

Population growth and sequestration of plant toxins along a gradient of specialization in four aphid species on the common milkweed *Asclepias syriaca*

Tobias Züst* and Anurag A. Agrawal

Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA

Summary

1. Dietary specialization in insect herbivores has long been hypothesized to predict tolerance of plant defences, with more specialized herbivores being highly tolerant of and sometimes sequestering plant secondary compounds. Plant variation in secondary compounds should thus play an important and predictable role in shaping the performance and distribution of insect communities.

2. We compared the performance of four naturally co-occurring aphid species on twenty genotypes of the common milkweed *Asclepias syriaca*. Genotypes of milkweed consistently differed in functional traits, including concentrations of toxic cardenolides, while the diet breadths of the four aphids ranged from broadly generalized to monophagous.

3. The two more generalized species had the highest population growth rate overall, while growth rates decreased with increasing specialization. In contrast, honeydew exudation as a measure of phloem consumption increased with specialization; thus, resource-use efficiency was lower in specialist aphids. The two more generalized aphids grew best on genotypes with the highest plant growth rate (as an approximation for resource availability), while specialist aphids were not affected by plant growth.

4. All four species contained apolar cardenolides in their bodies and excreted polar cardenolides, but only the most specialized aphid *Myzocallis asclepiadis* was negatively affected by increasing cardenolide concentrations of the host plant. Sequestration of cardenolides increased with diet specialization, with *M. asclepiadis* accumulating twice as much as any other species, perhaps explaining its susceptibility to plant cardenolides.

5. Heritable plant traits differentially impacted co-occurring insect herbivores within the same guild. Generalist aphids were susceptible to variation in plant vigour but not defensive compounds. Increased host specialization resulted in lower resource-use efficiency, increased phloem throughput and ultimately higher cardenolide sequestration. Variation in these traits is thus likely to determine the relative distribution of generalist and specialist herbivores on plants in natural communities.

Key-words: cardenolides, plant genotype \times insect species interactions, population growth, sequestration, specialization, trait variation

Introduction

Plants are often attacked by a diverse community of herbivores, and plant–herbivore interactions are a major driver of variation in traits such as the nutritional content of plant tissues or the production of toxic secondary metabolites (Ehrlich & Raven 1964; Gloss *et al.* 2013). Variation

in host plant traits directly influences the relative performance of herbivores (Awmack & Leather 2002), and more genetically diverse plant communities often have more complex insect communities, as distinct genotypes act as niches for different herbivores (Karban 1992; Johnson & Agrawal 2007; Underwood 2007; Whitham *et al.* 2008; Utsumi *et al.* 2011). Although such interactions between plant genotype and insect species are most pronounced for herbivores in different feeding guilds, even herbivores with

*Correspondence author. E-mail: tobias.zuest@cornell.edu

highly similar feeding behaviours can be divergent in their response to variable plant traits (Maddox & Root 1990; Johnson & Agrawal 2007; Züst *et al.* 2012).

Within a guild of herbivores, dietary specialists often compete with more generalist herbivores for the same limited resource. The degree of diet specialization in herbivores has been predicted to be linked to adaptations that enable tolerance of plant defences (Mitter, Farrell & Futuyama 1991; Termonia *et al.* 2001; Wahlberg 2001; Bramer *et al.* 2015). Thus, while more generalist herbivores have a larger number of potential hosts available, they often are more susceptible to the toxins of well-defended plants than specialists (Cornell & Hawkins 2003). Furthermore, the degree of specialization also correlates with sequestration of plant toxins by insect herbivores (Lampert, Dyer & Bowers 2014), providing additional benefits to specialist herbivores on defended plants. Differentially specialized herbivores should thus have different functional relationships between levels of host plant defence traits and performance (Ali & Agrawal 2012). Specifically, we predict a generalist herbivore will perform best at the lowest levels of plant defence and suffer decreasing performance with increasing defence. Conversely, a more specialized herbivore should be able to persist at higher defence levels than would be possible for a generalist. Plant genetic variation affecting levels of defence should thus have direct consequences for herbivore performance, which is hypothesized to predictably vary according to the herbivore's diet breadth.

Here, we compare direct effects of plant variation on the performance of four aphid species that commonly co-occur on a shared host plant, the common milkweed *Asclepias syriaca* L. (Apocynaceae). All four aphids share the plant's phloem as a common resource, but differ in their phylogenetic relatedness, life-history strategies, and diet breadths, ranging from an extreme generalist to a monophagous specialist (Smith, Mooney & Agrawal 2008). Milkweeds use an arsenal of toxic cardiac glycosides (aka cardenolides, Agrawal *et al.* 2012) as a defence against a range of herbivores and have substantial intraspecific genetic variation in additional functional traits (Agrawal 2005; Vannette & Hunter 2011; Woods *et al.* 2012). Cardenolides are present in the plant's phloem (Botha, Malcolm & Evert 1977); thus, all aphids are likely exposed to these compounds as part of their diet. At least one of the milkweed aphids, *Aphis nerii* Boyer de Fonscolombe, was previously found to sequester cardenolides in its body and exude them in its honeydew (Rothschild, von Euw & Reichstein 1970; Malcolm 1990).

Using a growth chamber experiment, we specifically addressed (i) whether plant genotypic variation, and especially increasing levels of cardenolide defence, changes the relative population growth of four differentially specialized aphids, (ii) whether the increasingly narrowing diet breadth of the aphids is associated with increasing tolerance of dietary cardenolides, and (iii) whether sequestration of cardenolides is a unique trait of *A. nerii* or is more commonly found among aphids on milkweed.

