

Trade-offs and tritrophic consequences of host shifts in specialized root herbivores

Jared G. Ali¹* and Anurag A. Agrawal²

¹Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA; and ²Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA

Summary

1. Trade-offs in an herbivore's ability to feed, avoid predation and succeed on alternative hosts are thought to be major driving factors in host specialization. In this study, we compared how two closely related milkweed beetles (*Tetraopes* spp.) that have specialized on separate *Asclepias* species respond to host switching to alternative milkweed plants. By additionally examining effects on the beetles' entomopathogenic natural enemies, we test whether host plant specialization is driven by plant–herbivore interactions alone or whether there is a role for the tritrophic interactions.

2. *Tetraopes* beetles feed on milkweed roots as larvae and on milkweed shoots as adults, and they sequester toxic cardenolides from their host plants. We predicted that growth and sequestration would be compromised on alternative hosts as a consequence of specialization. Larvae of each *Tetraopes* species were reared on their native host plant, the host plant of the other species and two closely related *Asclepias* species that do not typically host *Tetraopes*.

3. Regardless of beetle specialization, survival of both beetles was highest on *A. syriaca*, which has the second-to-lowest levels of root cardenolides and is the native host for *T. tetraophthalmus*. Larval growth was not affected by host plant for *T. texanus*, but *T. tetraophthalmus* grew best on *A. exaltata*, a close relative of *A. syriaca* that does not typically experience beetle feeding.

4. We found that larval sequestration of cardenolides largely mirrored host plant values in the roots, with the exception of *T. texanus* on *A. asperula*, which sequestered a low proportion of the cardenolides from this host. Although all larvae were susceptible to entomopathogenic nematodes (EPNs), the number of EPNs emerging from cadavers depended on the beetle–plant combination, with more EPNs produced when beetles were feeding on native compared to non-native hosts.

5. The observed consequences for tritrophic interactions did not fit expectations for specialized herbivores (i.e. EPN predation was highest when beetles were on their native hosts), suggesting that specialization may be driven primarily by a plant–herbivore interaction in this system, not by a tritrophic effect.

Key-words: co-evolution, entomopathogenic nematodes, plant defence, plant–herbivore interaction, specialists, tritrophic interactions

Introduction

Trade-offs are often predicted to maintain genetic variation within a species as well as to drive ecological specialization among species (Futuyma & Moreno 1988; Jaenike 1990; Thompson 1994; Joshi & Thompson 1995a). Whether trade-offs serve as by-products of pleiotropy (Roff & Fairbairn 2007), or act as forces in maintaining

specialization in co-evolutionary interactions, they are central to most evolutionary ecological theories and especially relevant to plant–insect interactions (Agrawal 2011). Recent work has further implicated trade-offs as critical in the evolution of multitrophic interactions, suggesting that host plant specialization may be in part driven by top-down effects of predators and parasites (Murphy 2004; Singer & Stireman 2005). However, measures of fitness trade-offs rarely consider tritrophic interactions, especially

*Correspondence author. E-mail: jga8@psu.edu

when herbivores sequester plant defence and may gain protection from natural enemies (Bowers 1980; Dyer 1995).

Evolutionary innovations in host defences and in the herbivores' ability to circumvent them seem to be drivers of adaptive radiations in both plants and insects (Ehrlich & Raven 1964). These junctions of plant–insect adaptation are likely to lead to host plant specificity (Scriber 2002). Indeed, most phytophagous insects are highly host specific, feeding only on a small fraction of the plants they encounter (Strong, Lawton & Southwood 1984; Mitter & Farrell 1991; Forister *et al.* 2015). One striking case of likely co-evolution already recognized by Ehrlich & Raven (1964) comes from milkweed plants in the genus *Asclepias* that produce toxic cardiac glycosides (cardenolides) as a characteristic trait. This genus comprising of more than 140 species is well known for its toxicity and highly specialized herbivores (Agrawal *et al.* 2012, 2015). Among these specialized herbivores, the genus *Tetraopes*, a group of North American long-horned beetles, comprises 25 species whose phylogeny matches that of their milkweed host plants, suggesting they may have diversified in parallel (Farrell 2001; Farrell & Mitter 2008). With this potential for parallel evolution of closely related insects and their host plants, we have an opportunity to evaluate mechanisms that promoted and maintain specialization.

