by extraction efficiency, we calculated the volume of solvent containing 10 mg of dry aphid excretions and transferred this volume into a new vial. These extracts were then spiked with 20 µg of digitoxin, freeze-dried, resuspended with 200 µl of 100% methanol and filtered through a 0.22-µm PTFE filter.

Ten microlitres of each sample prepared for HPLC was analysed with an Acclaim® 120 C18 4.6 × 250 mm column with 5 µm particle size (Thermo Fisher Scientific, CA, USA). Injections were eluted at a constant flow of 1.2 ml/min with a gradient of acetonitrile and water as follows: 0–1 min at 20% acetonitrile, 1–31 min from 20% to 30%, 31–47 min from 30% to 50%, 47–49 min from 50% to 95% and 49–54 min at 95%, followed by 6 min reconditioning at 20%. We identified cardenolides by their characteristic single absorption maximum between 214 and 222 nm, and quantified them at 218 nm. A compound was considered the same among different samples if retention time differed by less than 0.05 min. Concentrations of cardenolide compounds were calculated by relating peak areas to the area of the internal digitoxin standard.

3 | RESULTS

3.1 | Aphid growth and predation

We quantified population growth rate over the first four census points as a measure of aphid performance. Aphid population growth was best captured by a power law model that deviated from exponential growth (Figure 1). Aphid growth was strongly affected by differences in host plant, resulting in significant effects of plant species on both the initial aphid density N_0 ($F_{3,249}$ = 269.11, $p < 0.001$) and the rate parameter r ($F_{3,249}$ = 55.64, $p < 0.001$). In contrast, plant species effects on the scaling coefficient β were marginal ($F_{3,246}$ = 2.43, $p = 0.066$), and thus, we assumed a shared β for growth on all plant species in the final model. Even though aphid populations were initiated from five adult aphids on all plants, the estimated initial aphid density N_0 ranged from 1.91 ± 0.34 on *A. pulchra* (mean ± 1 *SE*) to 4.87 ± 0.52 on *A. curassavica*, indicating
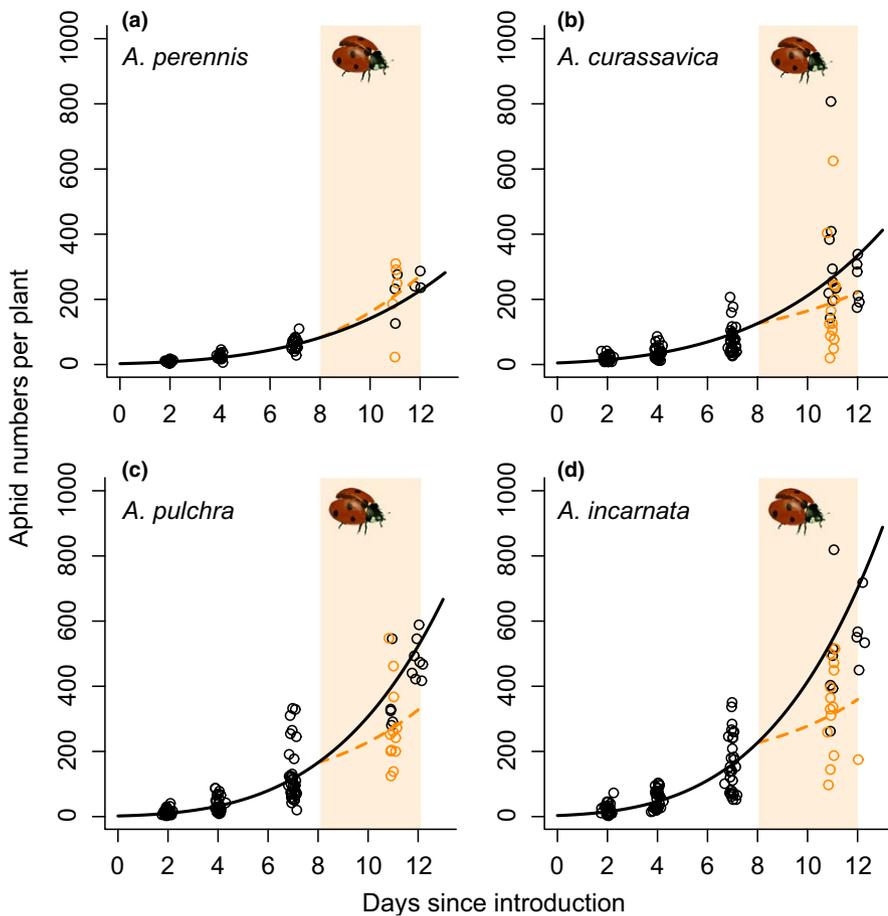
FIGURE 1 Average aphid population growth on four milkweed species. Black circles are individual populations in the control treatment and the predation treatment prior to introduction of ladybugs, and solid lines are the species-specific power law model fits. Shaded bars indicate duration of ladybug presence, and orange circles are the aphid population counts after 4 days of ladybug feeding. Orange dashed lines represent the population growth progression with an estimated species-specific daily aphid consumption k