Materials and methods

STUDY ORGANISMS

The common milkweed *A. syriaca* is a perennial plant native to eastern North America that reproduces both asexually by underground rhizome-like stems and sexually by flowers. Seeds of *A. syriaca* are sired by insertion of a single pollinium into a flower; hence, all seeds from a fruit pod represent a full-sibling genetic family (Gold & Shore 1995). Genetic diversity with respect to resistance to herbivores is substantial within local populations of *A. syriaca* (i.e. Agrawal 2005; Smith, Mooney & Agrawal 2008). We collected single seed pods from twenty patches of *A. syriaca* spread across four adjacent fields in Tompkins County, New York, USA. All seeds from a single pod are henceforth referred to as belonging to the same genotype.

Of the four aphids found on *A. syriaca*, *Myzus persicae* Sulzer (Aphidinae: Macrosiphini) is the most generalized and feeds on many herbaceous species, occasionally including milkweed (T. Züst, personal observation). The three other aphids are specialists on plants of the Apocynaceae with varying degrees of host specialization (Blackman & Eastop 2006). *Aphis nerii* (Aphidinae: Aphidini) is a broad specialist with >50 hosts across many genera and feeds in highly aggregated populations. The congeneric *Aphis asclepiadis* Fitch (Aphidinae: Aphidini) is a narrow specialist with <10 hosts (mostly *Asclepias* spp.) and feeds in smaller colonies that are usually tended by ants. Finally, *Myzocallis asclepiadis* Monel (Calaphidinae: Panaphidini) feeds on *A. syriaca* as its only confirmed host (A. A. Agrawal, personal observations), typically feeds in a dispersed pattern on the underside of lower leaves, is highly mobile, and only produces winged adults. *M. persicae*, *A. nerii*, and *A. asclepiadis* all feed gregariously on apical leaves of *A. syriaca*, and the three species produce predominantly apterous morphs that are relatively sessile, but all respond to adverse conditions and crowding by inducing production of winged offspring (Dixon 1998). We established colonies of all four aphid species on *A. syriaca* plants in a growth chamber using single aphids collected on *A. syriaca* in Tompkins County.

EXPERIMENTAL DESIGN

We grew 16 plants of each of twenty milkweed genotypes, resulting in a total of 320 plants (see Data S1 for details). After 20 days of growth, we began measuring stem height from the lowest to the topmost leaf node at 4-day intervals. Thirty days after planting, we harvested the two leaves below the topmost, fully expanded leaf pair for cardenolide analysis. Thirty-two days after planting, we initiated the aphid growth experiment by introducing five 4th instar aphids to each plant, which were then enclosed in a perforated cellophane bag that prevented movement of aphids among plants. Each aphid species was grown on four replicate plants per genotype, and all treatment combinations were evenly distributed between two identical growth chambers. We counted aphid numbers on all plants on days 37, 40, 43, 46 and 50 after planting, unless we were unable to complete a full count in 1 day, in which case aphids were counted the following day and the counting day was recorded. Plant height measurements were continued on all census days. After the fourth aphid census, we fixed a preweighed disc of aluminium foil (Ø 5 cm) underneath the major aggregation of aphids to collect discharged honeydew. Given the small size of plants, discs captured a large proportion of honeydew exuded by the aphids, even though aggregation patterns and feeding site of aphids affected capture success between replicates. Discs were removed before the final census, dried at 40 °C for 48 h and reweighed as an estimate of exuded honeydew.

APHID PERFORMANCE: GROWTH, HONEYDEW PRODUCTION AND SEQUESTRATION

We quantified aphid performance on different plant genotypes using a primary measure of the average population growth rate. Growth rates were estimated by modelling the exponential phase of aphid population growth using a linear function of log-transformed total aphid number through time (Paine *et al.* 2012). The slope of the loglinear function r_a is equivalent to the relative per capita population growth rate (RGR, unit aphids aphid⁻¹ time⁻¹). This approach ignores differences in age structure of populations, and thus, RGR is a compound measure of aphid fecundity and development time. Population growth was modelled using a mixed-effects model with plant identity specified as random effect to account for repeated aphid counts on the same plant. Aphid species and plant genotype (where statistically supported) were specified as fixed effects to estimate species- and genotype-specific average growth rates (see Data S1). Assessment of residuals indicated strong deviation from the exponential model at the final population census for both *A. nerii* and *M. persicae* on the majority of genotypes, and in fact, many populations of these species had crashed at this point. For *M. persicae*, we thus excluded the fifth census point for all genotypes, while for *A. nerii*, we could retain it for the five genotypes with the slowest aphid growth (Fig. 1).

Honeydew is a waste product and the exuded amounts typically reflect phloem consumption and respiratory rate (Dixon 1998). We estimated the daily per capita honeydew production for each population by standardizing the amount of accumulated honeydew with the cumulative number of aphids present during the collection interval. Cumulative aphid numbers were approximated

by linearly integrating aphid counts between the fourth and fifth census. We analysed the per capita honeydew production using an ANOVA with aphid species as explanatory variable after log-transforming honeydew weights to meet the assumption of homoscedasticity. As the amount of exuded honeydew was approximated by partial capture of discharged honeydew, the per capita honeydew production is a relatively crude metric, but still may be informative to understand species differences.

PLANT GROWTH AND NUTRITION

We quantified growth and nutritional status of the 20 plant genotypes as potential drivers of differences in aphid growth. Plant growth rate at time of aphid introduction was approximated from plant height measurements. Using the four measurements before and the first measurement after aphid introduction, we modelled early plant growth with an asymptotic (aka monomolecular) regression model of log-transformed plant height through time (Paine *et al.* 2012, see Data S1). By excluding the later time points after aphid introduction, we minimized potential effects of aphids on plant growth while avoiding predicting plant growth beyond the range of the data. We estimated the absolute stem growth rate (ASGR, unit mm time⁻¹) at time of aphid introduction to approximate relative differences in available plant resources. We next measured elemental nutrient content on a subset of 4–5 plants per genotype, using an aliquot of 3 mg dried leaf material collected for cardenolide analysis. Samples were analysed for percentage leaf carbon and nitrogen on a NC2500 elemental analyzer (CE Instruments Ltd., Wigan, UK) at the Cornell University Stable Isotope Laboratory.