Larvae of *Tetraopes* beetles feed on roots of milkweed, while adults feed on foliage of the same plants. Rasmann *et al.* (2011) showed that soil-dwelling entomopathogenic nematodes (EPNs) are effective agents of indirect defence against these root herbivores of milkweed and they are attracted to volatiles released by the roots as *Tetraopes* larvae feed. Cardenolides are expressed in milkweed roots and can act as a direct defence against herbivores, but many specialist herbivores of milkweed have gained the ability to manipulate and sequester these defences for their own benefit. For example, sequestration has been shown to effectively reduce predation and parasites of above-ground milkweed herbivores (Malcolm & Brower 1989; de Roode *et al.* 2008). Accordingly, there is the potential for a trade-off between direct and indirect defences, where high investment in direct defence by the plant may reduce the efficacy of indirect defences, especially if specialized herbivores are capable of sequestering the direct defence for their own benefit.

In this study, we examined benefits of specialization and trade-offs in host use among closely related herbivores. In particular, we tested whether the extent of sequestration (and its benefits) for specialized herbivores depends on the evolutionary history with their host plant. We selected two beetles from the genus *Tetraopes* that are closely related and both specialized on milkweeds, but have adapted to host plants with highly divergent levels of direct defence (root cardenolides). Although all of the chosen plants have the same class of toxic compounds, it has been consistently shown that plant species with increasing concentration of these toxins have a cost to specialist herbivores (reviewed in Agrawal *et al.* 2012, 2015), and for *Tetraopes* spp.

Specifically, such patterns were associated with specialization (Rasmann & Agrawal 2011a). Research has shown that the extent of sequestration and impacts on predators and parasites is likely to be variable, even among close specialist relatives (Brower & Brower 1964; Bowers 1992). We compared *Tetraopes* beetle survival, growth, sequestration of toxins and effects on the third trophic level through infection with EPNs. Each *Tetraopes* species was reared on its native host plant, the host plant of the other beetle species, and two additional *Asclepias* species that are close relatives of the native host plants but lack natural root herbivores (Fig. 1).

Specifically, we tested (i) assumptions of adaptive specialization and how they might be maintained among closely related species of herbivores and plants, (ii) the occurrence of fitness trade-offs associated with host switching and (iii) the potential roles of the third trophic level in maintaining native host relationships and its contribution to trade-offs in performance on alternate hosts. With this structure, we aim to assess whether host plant specialization is driven by trade-offs linked to plant–herbivore interactions alone, or whether there is a role for tritrophic interactions.

Materials and methods

PLANTS

Bioassay experiments were carried out on common milkweed (*Asclepias syriaca* L.; Apocynaceae), Green Antelopehorn Milkweed (*A. viridis* Walter), Spider Milkweed (*A. asperula* Decne) and Poke Milkweed (*A. exaltata* L.). These are perennial plants that occur in eastern (*A. syriaca* and *A. exaltata*), south-western (*A. asperula*) and south-eastern (*A. viridis*) North America (Seiber *et al.* 1986; Malcolm & Brower 1989). *Asclepias syriaca* and *A. exaltata* overlap in much of their range, whereas *A. asperula* and *A. viridis* primarily overlap in the south central USA. Milkweed utilizes a well-characterized suite of defences (Agrawal 2005). Cardenolides, a class of bitter-tasting cardiac glycosides, are present throughout milkweed tissues and function defensively by disrupting the exchange of sodium and potassium ions across animal cell membranes (Malcolm 1991; Agrawal *et al.* 2012). Latex, another key defence, is a sticky liquid exuded from above-ground, but not below-ground, plant parts following damage.

All bioassay experiments were conducted on 60- to 70-day-old plants grown from seed. Seeds of *A. syriaca* and *A. exaltata* were collected by the authors in Tompkins County, NY, USA, *A. viridis* was collected in Payne County, OK, USA, and *A. asperula* were obtained from Daggett County, UT, USA. Seeds were germinated at room temperature after being stratified at 4 °C on moist filter paper for 2 week. Seedlings were planted into potting soil (10-cm-diameter pots) and grown completely randomized in a growth chamber (14-h daylight, 26 °C day; 20 °C night). Plants were fertilized (N:P:K 21:5:20, 150 ppm N [$\mu\text{g g}^{-1}$]) once, 1 week after planting.

INSECT HERBIVORES

The red milkweed beetle *Tetraopes tetraophthalmus* Foster (Cerambycidae) is a univoltine specialist herbivore on common milkweed *A. syriaca*, and common throughout most of *A. syriaca*'s