an effect of host plant on aphid establishment. Nonetheless, differences in population growth were predominantly driven by plant effects on rate r, resulting in threefold differences in the absolute population growth rate (AGR, new aphids per day) by day seven: *A. perennis* = 19.02 ± 2.78, *A. curassavica* = 28.13 ± 2.48, *A. pulchra* = 42.65 ± 3.23, *A. incarnata* = 57.36 ± 4.34.

On day eight, adult ladybugs predators were introduced to half of all aphid populations to quantify a potential defensive benefit of cardenolide sequestration. While we did not directly monitor aphid consumption by ladybugs, we extrapolated the plant-specific aphid population growth prior to predator introduction, and inferred daily consumption from the difference between the predicted and actual aphid population densities after 3 days of predation. By estimating the same daily consumption for control plants (no predation), we could evaluate the sensitivity of this method to other potential factors influencing predicted growth trajectories and found that the estimated mean daily aphid consumption did not differ from zero for any controls (Figure 2). In contrast, for populations exposed to predators, estimated daily aphid consumption was positive for three plant species and significantly so for *A. pulchra* and *A. incarnata* (Figure 2), with predators removing close to half the aphids gained per day. However, a model testing the effects of predator treatment and plant species on daily aphid consumption k only revealed a marginal interaction term ($F_{3,81}$ = 2.21, p = 0.093), presumably due to the high variation in plant-specific values of k.

3.2 | Cardenolide content

We distinguished a total of 39 cardenolide compounds across the four milkweed species and the three sample types (leaf, aphid body and aphid excretions, Figure S1). Among the milkweed species, *A. curassavica* had the highest total amount of cardenolides, followed by *A. perennis*, *A. pulchra* and finally *A. incarnata* (Figure 3a). The amounts of foliar cardenolides were inversely related to aphid population growth, even though the slowest-growing aphid populations were on *A. perennis*, which had the second-highest cardenolide levels (Figure 3b). The total amount of cardenolides in aphid bodies and aphid excretions largely matched foliar cardenolides, with aphids on *A. curassavica* also accumulating and excreting the highest amounts (Figure 3c,d).