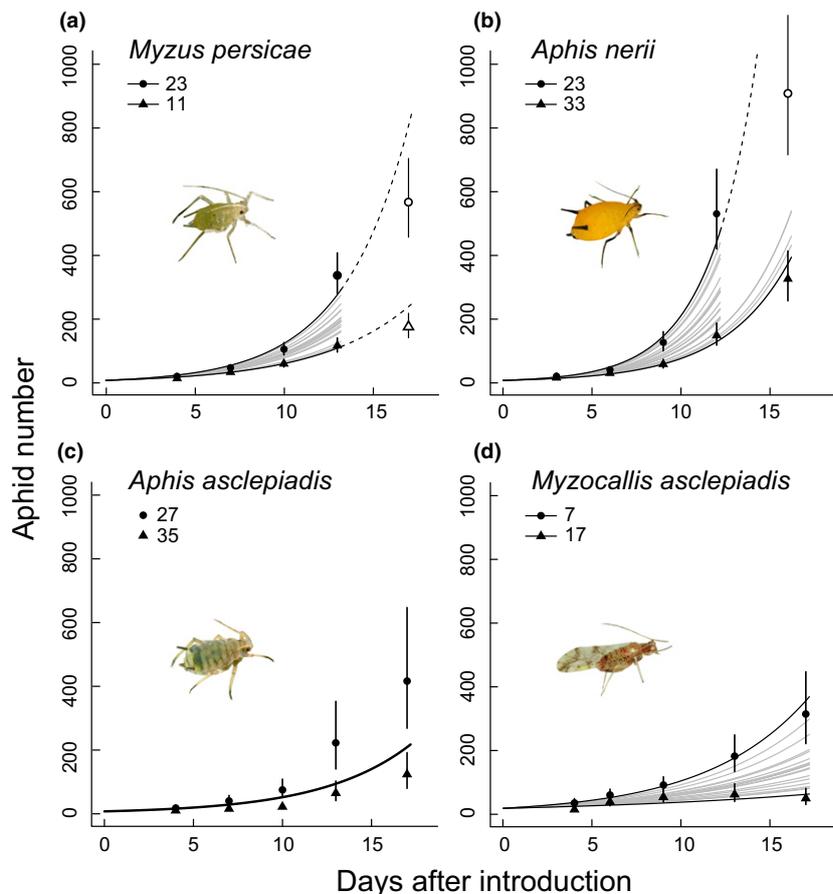


Fig. 1. Model fit of the exponential (loglinear) model for the four aphid species on *A. syriaca*. Grey lines are model predictions for aphid population growth on the twenty plant genotypes. Black lines are population trajectories on the two extreme genotypes for each species, and points are corresponding population means \pm 1 SE for the five population censuses. For *M. persicae* (a) and *A. nerii* (b), the model was only fitted to the first four census points (solid line, filled circles) when the fifth census point (empty circles) clearly deviated from the exponential trajectory (dashed line). Note that growth of *A. asclepiadis* (c) showed no support of a plant genotype effect; hence, only the species mean was fitted.

TRAITS AFFECTING APHID GROWTH AND SEQUESTRATION

We measured cardenolides in plant leaves, aphid bodies, and honeydew to link aphid performance with processes of cardenolide sequestration and exudation. Cardenolides in leaves, aphids, and honeydew were extracted in methanol, concentrated and filtered for HPLC analysis (see Data S1). Leaf and aphid extracts were subjected to an additional clean-up protocol following Züst, Rasmann & Agrawal (2015), which removes interfering phenolic compounds without affecting cardenolides. Despite clean-up, extracts of *A. nerii* bodies contained a large number of predominantly polar UV-absorbing compounds that could potentially mask polar cardenolides, while such matrix effects were much smaller in the other three species (Fig. S2, Supporting information). We therefore independently validated HPLC results for three of the aphid species using an enzymatic assay measuring the specific inhibition of the porcine Na^+/K^+ -ATPase by cardenolides in aphid extracts (Data S1).

For the three aphid species with statistical support for plant genotype-specific differences in growth rate, we regressed aphid growth rate against a set of plant traits as potential drivers of these differences. Specifically, we related aphid population growth rates on the twenty plant genotypes to the set of measured traits of these genotypes: plant ASGR, C:N ratio, and total cardenolide content. We also included separate variables for polar and apolar foliar cardenolide concentrations (based on HPLC retention times), which are the sums of the four most polar or the six most apolar cardenolides in *A. syriaca* (see results, Fig. 3). For each species, we began with a model including just the main effects for these four traits, since our sample size ($n = 20$ genotype means) was too small to allow reliable testing for interactions. Using Type III marginal SS specified in R with the function *drop1*, we removed the least-significant effects in a stepwise procedure, until either

only significant traits remained or models were left with a single trait.

