doi:10.1111/j.1558-5646.2012.01693.x

FOOD PLANT-DERIVED DISEASE TOLERANCE AND RESISTANCE IN A NATURAL BUTTERFLY-PLANT-PARASITE INTERACTIONS

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Received December 24, 2011
Accepted April 23, 2012
Data Archived: Dryad doi: 10.5061/dryad.82j66

Organisms can protect themselves against parasite-induced fitness costs through resistance or tolerance. Resistance includes mechanisms that prevent infection or limit parasite growth while tolerance alleviates the fitness costs from parasitism without limiting infection. Although tolerance and resistance affect host–parasite coevolution in fundamentally different ways, tolerance has often been ignored in animal–parasite systems. Where it has been studied, tolerance has been assumed to be a genetic mechanism, unaffected by the host environment. Here we studied the effects of host ecology on tolerance and resistance to infection by rearing monarch butterflies on 12 different species of milkweed food plants and infecting them with a naturally occurring protozoan parasite. Our results show that monarch butterflies experience different levels of tolerance to parasitism depending on the species of milkweed that they feed on, with some species providing over twofold greater tolerance than other milkweed species. Resistance was also affected by milkweed species, but there was no relationship between milkweed-conferred resistance and tolerance. Chemical analysis suggests that infected monarchs obtain highest fitness when reared on milkweeds with an intermediate concentration, diversity, and polarity of toxic secondary plant chemicals known as cardenolides. Our results demonstrate that environmental factors—such as interacting species in ecological food webs—are important drivers of disease tolerance.

KEY WORDS: **Host–parasite interactions, milkweed, monarch butterfly, *Ophryocystis elektroscirrha*, resistance, tolerance.**

Because parasites pose a major threat to free-living species, natural selection should strongly favor the evolution of host defenses that limit parasite-induced fitness loss (Combes 2001). In principle, hosts can evolve two distinct defense mechanisms: resistance and tolerance (Råberg et al. 2007, 2009; Boots 2008). Resistance encompasses behavioral, physiological, and genetic mechanisms that reduce infection probability or parasite growth upon infection. In contrast, tolerance mechanisms do not reduce parasite infection or growth, but instead alleviate the fitness consequences of parasite infection. Both types of defense limit fitness costs to the host from parasitism but they vary critically in their effects on parasites. Specifically, resistance limits parasite fitness while tolerance does not (Boots 2008; Svensson and Råberg 2010).

These varying effects have important consequences for the long-term coevolution of hosts and parasites (Boots and Bowers 1999; Roy and Kirchner 2000; Rausher 2001; Restif and Koella 2004; Miller et al. 2005, 2006; Svensson and Råberg 2010).

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Theoretical models of the evolution of host defenses predict that genetic variation in resistance will be maintained but tolerance mechanisms will become fixed (Boots and Bowers 1999; Roy and Kirchner 2000; Miller et al. 2006; but see Best et al. 2008). The reason for this difference is that resistance results in a negative epidemiological feedback where parasite infection selects for resistant hosts and this reduces parasite prevalence in the population. Assuming that resistance is costly, low parasite prevalence then reduces selection for resistance and susceptible hosts are favored. In contrast, tolerance evolution results in positive feedback where parasite infection selects for tolerant hosts. Tolerant hosts increase parasite transmission, which results in greater parasite prevalence and continuing selection for tolerant hosts. Because tolerance does not reduce parasite infection or transmission, it has been suggested that disease treatments based on tolerance are less likely to select for countermeasures in parasites than are treatments based on disease resistance (Roy and Kirchner 2000; Rausher 2001; Schneider and Ayres 2008). It has also been suggested that increased host tolerance may lead to increased parasite virulence (Restif and Koella 2004; Miller et al. 2006), and additional work will be necessary to determine how tolerance affects host–parasite coevolution dynamics (Little et al. 2010).