Using HPLC retention time as an approximate measure for compound polarity, aphids accumulated mostly apolar cardenolide compounds, while excreted cardenolides were mostly more polar (Figure S1). For aphids feeding on *A. curassavica* and *A. perennis*, several abundant apolar cardenolide peaks in aphid bodies corresponded to apolar cardenolide peaks in leaf tissue (Figure S1). In addition, all aphids contained considerable amounts of two cardenolide compounds, eluting at 36.2 and 40.6 min, that had no corresponding compounds in plant samples (Figure S1). In fact, these two compounds constituted the main cardenolides present in aphids
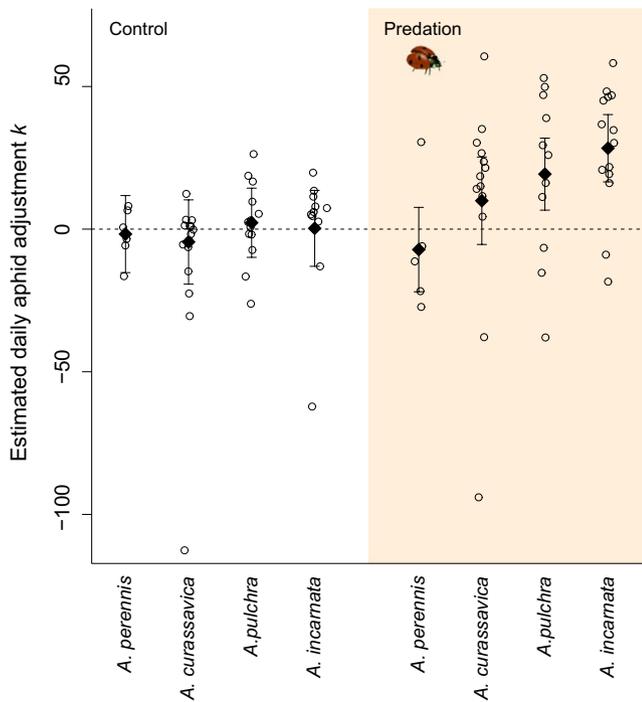
FIGURE 2 Estimated daily aphid adjustment k required to reach the final population size, assuming continued constant population growth between census 3 and census 4. For controls (left), no aphids were consumed; thus, estimates of k should be zero if population growth followed predicted trajectories. For populations with ladybug predators (right), k is the number of the daily removed aphids for the predicted population size to match actual population size after 3 days of predator feeding. Each circle represents a plant-specific estimate based on the deviation from predicted population growth trajectories in the absence of predators (see also Figure 1). Filled diamonds represent the species means, with error bars denoting 95% confidence intervals

feeding on the low-cardenolide plants *A. pulchra* and *A. incarnata*, resulting in a much higher ratio of sequestered vs. plant cardenolide concentrations (per unit dry weight aphid/plant) for these host species (*A. incarnata*: 2.02, *A. pulchra*: 0.49, *A. perennis*: 0.16, *A. curassavica*: 0.21). This suggests that these compounds are metabolic conversion products of plant compounds that may be preferentially accumulated or retained by the aphid. As further evidence of metabolic conversion of cardenolides, aphid excretions also contained several polar compounds not present in the host plant or aphid bodies (Figure S1).

Given the potential for at least partially active manipulation of cardenolides by the aphid, we compared the cardenolide content of aphid bodies and aphid excretions in the presence and absence of predators. Plant species clearly determined cardenolide content of aphid bodies ($F_{3,30} = 10.25$, $p < 0.001$) and aphid excretions ($F_{3,47} = 48.73$, $p < 0.001$). The predation treatment had no effect on the amount of cardenolides in aphid bodies (Figure 3c, $F_{1,30} = 0.19$, $p = 0.667$), and there was no evidence of an interaction with plant species ($F_{3,30} = 0.53$, $p = 0.663$). In contrast, the cardenolide content of aphid excretions was marginally higher in the presence of

predators (Figure 3d, $F_{1,47} = 3.37$, $p = 0.073$), and this effect was independent of plant species ($F_{3,47} = 1.65$, $p = 0.190$).

Quantification of cardenolides by HPLC relies on characteristic absorption of these compounds at 218 nm, but compounds could potentially be misidentified, or matrix effects could have masked cardenolides if other substances co-eluted at the same time. We therefore cross-validated our HPLC quantification of leaves and aphid bodies (the sample types with large matrix effects) using an enzymatic activity assay that quantifies the total amount of cardenolides in an extract from the highly cardenolide-specific inhibition of animal Na$^+$/K$^+$-ATPase (measured in equivalents of the standard cardenolide ouabain). We found that the two methods of quantification produced highly correlated results for both sample types (Figure 4, leaves: Pearson's $r = 0.97$, $p < 0.001$, aphid bodies: $r = 0.60$, $p < 0.001$). For both sample types, quantification by Na$^+$/K$^+$-ATPase resulted in higher total amounts than quantification by HPLC, presumably because milkweed cardenolides are more toxic than the standard cardenolide ouabain. This effect was much stronger for aphid samples than for plant samples (Figure 4), which suggests that the (mostly apolar) cardenolides that accumulate in aphid bodies are particularly efficient inhibitors of animal Na$^+$/K$^+$-ATPase.