Results

APHID PERFORMANCE AND PLANT-DRIVEN VARIATION

We first quantified rates of aphid population growth as the primary measure of aphid performance. All four aphid species maintained exponential growth for at least four out of the five population censuses (Fig. 1), but mean species growth rates did not follow the order of diet specialization (Fig. 2a). The generalist *M. persicae* had the second-highest growth rate, resulting in an average population doubling time (DT) of 3.01 days (± 0.09 , 1 SE), while the broad specialist, *A. nerii*, had the highest mean growth rate of all species (DT 2.21 ± 0.05 days). The narrow specialist *A. asclepiadis* grew slightly, but significantly slower than *M. persicae* (DT 3.33 ± 0.10 days, conditional *t*-test: $t = -2.34$, d.f. = 1001, $P = 0.017$), while the monophagous *M. asclepiadis* grew the slowest of all species (DT 5.19 ± 0.25 days). Faster growth generally requires more resources and thus higher phloem throughput, and accordingly, honeydew exudation largely mirrored the patterns of growth (Fig. 2b). Nonetheless, *M. asclepiadis* strongly deviated from this pattern and produced by far the largest per capita quantities of honeydew despite its slow growth. This suggests that the extremely specialized *M. asclepiadis* employs a distinctly different food processing strategy.

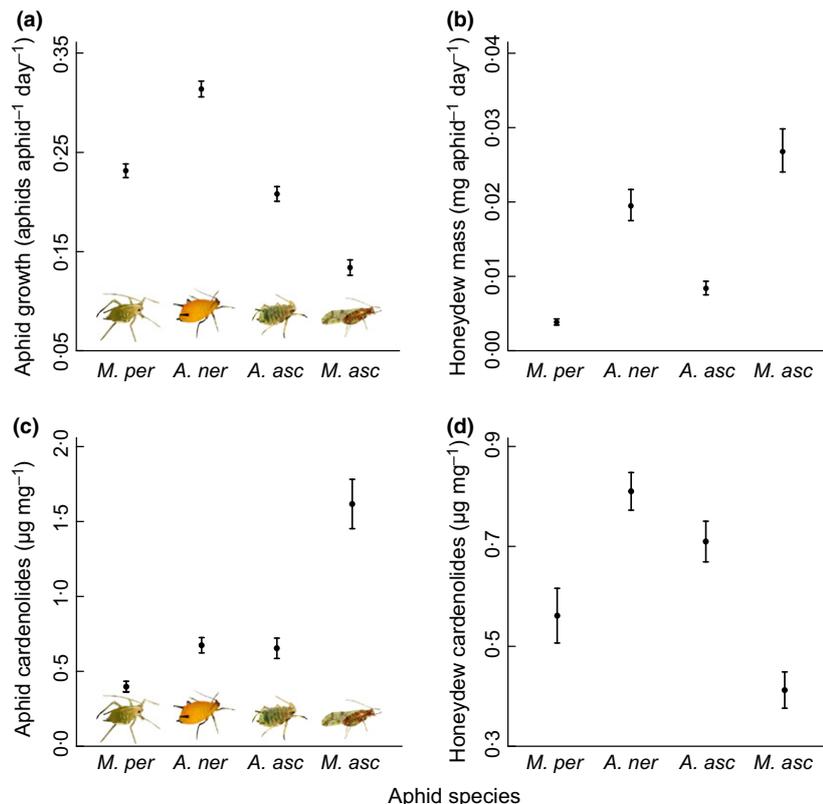


Fig. 2. Species trait means of the four aphids, ranked from least to most specialized (*M. per*: *Myzus persicae*, *A. ner*: *Aphis nerii*, *A. asc*: *Aphis asclepiadis* and *M. asc*: *Myzocallis asclepiadis*). Upper panels: (a) population growth, averaged across all twenty plant genotypes and (b) mean dry weight of honeydew produced per aphid individual and day. Lower panels: average cardenolide content in (c) aphid bodies and (d) honeydew. Cardenolide content is presented as percentage dry weight of aphid bodies or honeydew. All values are means ± 1 SE.

Within aphid species, plant genotype accounted for 11.7% of total variation in population growth rate for *M. persicae*, 15.4% for *A. nerii* and 9.8% for *M. asclepiadis*. In contrast, genotype did not account for any variation in growth rates of *A. asclepiadis*. There were significant effects of plant genotype on the growth rate parameter r_a for *M. persicae* (conditional F -test: $F_{19,273} = 2.11$, $P = 0.006$), *A. nerii* ($F_{19,220} = 1.96$, $P = 0.011$) and *M. asclepiadis* ($F_{19,224} = 1.74$, $P = 0.032$). Corresponding to the lack of a genotype contribution to total variation in *A. asclepiadis*, there was no significant effect of genotype on r_a ($F_{19,251} = 1.15$, $P = 0.305$); thus, we did not estimate genotype-specific growth rates for this species. The estimated initial population size varied between species, with 8.6 ± 0.4 aphids for *M. persicae*, 5.2 ± 0.3 for *A. nerii*, 6.5 ± 0.3 for *A. asclepiadis* and 16.2 ± 1 for *M. asclepiadis*, but there was no support for a genotype effect on the estimated initial population size ($P > 0.05$ for all species). As all populations were initiated with five adult aphids, estimated initial population size likely reflects differences in time to initiate feeding.

For *M. persicae*, average DT ranged from 2.59 ± 0.15 days to 3.54 ± 0.28 days between the two extreme plant genotypes (Fig. 1a), while for *A. nerii*, DT ranged from 1.86 ± 0.11 days to 2.57 ± 0.21 days (Fig. 1b). Thus, for both species, populations growing on the least-suitable plant genotype needed almost one additional day (>35% longer) to double in size compared to populations on the best genotype. In contrast, DT of *M. asclepiadis* ranged from 3.95 ± 0.39 days to 9.57 ± 3.48 days (Fig. 1c). Genotype-specific growth rates of *M. persicae* and *A. nerii* were positively correlated, while *M. asclepiadis* varied independently (Table S1).