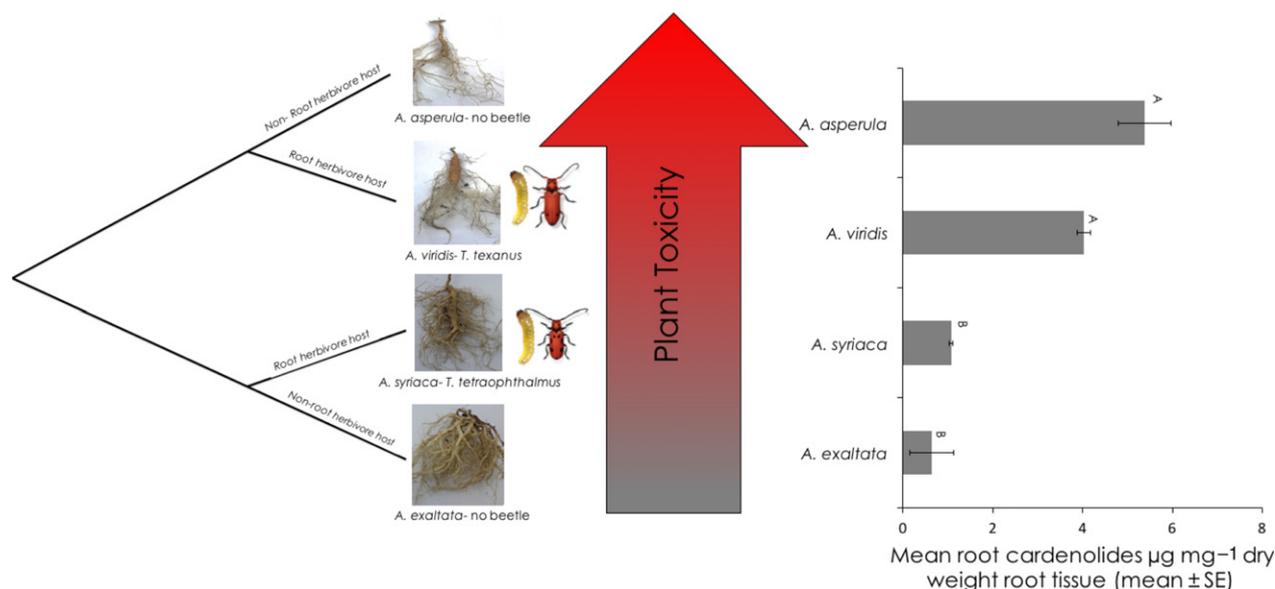


Fig. 1. Left panel: Depiction of plant–beetle relationships and plant toxicity. Sister taxa *Asclepias asperula* and *A. viridis* along with sister taxa *A. syriaca* and *A. exaltata* were used in this study. *Asclepias asperula* and *A. exaltata* have no known association with *Tetraopes* root herbivores. *A. viridis* and *A. syriaca* are the native hosts of *T. texanus* and *T. tetraophthalmus*, respectively. Right panel: root cardenolides (Mean \pm SE) extracted from plant used in this experiment. $N = 10$ per species. Different letters (e.g., A,B, etc.) correspond to means with P -value significance < 0.05 . Levels not connected by same letter are significantly different.

range (Wyatt 1996; Rasmann & Agrawal 2011a). *Tetraopes texanus* Horn is typically found throughout the southern, central and Big Bend regions of Texas and is known to utilize *A. viridis* as its native host plant (Chemsak 1961). Adult beetles emerge from the soil in late spring or early summer and begin to feed on flowers and foliage. Females oviposit clutches of 10–15 eggs in dry stems of nearby thick-bodied grasses and thick, hollow forbs (Masters 1995; Strong *et al.* 1995; Agrawal 2004). Larvae hatch after approximately 10 days, drop to the ground and begin to feed on milkweed roots and rhizomes (Farrell & Mitter 2008). Adults and larvae co-occur on individual milkweed plants from mid- to late summer. Before and after this period, the two life stages are separated in time and in space, with adults living above-ground early in the season and larvae inhabiting the rhizosphere in later summer and fall (Erwin *et al.* 2014). Larvae overwinter in earthen cells as prepupae (Agrawal 2004).

Traits of common milkweed are known to influence fitness measures of adult and larval *T. tetraophthalmus*. Above-ground, the abundance of adult beetles is positively correlated with plant height and flowering, while negatively correlated with the amount of latex exudation and foliar trichome density (Agrawal 2004). Below-ground, larval survival is negatively correlated with root cardenolides; this pattern has been observed among genotypes of common milkweed (Rasmann & Agrawal 2011b) as well as different milkweed species (Rasmann & Agrawal 2011a). Although it is known that adults contain cardenolides from their milkweed host plants (Duffey & Scudder 1972), it is unclear to what extent these cardenolides are directly sequestered by adults or transferred from larval root feeding to the adult stage. Cardenolides are present in adult elytra, suggesting that some transfer from larvae to adults occurs (A.A. Agrawal, unpublished data).