4 | DISCUSSION

Population growth of *A. nerii* varied up to threefold among the four closely related milkweed host species, with increasing foliar concentrations of cardenolides reducing aphid growth. Aphids on all host plants accumulated two apolar cardenolide compounds not detectable in plants, likely through metabolic conversion of plant cardenolides and preferential accumulation or retention of these products. In addition, aphids on high-cardenolide host plants sequestered several apolar cardenolides from their host plant by a (likely passive) concentration-dependent mechanism. Concentration-dependent passive sequestration of plant toxins (e.g., cardenolides, alkaloids) appears to be common among aphids (Züst & Agrawal, 2016a); thus, many aphids may face a dilemma in choosing their host plant: high-toxin host plants will provide the best defence for the aphids, but the resulting hightoxin load may impair their performance.

Accumulation of high levels of cardenolides was sufficient to reduce predation by a generalist ladybug under no-choice conditions. Under more natural conditions, effects would likely be amplified by predator choice. Indeed, on cardenolide-rich host plants, *A. nerii* is rarely eaten by ladybug predators (Iperti, 1965; Omkar, 2005), with the exception of the putatively cardenolide-adapted ladybug *Hippodamia variegata*, which is frequently seen attacking *A. nerii* (Iperti, 1965; Pasteels, 2007; T. Züst, personal observation). Therefore, sequestration of even quite low levels cardenolides on *A. incarnata* and *A. pulchra* may provide considerable defensive benefits against generalist predators. In contrast, even high levels of sequestered cardenolides may be ineffective against specialized predators or parasitoids (Helms, Connelly, & Hunter, 2004; Pasteels, 2007).
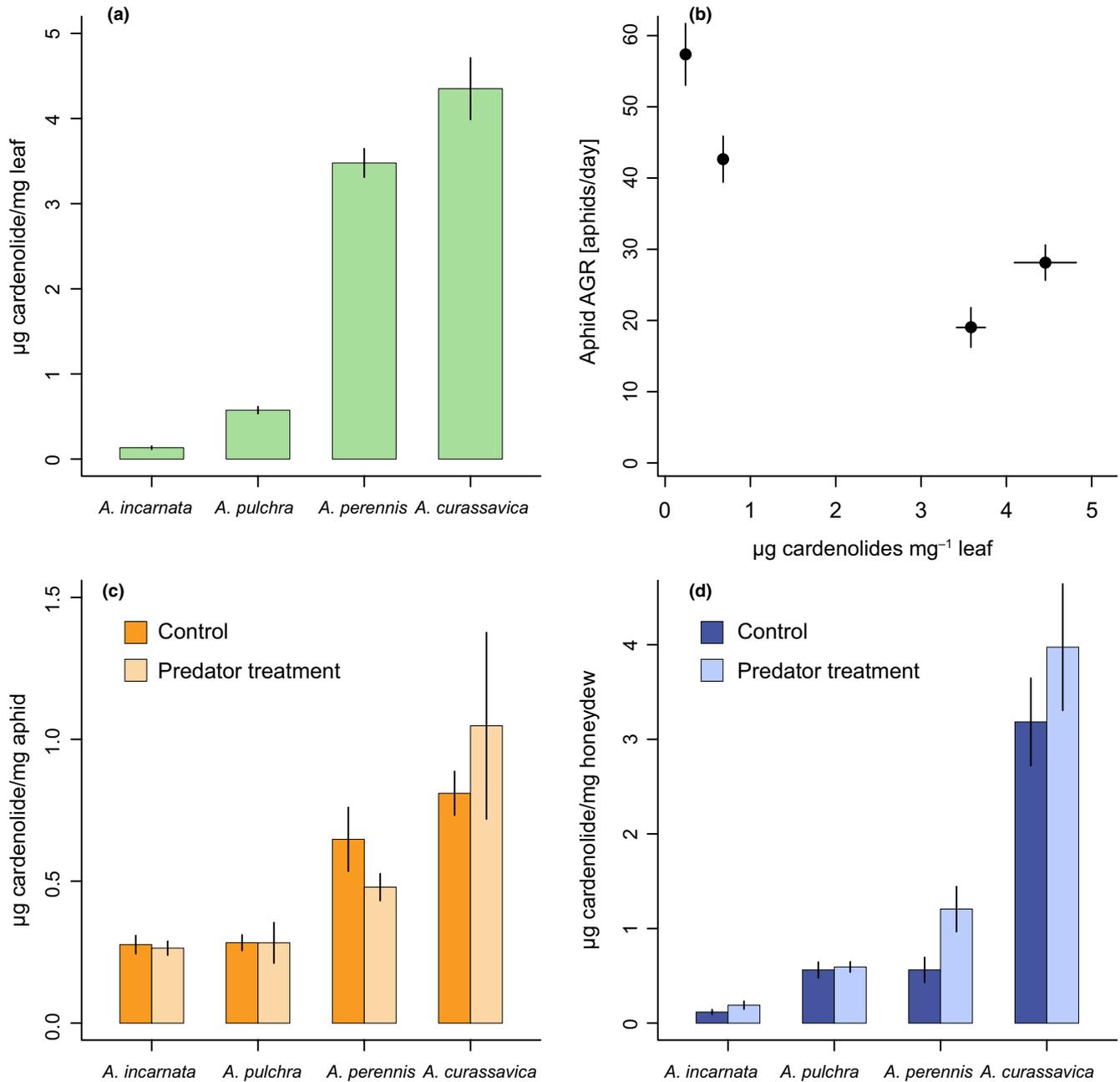
FIGURE 3 Cardenolides in milkweed leaves and aphids and their interaction with aphid performance. (a) Mean cardenolide content of leaves, and (b) negative association between mean absolute growth rate of aphid populations (AGR) at day 7 and leaf cardenolide content. (c) Cardenolide content of aphid bodies and (d) aphid honeydew and other excretion products. Error bars show ± 1 *SE*