CARDENOLIDE SEQUESTRATION AND EXUDATION

As one potential mechanism underlying the differential responses of aphids to plant genotypes, we next compared the processing of plant cardenolides among the four aphid species. All four species contained significant amounts of cardenolides in their bodies and excreted cardenolides in their honeydew, and the amount of cardenolides in aphid bodies increased with host plant specialization. The generalist *M. persicae* contained the lowest amount of cardenolides per unit dry mass (Fig. 2c), while the more specialized congeneric *A. nerii* and *A. asclepiadis* both contained intermediate amounts that were statistically indistinct from each other (conditional t -test: $t = 0.12$, d.f. = 76, $P = 0.903$). The monophagous *M. asclepiadis* contained more than twice the cardenolides as any of the other species. Cardenolide content in honeydew largely mirrored the pattern in aphid bodies (Fig. 2d), except for the monophagous *M. asclepiadis* which - despite having the highest cardenolide levels in its body - exuded the lowest amounts of cardenolides in its honeydew.

Comparing cardenolide profiles between different sample types, we found that aphid bodies and honeydew

contained distinct subsets of plant cardenolides (Fig. 3). In leaves of *A. syriaca*, we detected 11 cardenolide compounds that loosely clustered into a polar and an apolar group as determined by HPLC retention time (Fig. 3a). Aphid bodies only contained a subset of three compounds from the apolar cluster (Fig. 3b), with the exception of *A. nerii* in which we detected an additional polar cardenolide that was not present in plant leaves (#8.4). Corresponding to HPLC results, body extracts of *M. persicae* caused the lowest inhibition on porcine Na^+/K^+ -ATPase, while extracts of both *A. nerii* and *A. asclepiadis* caused considerably stronger but equivalent inhibition (Fig. S3), giving no indication of significant amounts of cardenolides 'hidden' in the matrix of *A. nerii* extracts. The honeydew of all aphid species contained 22 distinct, predominantly polar cardenolides (Fig. 3c), while most apolar cardenolides found in aphid bodies were absent from honeydew. The disparity between aphid bodies and honeydew strongly suggests that measurements of aphid bodies represent the cardenolide content of the haemocoel or the integument, rather than simply reflecting gut contents.

EFFECTS OF PLANT TRAIT VARIATION

We found support for an effect of genotype on plant growth rate before aphid introduction (conditional F -test: $F_{19,1204} = 1.91$, $P = 0.010$). The differences in plant growth rate resulted in up to 1.5-fold differences in height among plant genotypes at the time of aphid introduction. Additionally, we found significant heritable variation in plant nutritional and defence traits, with genotype explaining a substantial proportion of the total variance for C:N ratio ($F_{19,79} = 2.77$, $P < 0.001$; full-sib heritability $H^2 = 0.44$) and total cardenolide concentration of leaves ($F_{19,263} = 3.05$, $P < 0.001$; $H^2 = 0.19$). The rate of absolute stem growth of genotypes (ASGR) was not significantly correlated with nutrition (C:N ratio: Pearson's $r = -0.36$, $P = 0.123$) or defence (cardenolides: $r = 0.36$, $P = 0.117$), but C:N ratio was negatively correlated with cardenolide concentration ($r = -0.61$, $P = 0.004$). Thus, in this study, plant growth varied independently of cardenolide levels and nitrogen content, but plants with a higher nutritional quality (i.e. low C:N ratio) also had higher levels of cardenolides.

Regressing mean aphid performance with trait means of the twenty genotypes (plant ASGR, C:N ratio, total cardenolide content, polar and apolar cardenolide content; see Table S1), we found plant ASGR to be the only remaining term after model simplification for *A. nerii* and *M. persicae*. The mean effect of ASGR on growth of *M. persicae* was not significant (Fig. 4a, $F_{1,18} = 0.18$, $P = 0.674$), whereas ASGR had a significant positive effect on growth of *A. nerii* (Fig. 4b, $F_{1,18} = 7.01$, $P = 0.016$). In contrast, apolar cardenolide content was the only remaining term for *M. asclepiadis*, with mean apolar cardenolide content of plant genotype having a negative effect on aphid growth (Fig. 4c, $F_{1,18} = 6.25$, $P = 0.022$). The genotype means of