Tetraopes tetraophthalmus and *T. texanus* larvae used in experiments were hatched in the laboratory from field-collected adults from multiple sites in Tompkins Co., NY, USA and Montgomery Co., AL, USA, respectively. Adults were placed in containers with dried grass stems for mating and oviposition. Stems were inspected for oviposition markings and removed from adult

colony containers as needed. Emerging neonates were collected and placed on plant roots in experiments outlined below.

ENTOMOPATHOGENIC NEMATODES

The entomopathogenic nematodes, *Heterorhabditis bacteriophora* used in our experiments, were descendants of field-collected specimens from caged sentinel larvae in milkweed fields with *Tetraopes* beetle larvae feeding on plant roots during the fall of 2013 (Tompkins County, as described in Erwin *et al.* 2014). Using these specimens assured we were evaluating populations of natural enemies that actively hunt and kill *Tetraopes* larvae. All nematodes were cultured in last-instar larvae of the greater wax moth, *Galleria mellonella*, at approximately 25 °C according to procedures described in Kaya & Stock (1997). Infective juveniles (IJs) that emerged from insect cadavers into White traps (White 1927) were stored in shallow water in transfer flasks at 15 °C for up to 2 week prior to use. *Heterorhabditis bacteriophora* are more attracted to *T. tetraophthalmus*-damaged plants than to healthy plants, and this was reflected in the 80% higher infection rate of sentinel larvae near *T. tetraophthalmus*-damaged plants compared to controls (Rasmann *et al.* 2011). In field experiments, *Tetraopes* larvae reduced plant biomass by 40% compared to unattacked plants, but this effect was almost completely attenuated in the presence of nematodes (30 000 infective juveniles m⁻²) (Rasmann *et al.* 2011).

EXPERIMENTAL DESIGN

Six neonate larvae of each beetle species were applied to the soil surrounding roots of each milkweed plant from the four plant species that were potted separately (four plant species \times 2 herbivore species \times 10 replicates = 80 replicate plants). Six weeks after applying the larvae, we destructively harvested all plants and larvae. Below-ground root material was gently washed in water and larvae were separated out. The number of recovered larvae divided by the number of introduced larvae represents the per cent larval survival. All root material was oven-dried at 50 °C for 3 days and

then ground to powder in a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA). The total cardenolide concentration of the roots was measured by analysing 100 mg of ground powder via HPLC (see below). All recovered larvae were first weighed alive and then were either allocated to an EPN performance assay (live) or frozen for cardenolide analysis. We processed a maximum of ten larvae for each plant–herbivore combination. Larvae used for cardenolide analysis were kept frozen at -80°C for 3 days and then oven-dried at 50°C as described for root tissue. The oven-dried larvae were weighed and ground prior to extraction using methods described above.

Larvae used for nematode bioassay were rinsed and placed in Petri dishes with moistened filter paper individually. Hundred infective juvenile nematodes were applied to each larva. The Petri dishes were closed and sealed with parafilm (Menasha, WI, USA). Larvae and nematodes were monitored for 4 weeks. The resulting total EPN emerged were counted and standardized by dividing the total number EPN emerged by the initial fresh mass of the larva on the date of inoculation.

HPLC ANALYSIS OF CARDENOLIDES

Ground tissue was extracted with 1.9 mL of 95% ethanol in a sonicating water bath at 55°C for 20 min and spiked with $20\ \mu\text{g}$ of digitoxin (Sigma, St. Louis, MO, USA) as an internal standard. Samples were analysed by HPLC using a Gemini C18 reversed-phase column ($3\ \mu\text{m}$, $150 \times 4.6\ \text{mm}$; Phenomenex, Torrance, CA, USA) and an Agilent 1100 series instrument with diode array detection. The $15\text{-}\mu\text{L}$ injection was eluted at a constant flow of $0.7\ \text{mL min}^{-1}$ with a gradient of acetonitrile and 0.25% phosphoric acid in water as follows: 0–5 min 20% acetonitrile; 20 min 70% acetonitrile; 20–25 min 70% acetonitrile; 30 min 95% acetonitrile; and 30–35 min 95% acetonitrile. Absorbance spectra were recorded from 200 to 400 nm and peaks were detected at 218 nm. Peaks showing a characteristic symmetrical absorption band with a maximum between 217 and 222 nm were recorded as cardenolides (Malcolm & Zalucki 1996). Concentrations were calculated by relating peak areas to the internal standard.