Negative effects of cardenolides on the performance of *A. nerii* have not been consistently found in the past (Malcolm, 1992; Martel & Malcolm, 2004; Züst & Agrawal, 2016b), but the emerging consensus is that high levels of cardenolides in the host plant impair this aphid's performance (Agrawal, 2004; Birnbaum et al., 2017), either as a consequence of direct toxicity or due to the costs of detoxification (Birnbaum et al., 2017). Overall, our results from four milkweed species with large differences in their cardenolide concentrations match this consensus; the exception was *A. perennis*, which—despite having lower foliar cardenolide concentrations than *A. curassavica* in our study—supported the

lowest aphid population growth. However, cardenolides are only one of several factors determining aphid performance, and plant quality effects are often found to be of high importance (Zehnder & Hunter, 2008; Züst & Agrawal, 2016b). The *A. perennis* plants used here originated from stem cuttings, and in preparation for the experiment received one more cutting-back than the seed-grown species. Although we intended this to reduce effects of the different plant origins, the repeated cutting may have depleted the plant's resources, as indicated by the occurrence of a few *A. perennis* plants with growth defects (removed from the experiment). Hence, while aphids on *A. curassavica* experienced the highest
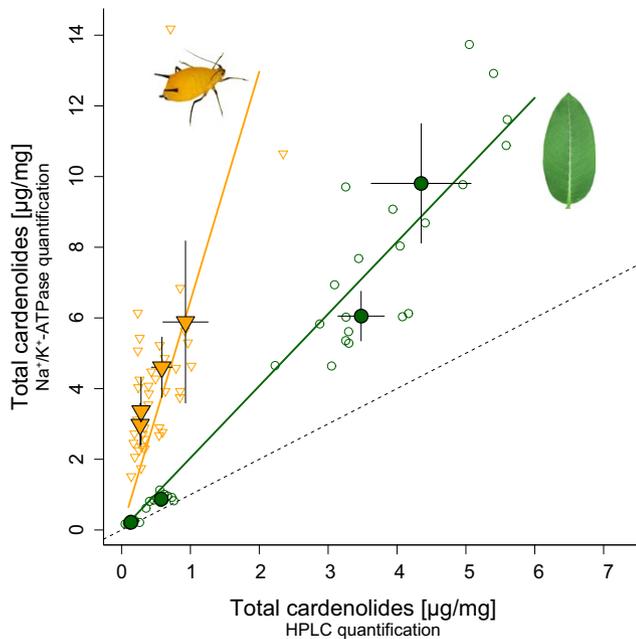
FIGURE 4 Aphids sequester a more toxic subset of cardenolides than present in plants. Shown is the relationship between two methods of cardenolide quantification of milkweed leaves (green circles) and aphid bodies (orange triangles). For HPLC, cardenolides are quantified from a compound-specific light absorption signal, which increases linearly with increasing concentrations. In contrast, the Na^+/K^+-ATPase assay quantifies cardenolides in units of a reference compound (ouabain); thus, more toxic cardenolides result in a higher quantification. Empty symbols are individual plant or aphid samples, and filled symbols are the species means ± 95% confidence intervals. Solid lines represent fits of linear regressions, while the dotted line represents a 1:1 relationship (equivalent quantification by both methods)