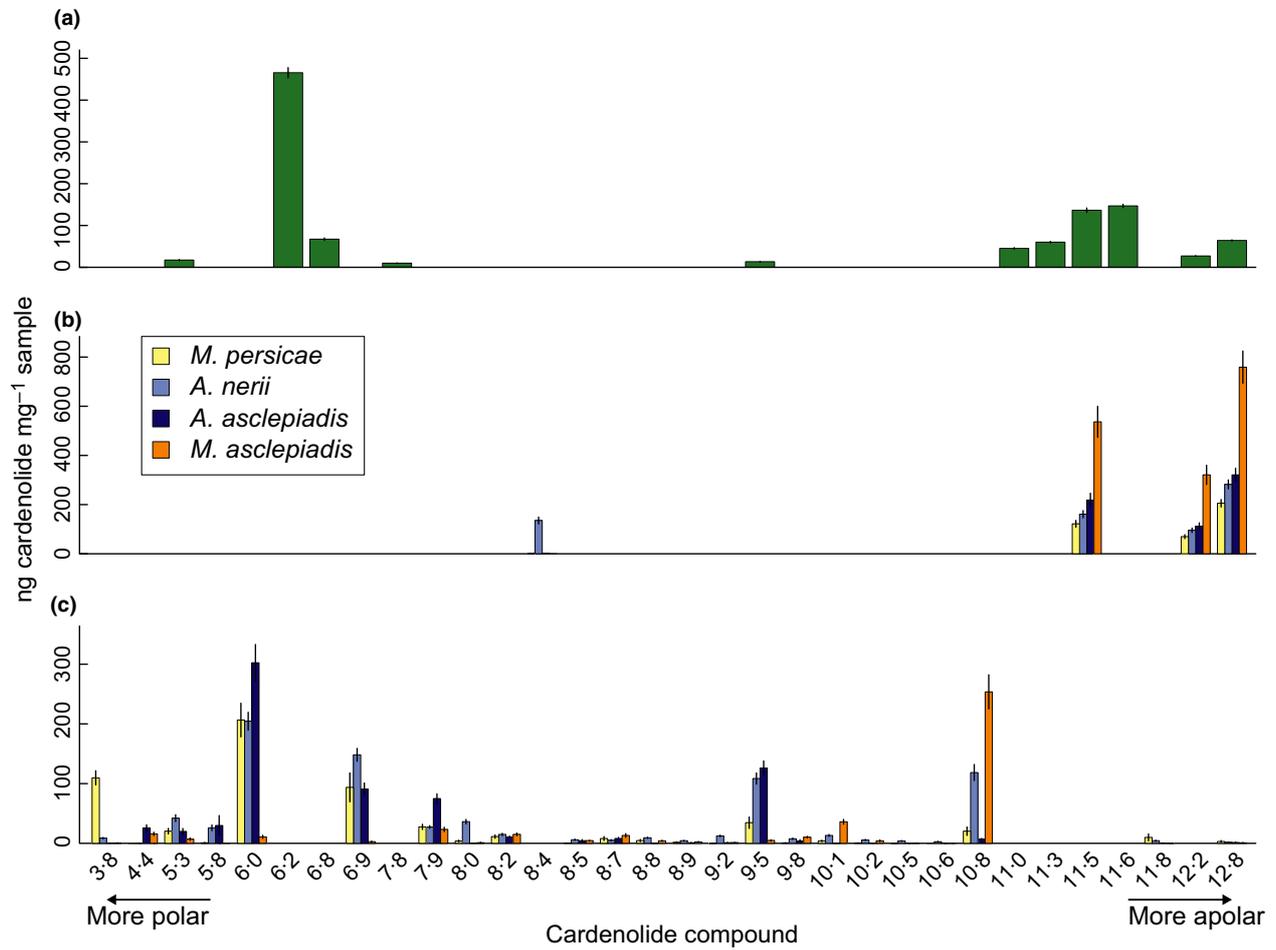


Fig. 3. Cardenolide profiles of (a) plant samples, (b) aphid bodies and (c) honeydew. Each bar or group of bars represents one distinct cardenolide compound detected by HPLC (mean \pm 1 SE). Compounds are ordered according to polarity, with numbers referring to HPLC elution time. Putatively identical compounds in different tissues are aligned vertically.

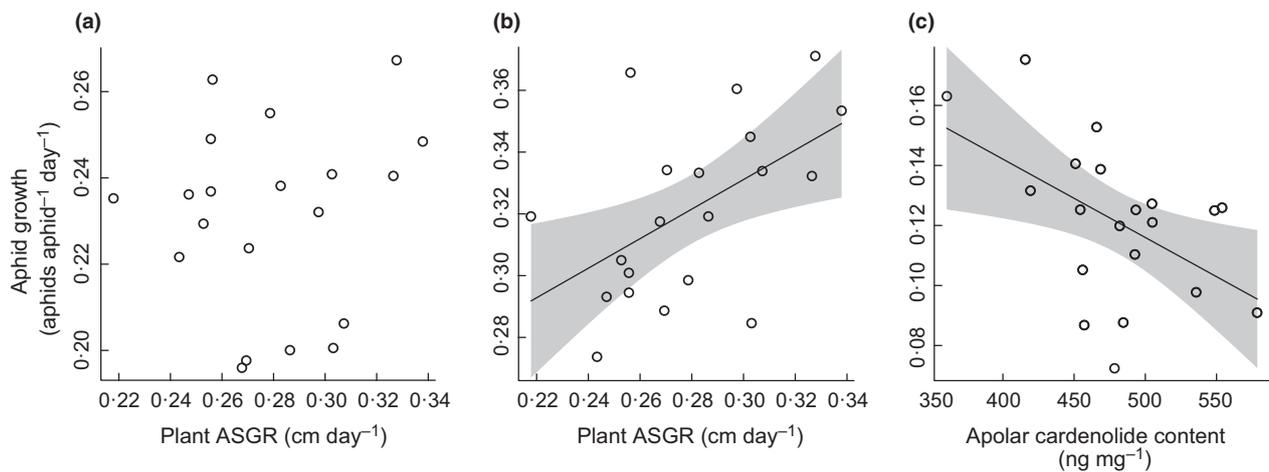


Fig. 4. Correlations between aphid growth rate and the most important plant trait for (a) *Myzus persicae*, (b) *Aphis nerii*, and (c) *Myzocalis asclepiadis*. Points are mean trait values on plant genotypes, while lines represent linear regression between the two respective variables. The grey area is the 95% confidence interval around each regression. Out of the traits evaluated, the absolute stem growth rate (ASGR) at time of introduction was the last remaining term in a model of aphid growth rate for both *A. nerii* and *M. persicae*, even though for the latter, the relationship was not significant. In contrast, the subset of apolar leaf cardenolides correlated most strongly with growth rates of *M. asclepiadis*.

total foliar cardenolides were uncorrelated with body content for any of the aphid species (Pearson's $r = -0.15$ to 0.33 , $P > 0.1$ for all species). Instead, the cardenolide content of aphid bodies was positively affected by the rate of honeydew exudation ($F_{1,75} = 4.09$, $P = 0.047$), with higher exudation (and thus phloem throughput) resulting in higher cardenolide accumulation (Fig. S4).