STATISTICAL ANALYSIS

We compared effects of host switching in two ways. The first analysis compared the beetle performance on all four plant species separately using a one-way ANOVA. We also examined the effects and differences in responses of the two beetles when swapped on their reciprocal native hosts. A two-way ANOVA was used to analyse the impacts of beetle species, plant species and their interaction on each of the responses (per cent survival, mass of survivors, larval cardenolide concentration and EPN numbers) when evaluating effects from the two beetles on their native and alternate host plants. This latter analysis specifically tests the role of host plant species for trade-offs in performance between the two beetle species. Minor differences in degrees of freedom between treatments and response variables resulted from differential recovery of bioassay insects or lost plant samples. All analyses were conducted using JMP (Version 11.2; SAS Institute Inc., Rockville, MD, USA). Tukey HSD was used to compare means between treatments. Cohen's d was calculated to measure effect size.

Results

Root cardenolides were about four times higher in *A. asperula* and *A. viridis* than in *A. syriaca* and *A. exaltata* (plant species, $F_{(1,63)} = 0.07$, $P < 0.0001$, Fig. 1), and levels were not changed by feeding of the different beetle species in

comparison with undamaged plants nor the interaction between beetle species identity and plant species ($F_{(1,63)} = 0.31$, $P = 0.57$, Plant \times Beetle $F_{(3,63)} = 23.5$, $P = 0.97$).

Larvae of *T. tetraophthalmus* had marginally higher survival than larvae of *T. texanus* ($F_{(1,70)} = 3.43$, $P = 0.07$, Fig. 2), and there were significant differences in per cent survival across the four plant species for both beetles ($F_{(3,70)} = 18.37$, $P < 0.0001$). Overall, both beetle species survived best on *A. syriaca* (46%), followed by *A. exaltata* (29.5%) and *A. viridis* (23%), and the fewest larvae surviving on *A. asperula* (13%). However, performance of *T. tetraophthalmus* was strongly impacted by plant species, while *T. texanus* survival was quite consistent, but lower, across the four host plants, resulting in a highly significant interaction term between plant and beetle species ($F_{(3,70)} = 4.52$, $P = 0.0059$, Fig. 2).

The weight of the surviving *T. tetraophthalmus* larvae was highest on *A. exaltata*, while larvae weighed less on the remaining three plant species with no significant difference between them ($F_{(3,32)} = 20.40$, $P < 0.0001$, Fig. 3a). Larval weights of *T. texanus* followed a similar, but only marginally significant pattern ($F_{(3,32)} = 2.39$, $P = 0.08$, Fig. 3b), thus essentially growing equally well on all four plant species. When we compared the effects of native host switching between the two beetle-associated plants, we found that effects on growth were only determined by beetle species, with *T. tetraophthalmus* weighing 54% more than *T. texanus* ($F_{(1,29)} = 41.055$, $P < 0.0001$) regardless of plant species ($F_{(1,29)} = 0.50$, $P = 0.49$) and the interaction ($F_{(1,29)} = 1.14$, $P = 0.29$).

SEQUESTRATION AND TRITROPHIC INTERACTIONS

We found sequestered cardenolides in all beetle larvae; nonetheless, there were significant differences in the

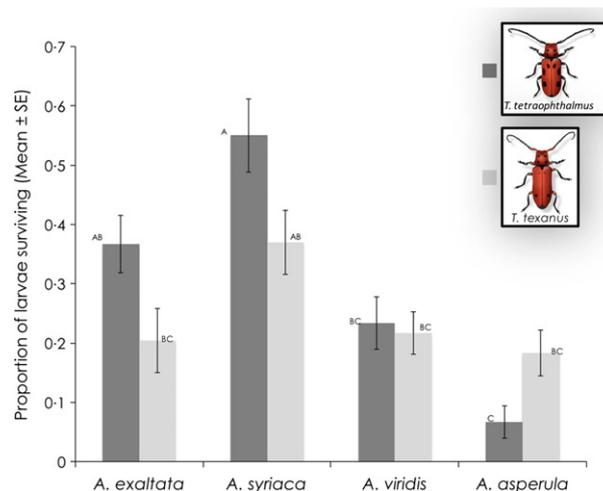


Fig. 2. The survival of the two milkweed beetles (*Tetraopes tetraophthalmus* and *T. texanus*) on four species of milkweed. Shown are means \pm SE. Different letters (e.g., A,B, etc.) correspond to means with P -value significance < 0.05 . Levels not connected by same letter are significantly different.