honeydew using thin-layer chromatography. There, cardenolide profiles were more similar between the three tissue types than in our study, but the relative abundance of apolar and polar cardenolides in aphid bodies and honeydew broadly matches our findings. In addition, Malcolm (1990) reported significant shifts in the chromatographic retention of cardenolides in aphid bodies relative to plant tissue, which may also be an indication of metabolic modification of these compounds. Surprisingly, we found no evidence for the accumulation of novel cardenolides by *A. nerii* on *A. syriaca* in our previous work (Züst & Agrawal, 2016b). However, it is important to note that the four species used here are closely related, whereas *A. syriaca* is a more distant relative (Fishbein et al., 2011). Therefore, *A. syriaca* differs not only in its cardenolide profile, but also in the chemical structures of its primary cardenolides (Araya, Kindscher, & Timmermann, 2012; Zhang et al., 2014). It is thus feasible that potential mechanisms for cardenolide modification in *A. nerii* have specifically evolved for compounds in *A. curassavica* and its relatives.

Structural elucidation will be required to identify the types of modifications involved, and further quantification and characterization of cardenolides in different aphid tissue types (e.g., head vs. gut vs. haemocoel) are required to fully elucidate cardenolide sequestration by *A. nerii*. There are however surprising parallels between this aphid and other sequestration systems; for example, monarch caterpillars match the concentration of their host plant at intermediate levels, but concentrate the toxins from cardenolide-poor hosts (Agrawal, Ali, Rasmann, & Fishbein, 2015), and cabbage aphids preferentially sequester specific glucosinolate compounds from a range of structurally similar compounds in their host plant (Goodey, Florance, Smirnoff, & Hodgson, 2015). Therefore, many sequestering herbivores with variable predator pressures may face the same dilemma of conflicting demands when choosing their host plant.

Although *A. nerii* was the first aphid species for which sequestration of host plant toxins was reported (Rothschild et al., 1970), a deeper understanding of the mechanisms of sequestration used by this specialist herbivore has been lacking. Our study reveals a nuanced story involving two mechanisms of sequestration where high concentrations of toxins in the host plant impair aphid population growth, but also determine the ease and efficiency by which toxins are sequestered, and thus the magnitude by which predation pressure is reduced. Therefore, variation in plant toxins is of central importance for co-evolutionary plant–insect interactions, and in choosing their host plant, sequestering herbivores must navigate a fine line between self-intoxication and protection from predators.

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cardenolide burden, the otherwise more vigorous plant quality may have counterbalanced some of these negative effects.

Both mechanisms of sequestration employed by *A. nerii* resulted in the accumulation of mostly apolar cardenolides, which on average had a higher toxicity than the full set of plant cardenolides. This confirms a proposed link between polarity and toxicity of cardenolides (Rasmann & Agrawal, 2011b), yet it is unclear whether *A. nerii* sequesters apolar cardenolides to maximize its defences or whether this pattern is simply the consequence of physical properties of the compounds. At least for concentration-dependent sequestration, uptake likely depends on passive diffusion of apolar, lipophilic compounds across the aphid's gut membrane, whereas polar, hydrophilic compounds may never enter the aphid's body cavity or can be easily excreted by the aphid's catabolism. In support of passive uptake of apolar cardenolides, four aphid species—including *A. nerii* and ranging from a generalist to a highly specialized species—all accumulated the same subset of apolar cardenolides from the host plant *A. syriaca*, while polar cardenolides were excreted (Züst & Agrawal, 2016b).

In a similar study, Malcolm (1990) characterized the cardenolide profiles of *A. curassavica* plants, *A. nerii* bodies and the aphid's

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

T.Z., S.M. and A.A.A. conceived the study and designed the methodology; T.Z. and S.M. collected the data and T.Z. analysed the data; and T.Z. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data and R-code available from the Dryad Digital Repository https://doi.org/10.5061/dryad.3512gh3 (Züst, Mou, & Agrawal, 2018).