Discussion

The levels of sequestration and population growth rates of the four aphid species on milkweed generally followed the order of host plant specialization, but in contrasting ways. The amount of sequestered cardenolides per body weight increased with specialization, although the two intermediately specialized aphids did not differ in their cardenolide content. With the exception of the broad specialist *A. nerii*, aphid population growth decreased with specialization. Thus, while increasing diet specialization corresponded to levels of sequestration, there was no clear evidence for a concurrent increase in resource-use efficiency.

Aphid performance and interactions with plant quality are rarely assessed in more than two species concurrently (e.g. the problem of one 'generalist' and one 'specialist', Ali & Agrawal 2012). However, host plant specialization often changes more gradually among aphids, and many aphid species share a single host plant. The community of aphids on tansy (*Tanacetum vulgare* L.), for example, includes up to eight species of aphids that differ in various life-history traits (Stadler, Dixon & Kindlmann 2002; Stadler 2004; Woodring *et al.* 2004). Here too, the most generalist aphids tend to have the fastest growth rates (Stadler, Dixon & Kindlmann 2002), but only on high-quality plants, while decreasing quality disproportionately impaired the generalists without affecting specialists. Thus, across at least two systems, more generalist aphids tend to have higher growth than specialists on plants with high nutritional quality, while specialization increases tolerance of low nutritional quality. Strategies for persistence of specialists are thus likely to shift away from explosive population growth to mutualisms with ants or increased sequestration of plant compounds.

The four milkweed aphids in our experiment clearly differed in their responses to intraspecific trait variation of *A. syriaca*. Genotypic differences among the plants used in our experiment had significant effects on the performance of three out of the four aphid species. For *M. persicae* and *A. nerii*, the two most generalist aphids, populations growing on the least-suitable genotype, required >35% more time (an extra day) to double their population compared to populations on the best genotype. For both species, population growth rate was positively correlated with the mean growth rate of plant genotypes, even though this effect was only significant for *A. nerii*. While genotype effects were too weak to impair the generalist's performance to lower levels than the specialist's, this finding supports a higher susceptibility of generalist aphids to low

plant nutritional quality. Plant growth is a good approximation for the resources available to the aphid and often correlates positively with aphid growth (Züst *et al.* 2011). Indeed, Zehnder & Hunter (2008) demonstrated that nitrogen fertilization of *Asclepias tuberosa* L. directly increased the population growth of *A. nerii*. In aphids, embryo initiation and maturation continue throughout the adult's reproductive life; thus, fecundity is directly affected by the quality of the host plant (Awmack & Leather 2002).

The most specialized *M. asclepiadis* was strongly and directly affected by plant variation, with a difference in population doubling time between the two extreme genotypes of about 5 days that was linked to plant cardenolide content. In contrast, we found no effect of plant genotype on the performance of the narrow specialist *A. asclepiadis*, while a similar experiment using a different set of genotypes did report such effects (Smith, Mooney & Agrawal 2008). In the field, *A. asclepiadis* is frequently tended by ants, and ant tending can have strong positive effects on aphid growth (Flatt & Weisser 2000). However, the benefit of ant tending changes between different plant genotypes (Mooney & Agrawal 2008), perhaps as plant quality determines the quality of honeydew rewards for ants. Thus, even though direct effects of plant variation may be variable and not appear to be important for *A. asclepiadis*, they still may affect aphid performance via ecological interactions.

Genotypes of *A. syriaca* in our experiment significantly differed in functional traits related to growth and defence. Plants differed strongly in the rate of stem growth and both nitrogen availability and cardenolide content, but surprisingly growth did not significantly correlate with either of the other quality measures, despite our recent demonstration of costs of cardenolides in the common milkweed (Züst, Rasmann & Agrawal 2015). In comparison with the more sophisticated methods to quantify growth we used in Züst, Rasmann & Agrawal (2015), rate of stem elongation is a simpler approximation of shoot growth; additionally, the genotypes in the current study had roughly half the variation in cardenolide content compared to Züst, Rasmann & Agrawal (2015).

SEQUESTRATION AND TOLERANCE OF CARDENOLIDES

While we did not directly measure cardenolides in phloem, aphid bodies and honeydew together contained the full range of foliar cardenolides, which is a strong indication for the phloem mobility of these compounds (Molyneux, Campbell & Dreyer 1990). Apolar cardenolides are considered more toxic (Agrawal *et al.* 2012), as apolarity of a compound determines whether it can pass through cell membranes via passive diffusion. In contrast, polar compounds often require active carrier molecules to cross membranes (Frick & Wink 1995) and can be excreted more easily. All four aphid species contained the same subset of apolar cardenolides from *A. syriaca*, although at different body concentrations, while polar cardenolides were mostly

metabolized or excreted in honeydew. Our results mirror the findings of Malcolm (1990), who found that *A. nerii* feeding on *Asclepias curassavica* L. contained high amounts of apolar cardenolides in its body, while its honeydew contained proportionally more polar cardenolides. Concordant with a passive mode of sequestration, we found a positive correlation between honeydew exudation and aphid body cardenolide content both among and within species, most likely as higher phloem throughput resulted in higher cardenolide exposure. Sequestration by aphids thus differs from sequestration by other insects. The monarch butterfly (*Danaus plexippus* L.), for example, preferentially sequesters a subset of mostly polar cardenolides to concentrations up to twofold higher than found in its host plant (Malcolm 1990).

Using two different analytical methods, we did not find a significant difference in sequestration between the aposymbiotically coloured *A. nerii* and the more cryptically coloured *A. asclepiadis*. In contrast, Mooney, Jones & Agrawal (2008) found a strong relative preference of predators for *A. asclepiadis*, which indicates higher sequestration by *A. nerii*. Even though the cardenolide quantification of Mooney, Jones & Agrawal (2008) was a cruder, relatively unspecific photometric method that is sensitive to matrix effects, it is possible that the sequestration of cardenolides might be dependent on environmental conditions and plant quality. For example, in our experiment, the input of new cardenolides might have been much reduced in the days prior to aphid collection due to decreasing plant quality (e.g. Wink & Witte 1991), which could have affected the fast-growing *A. nerii* more strongly than *A. asclepiadis*.