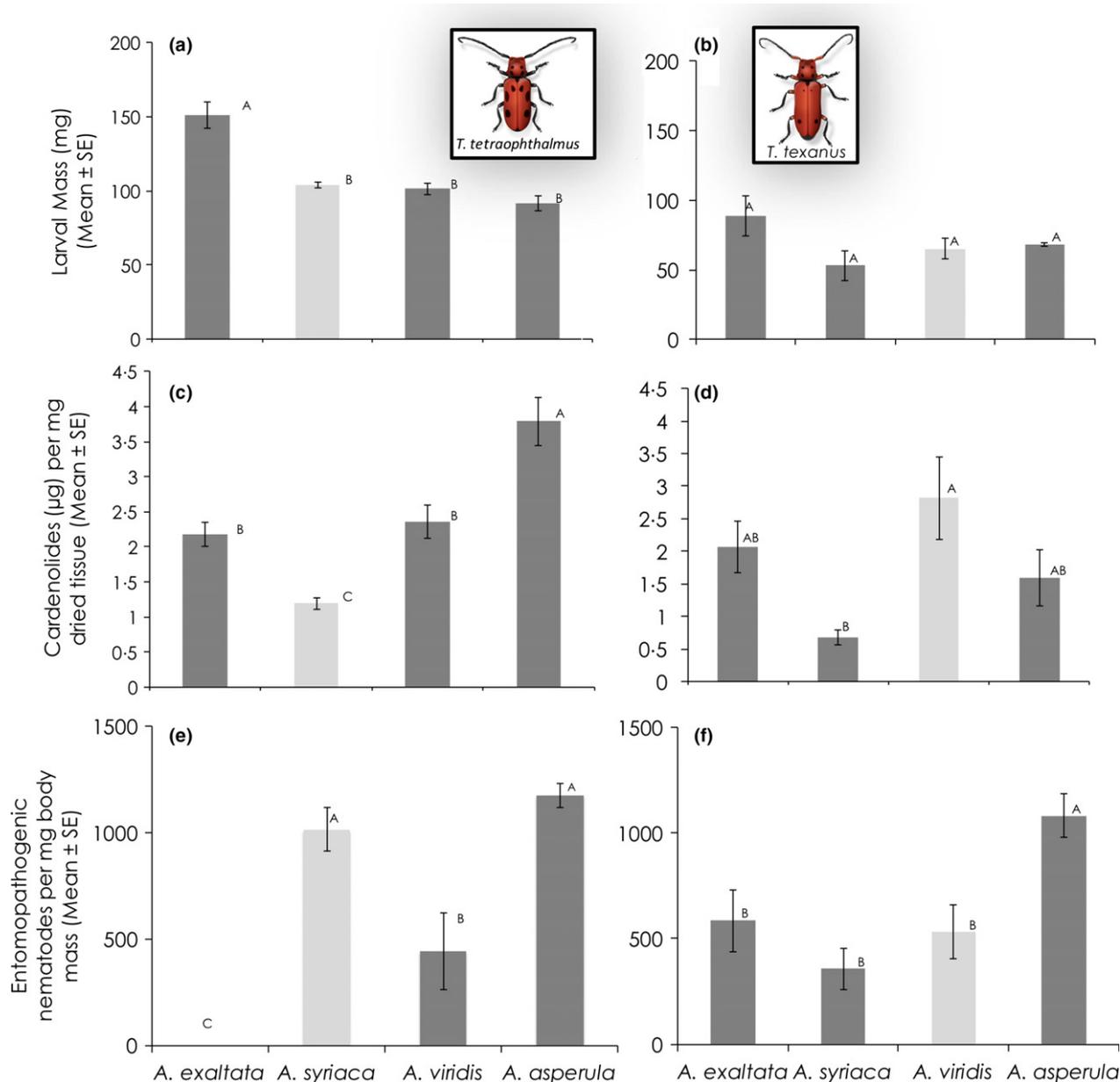


Fig. 3. Measurements of performance traits from milkweed beetles (*Tetraopes tetraophthalmus* (left column), and *T. texanus* (right column)) on four species of milkweed. Error bars are mean \pm SE. Plants are arranged in ascending order of toxicity from left to right (*Asclepias exaltata*, *A. syriaca*, *A. viridis*, *A. asperula*). The native host species is indicated by grey shading. (a, b) Mass of larvae feeding on the four species. (c, d) Mean cardenolides isolated from larvae feeding on the four milkweed species. (e, f) Mean number of entomopathogenic nematodes recovered from individual larvae standardized by larval weight. Different letters (e.g., A,B, etc.) correspond to means with P -value significance < 0.05 . Levels not connected by same letter are significantly different.