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SUPPORTING INFORMATION

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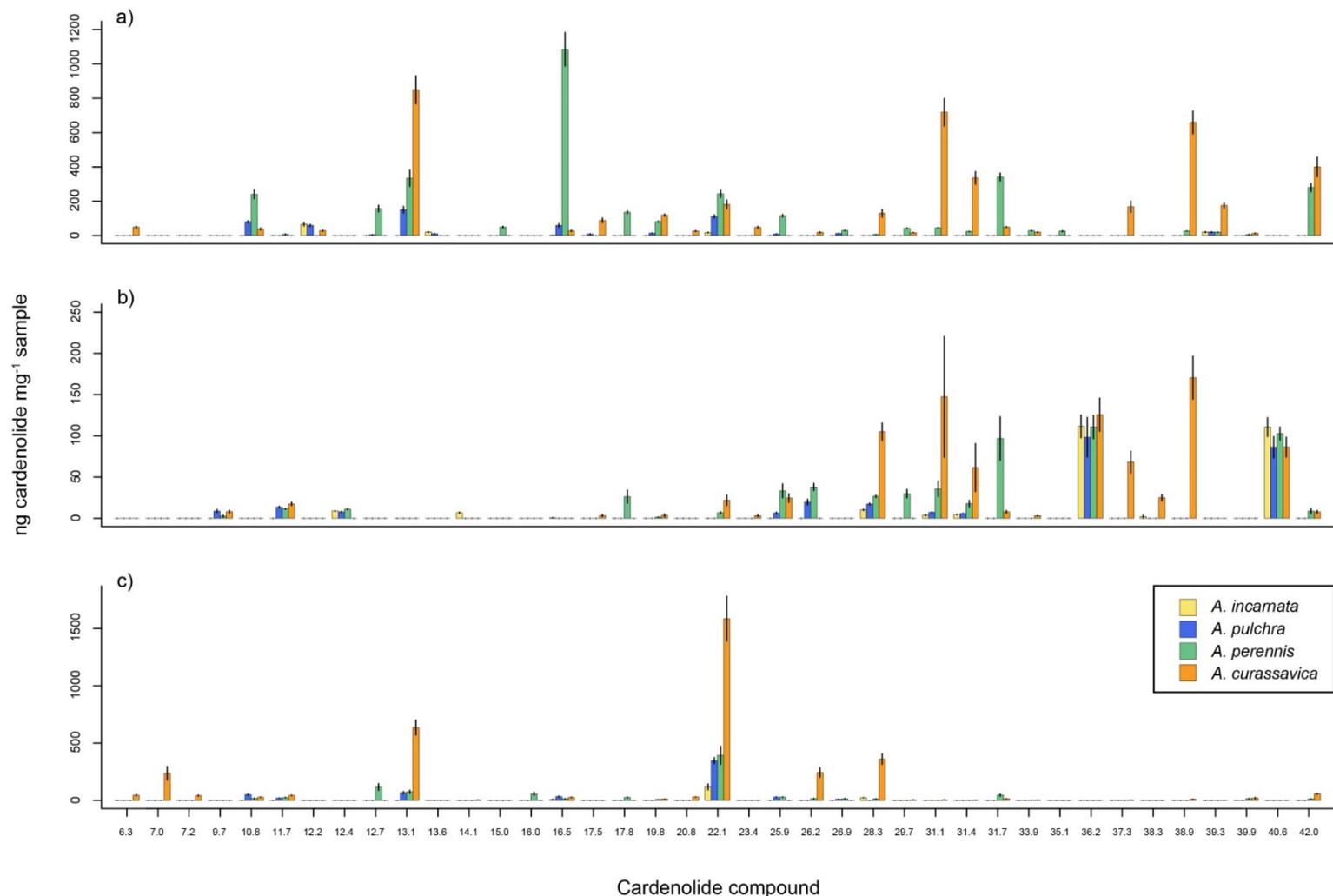
Fig. S1. Concentration of 39 cardenolides in a) plant leaves, b) aphid bodies) and c) honeydew and other excretion products. Each bar represents the mean concentration within species ± 1 SE. Numbers on the x-axis correspond to HPLC retention time, sorting compounds from polar (left) to apolar (right).