In general, there was no correlation between cardenolide concentrations measured in leaves and aphid bodies for most species. While we showed that the full range of foliar cardenolides must be present in the phloem, it is unclear how concentrations of cardenolides in phloem relate to concentrations in leaf tissue. In addition, foliar cardenolides were measured before aphid introduction. While aphid feeding generally has little effect on foliar cardenolide levels (Ali & Agrawal 2014), it is unclear how phloem concentrations might change in response to aphid feeding. Current methods for extracting phloem such as aphid stylectomy are promising avenues for future research, but are currently limited by their variable success on different species and the difficulties associated with collecting substantial amounts of phloem sap (T. Züst, unpublished).

In an earlier study, Agrawal (2004) demonstrated that the broad specialist *A. nerii* can be impaired by very high cardenolide concentrations found in some milkweed species. Cardenolides are specific inhibitors of animal Na^+/K^+ -ATPase, and several specialized herbivores have evolved cardenolide resistance through convergent modifications of their Na^+/K^+ -ATPase (Dobler *et al.* 2012; Zhen *et al.* 2012). Interestingly, Zhen *et al.* (2012) demonstrated that both specialist and generalist aphids have a basal modification of their Na^+/K^+ -ATPase not found in

other insects, and with unknown function for cardenolide resistance. Thus, while cardenolides might be harmless at low to average concentrations found in many milkweed species (Malcolm 1992; Martel & Malcolm 2004), cardenolide exposure at higher levels may well be toxic for aphids.

In our study, the most specialized aphid *M. asclepiadis* accumulated the highest levels of cardenolides in its body and was the only species susceptible to variation in foliar cardenolide concentration. The high accumulation of cardenolides can be explained at least in part by the extremely high honeydew exudation and thus phloem throughput of *M. asclepiadis*. The tremendous amounts of honeydew exuded by this species are indicative of either high energy requirements or low metabolic efficiency. Aphids of the genus *Myzocallis* predominantly feed on trees (Blackman & Eastop 2006) and appear to have a relatively simple community of bacterial endosymbionts (Michalik *et al.* 2014). Thus, it is possible that *M. asclepiadis* lacks some of the metabolic adaptations to make it an efficient feeder of forbs. Alternatively, its life-history strategy might constrain *M. asclepiadis* to less nutritious feeding sites. Milkweeds have bicollateral vascular bundles, and *A. nerii* was shown to preferentially feed on the nutrient-rich internal (adaxial) phloem system (Botha, Malcolm & Evert 1977). To maintain its high mobility, *M. asclepiadis* might feed on the less nutritious external (abaxial) phloem system, as it is closer to the leaf surface and might be quicker to reach. In support of the latter, a comparison of *M. asclepiadis* and *A. nerii* between plant genotypes sustaining equivalent rates of honeydew exudation reveals a consistent difference in body cardenolide content independent of phloem throughput.

We demonstrated that all aphids feeding on milkweed sequester cardenolides, including the generalist aphid *M. persicae*. Despite the apparently mostly passive uptake of cardenolides by the aphids, sequestration of these compounds appears to be beneficial for *A. nerii* at levels where aphid growth is not impaired, as several studies that grew *A. nerii* on low vs. high cardenolide plants showed increased survival or lower predation on high-cardenolide plant species (Omkar 2005; Desneux *et al.* 2009). While the benefit of sequestration needs to be demonstrated in the other three species, findings from Desneux *et al.* (2009) suggest that at least *A. asclepiadis* benefits from sequestration as well. Cardenolide sequestration at intermediate levels is thus likely beneficial at no cost to the aphid. Even the negative impact of cardenolides on *M. asclepiadis* may be balanced by increased protection against natural enemies that are among the most important drivers of natural aphid population dynamics and distributions (Dixon 1958; Cappuccino 1987; Härrä, Krauss & Müller 2008). Importantly, these findings seem to be more broadly applicable across different feeding guilds. For example, Lampert, Dyer & Bowers (2014) compared the performance of a guild of three differentially specialized nymphalinea caterpillars on plants producing iridoid glycosides and iridoid glycoside-free controls in a recent study. In parallel to our

results, they found the most generalist caterpillars to grow the fastest, but sequestration of iridoid glycosides increased with specialization (Lampert, Dyer & Bowers 2014).

CONCLUDING SPECULATION

In conclusion, heritable plant traits can differentially affect co-occurring herbivores within the same guild. Co-occurring herbivores commonly differ in their degree of host plant specialization, and increased tolerance of dietary plant toxins by specialists is often proposed as a mechanism that mediates coexistence in an environment of variable plant defence. Among the guild of naturally co-occurring milkweed aphids, diet breadth predicted the extent of sequestration but was inversely related to population growth. This indicates a benefit of host plant specialization for defence in a multitrophic context, but not for tolerance of dietary toxins or resource-use efficiency.

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Data accessibility

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.35nn0>, (Züst & Agrawal 2015).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Data S1. Methods.

Fig. S1. Timeline of the aphid growth experiment.

Fig. S2. Representative HPLC chromatograms of aphid bodies to highlight differences in matrix effects among species.

Fig. S3. Cardenolide quantification of aphid bodies using the specific inhibition of extracts on the porcine Na⁺/K⁺ ATPase.

Fig. S4. Relationship between exuded honeydew mass and aphid body cardenolide content.

Table S1. Correlation coefficients among plant genotype means of aphid growth rates and plant traits.