amount beetles sequestered from each plant species for both *T. tetraophthalmus* ($F_{(3,31)} = 21.68$, $P = < 0.0001$) and *T. texanus* ($F_{(3,26)} = 3.45$, $P = 0.03$). *Tetraopes tetraophthalmus* had the highest level of cardenolides when feeding on *A. asperula* (threefold higher than lowest plant), intermediate levels when feeding on *A. exaltata* and *A. viridis* and the lowest levels on its preferred host *A. syriaca* showing a strong overall effect size for cardenolide concentration (Cohen's $d = 1.54$) (Fig. 3c). In contrast, *T. texanus* sequestered the most cardenolides on its native host, *A. viridis* (fourfold higher

concentration than the lowest plant (Cohen's $d = 0.88$), although not significantly more than either *A. exaltata* or *A. asperula*, all of which were higher levels than those recovered from *T. texanus* when feeding on *A. syriaca* (Cohen's $d = -0.96$) (Fig. 3d). When we compare just the two-way ANOVA effects between the two beetles feeding on the two beetle-associated plants, we found that effects of cardenolide sequestration are plant driven, where beetles feeding on the higher cardenolide containing plant sequester more ($F_{(1,28)} = 15.26$, $P = 0.0005$). We found no significant effects for beetle

species ($F_{(1,28)} = 0.007$, $P = 0.94$) or an interaction of beetles with plant species ($F_{(1,28)} = 1.31$, $P = 0.26$).

Our results for the effects of host plants on nematode emergence indicate that EPNs developed and were lethal on seven of the eight plant–beetle combinations; the sole exception was for *T. tetraophthalmus* when feeding on *A. exaltata* (Fig. 3e). Overall, we found the highest production of EPNs in *T. tetraophthalmus* that had been feeding on *A. asperula* and *A. syriaca*, with 80% lower numbers emerging from *A. viridis* ($F_{(3,35)} = 37.09$, $P < 0.0001$). For *T. texanus*, we again recovered most nematodes from larvae fed *A. asperula*, but larvae on *A. exaltata*, *A. syriaca* and *A. viridis* had 75% less nematodes emerging and were not significantly different from one another ($F_{(3,32)} = 6.39$, $P = 0.0016$) (Fig. 3f). When we compared the effect of switching the native-plant–beetle-associated pairs, we found an interactive effect of beetle and its host plant species: *T. tetraophthalmus* had significantly more nematodes produced when feeding on its native host (*A. syriaca*) in comparison with the larvae that fed on *A. viridis* and *T. texanus* on *A. syriaca* (Beetle, $F_{(1,32)} = 4.87$, $P = 0.0359$; Plant, $F_{(1,32)} = 2.29$, $P = 0.14$; Beetle \times Plant, $F_{(1,32)} = 8.29$, $P = 0.0074$).

Discussion

Trade-offs in host plant use driven by plant chemistry have been argued to play a central role in herbivore specialization (Schultz 1988; Dyer & Floyd 1993; Bowers & Stamp 1997). Sequestering is typically a double-edged sword for specialist herbivores, and increased levels of toxins are often not linearly correlated with benefits (Ali & Agrawal 2012). In systems where adapted herbivores rely on secondary compounds as sequestered defences, an herbivore's ability to maximize fitness on alternative hosts is expected to be reduced. In other cases, adapted herbivores may be susceptible to the negative effects of plant defences even though they prefer those plants (Adler, Schmitt & Bowers 1995), and tritrophic factors, such as reduced predator pressure, may be more important than optimal growth when specialization occurs on a particular host (Janzen 1985; Bernays & Graham 1988; Joshi & Thompson 1995b; Fry 1996; Björkman, Larsson & Bommarco 1997). We found that two specialist beetles feeding on milkweeds had very distinct responses to host switching, and the traits leading to specialization in this system are not easily understood and other factors such as alternate secondary compounds or nutrients may be playing a role in these different plant species. However, we have some evidence, from both survivorship and growth, that herbivores that have an evolutionary history feeding on more toxic plants are less affected by alternative plants than those species which are commonly associated with less toxic host plants.

We found that two beetles feeding on their preferred host did not meet expectations for optimal growth and defence in tritrophic interactions. Although survival of *T. tetraophthalmus* was highest for larvae feeding on its native

host *A. syriaca*, these larvae had only average mass, sequestered low levels of cardenolides and were poorly protected from EPNs. In contrast, larvae feeding on *A. exaltata* had a higher mass and were not susceptible to parasitization by EPNs, suggesting that *A. exaltata* should be a better host for *T. tetraophthalmus*. However, even though *A. exaltata* has a largely overlapping geographic distribution with *A. syriaca*, it is not attacked by any *Tetraopes* sp. in the field, perhaps because it typically grows in the forest understorey where *Tetraopes* does not forage. For *T. texanus*, host switching had little effect on the herbivore's performance, with the exception of lower sequestration on alternate host plants compared to its native *A. viridis*. Below we discuss how these trends reveal features of host specialization in these two related beetles.

First, we see that host shifting affected *T. texanus* < *T. tetraophthalmus*. *Tetraopes texanus* diverged earlier in the lineage than *T. tetraophthalmus*, feeding on an earlier diverging milkweed (Farrell & Mitter 2008). Our findings contribute to the understanding of phylogenetic trends in host defence, where more derived milkweed species were found to produce progressively lower concentrations of cardenolides (Agrawal & Fishbein 2008). Here we report that the beetle which has adapted to the more basal (and more toxic) plant species is slower growing in the absence of natural enemies, but has the ability to tolerate plant toxicity (cardenolides). Nonetheless, when feeding on non-host plants with lower cardenolide levels, larvae were found to sequester lower levels than the range typically experienced when feeding on its preferred host. *Tetraopes tetraophthalmus*, which specializes on a derived (and far less toxic) plant species, experienced elevated levels of the plant toxins when feeding on non-hosts. It seems as though having sequestered these elevated levels of toxins had negative consequences for growth and survival (in the absence of natural enemies). Rasmann *et al.* (2009) found a similar trend leading to decreased survival and performance of *T. tetraophthalmus* on alternative hosts with increasing cardenolides. Thus, the more derived *T. tetraophthalmus* appears to have evolved a strategy of faster growth on a less toxic diet, and as is discussed below, greater sensitivity to the third trophic level may be a consequence of this strategy. Growth rate in herbivores is an important response variable linking top-down (i.e. predators) to bottom-up (i.e. plant chemistry) pressures (Benrey & Denno 1997). Although we did not measure development time *per se*, by utilizing growth of larvae that were all started on the same time on different plants, larval mass at the time of infection represents vigour and serves as an indirect measure of development time.

Studies on tritrophic interactions between phytophagous insects, their food plants, predators and parasites have highlighted the role of environmental factors on parasite infection and virulence (reviewed in Cory & Hoover 2006). For example, studies on baculoviruses have shown that both host mortality rate (e.g., Keating, Yendol & Schultz 1988; Forschler, Young & Felton 1992; Farrar & Ridgway

2000) and viral replication (Ibrahim Ali *et al.* 2002; Raymond *et al.* 2002) can vary depending on the host plant on which infection occurs. We observed similar trends in our system, where a particular combination of plant and herbivore species resulted in no parasite-associated mortality, most likely independent of cardenolide sequestration and more related to general vigour of larvae in this treatment combination. However, we note that nematodes are not the only 'enemies' of these root herbivores. Furthermore, our current understanding of the milkweed-insect sequestration systems leads us to believe that aposematic coloration and sequestration may be primarily targeted at vertebrate members of the third trophic level. In other words, faster growth (and low sequestration) may still trade-off with resistance to enemies in some tritrophic pairings other than EPNs.

Yet, a paradox still exists in sequestering specialist insects, which may be protected from some, but certainly not all enemies. Although it has been suggested that sequestration by insects leads to reduced establishment, preference and performance of their parasites (Cory & Hoover 2006), it has also been proposed that these parasites should evolve a preference for defended hosts that represent a safe haven, especially from predators (enemy free space, Dyer 1995). We found that overall higher amount of toxins sequestered by beetle larvae resulted in better success of EPNs, suggesting that EPNs are unaffected by cardenolides or that the beetle's immune system may have been compromised when dealing with the plant toxins. Indeed, sequestration of toxins may lead to a reduction in the herbivores' immunocompetence, even if a specialist can tolerate these secondary metabolites (Smilanich *et al.* 2009).

Our observations with larvae feeding on the very toxic *A. asperula* suggest effects from direct defences (i.e. cardenolides) and indirect defences (i.e. EPN) do not necessarily trade off, but can have a reciprocal augmentative effect. Thus, it is not surprising given that this plant species is not selected as a host for any *Tetraopes* species. Meanwhile, our observation on *A. exaltata* shows the opposite effect, suggesting that relationships between both direct and indirect defences differ by plant species, and limitation in an herbivore's utilization of a host in this system is not directly linked to escaping predator/parasite pressures. Considering the consequences of plant provisioning of defences on the performance of the third trophic level, including diverse predators and parasites, should be considered in studies which attempt to understand adaptive host relationships.

The observed consequences for tritrophic interactions between milkweeds, highly specialized root herbivores and entomopathogenic nematodes did not fit optimal predictions for herbivores that have specialized on a host, suggesting that specialization in this system is driven primarily by a plant-herbivore interaction. The extent to which the third trophic level is impacted by plant toxins such as cardenolides is critical in understanding the extent to which

tritrophic trade-offs will occur (Price *et al.* 1980). Cardenolides are known to impact many vertebrates and invertebrates, but not all (Agrawal *et al.* 2012), and thus, EPNs may be a natural enemy that does not enhance trade-offs in the evolutionary interactions between milkweed and *Tetraopes*.

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Data accessibility

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.3r041> (Ali & Agrawal 2016).

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