The distinction between resistance and tolerance has long been recognized in plants that suffer attack from herbivores (e.g., Fineblum and Rausher 1995; Mauricio et al. 1997; Tiffin and Rausher 1999; Simms 2000) and parasites (e.g., Simms and Triplett 1994; Koskela et al. 2002; Kover and Schaal 2002; Carr et al. 2006). That animals also show both resistance and tolerance to enemies has received attention only recently (e.g., Corby-Harris et al. 2007; Ayres and Schneider 2009). Because tolerance per se is difficult to measure (Råberg et al. 2007; Boots 2008; Råberg et al. 2009) studies have mainly investigated whether host genotypes vary in their levels of tolerance, usually measured as variation in the slopes of the relationships between host fitness and parasite burden (Råberg et al. 2007; Blanchet et al. 2010; Rohr et al. 2010; Lefèvre et al. 2011; Soler et al. 2011). Although these studies are a noteworthy step forward, they are entirely focused on tolerance as a genetically determined trait. This is a major limitation because, in addition to varying genetically, hosts and parasites in nature interact within a larger ecological community (Lafferty et al. 2006). Interacting species can affect traits such as host resistance and parasite virulence (Wolinska and King 2009; De Roode et al. 2011b; Parker et al. 2011; Sternberg et al. 2011) and it is possible that tolerance is also affected by such interactions. By isolating hosts and parasites from their environment, we may erroneously conclude that hosts do not use tolerance as a defense or that there is no variation in this trait.

Here we explicitly test how the environment in which hosts and parasites interact can provide hosts with tolerance and resistance to their parasites. We focus on monarch butterflies (*Danaus plexippus*) and their naturally occurring protozoan parasite *Ophryocystis elektroscirrha* (McLaughlin and Myers 1970). In this system, infections occur when larvae ingest parasite spores on eggs or milkweed plants (genus *Asclepias*). Spores lyse in the gut and parasites penetrate the intestinal wall to undergo asexual and sexual replication in the hypoderm; parasites then form spores around the scales of the developing butterfly, such that adult monarchs emerge covered with dormant spores on the outsides of their bodies (McLaughlin and Myers 1970). Parasites do not replicate on adults, and spores must be ingested by larvae to cause new infections. Most parasite transmission occurs from infected butterflies to their offspring, when females scatter spores on eggs and milkweed during oviposition (Altizer et al. 2004).

The monarch-parasite system is ideally suited for testing the effect of environment on host resistance and tolerance because monarchs and their parasites have an obligate interaction with milkweed plants, which monarchs use as their larval food plants (Ackery and Vane-Wright 1984). Previous work has shown that certain milkweed species reduce infection and growth of *O. elektroscirrha* in monarch larvae, most likely due to the presence of milkweed toxic secondary chemicals known as cardenolides (De Roode et al. 2008a; Lefèvre et al. 2010; De Roode et al. 2011a,b). Here we infected and reared monarch larvae on 12 species of milkweed, and we quantified the cardenolides present in milkweed foliage. We show that there is a gradient of resistance to *O. elektroscirrha* conferred by the 12 milkweed species, and that the cardenolide composition of the milkweed plants affects the fitness of both infected and uninfected monarchs. Importantly, we show that milkweed species can provide disease tolerance to monarch butterflies, and that this tolerance is associated with milkweed cardenolides. Hence, we demonstrate that an environmental variable can confer disease tolerance to an animal host.

Methods

EXPERIMENTAL PROCEDURE

The monarchs used in this experiment were the noninbred grand-progeny of monarchs collected from Pismo Beach, CA, USA These monarchs are part of a large panmictic genetic population inhabiting North America (Lyons et al. 2012). Mated females were provided with *A. incarnata* for oviposition and eggs were manually transferred to leaves from one of 12 food plant species. The species of plants used were: *A. curassavica*, *A. eriocarpa*, *A. erosa*, *A. fascicularis*, *A. incarnata, A. physocarpa*, *A. purpurascens*, *A. speciosa*, *A. sullivantii*, *A. syriaca*, *A. tuberosa*, and *A. verticillata*. With the exception of *A. physocarpa*, all of these species are widely distributed throughout North America (Woodson 1954; Hickman 1993), thus making them ecologically relevant species for the North American monarch

population (Malcolm and Brower 1989). All plants used in this experiment were grown under uniform conditions in a climate-controlled greenhouse, from seeds obtained from Butterfly Encounters, CA.

Upon hatching, larvae were randomly assigned a single, unique plant and transferred to petri dishes with leaves from their plant. Two days after hatching, larvae were transferred into fresh petri dishes containing leaf discs from their assigned plants. Larvae were inoculated by manually depositing 10 parasite spores on the leaf discs, while control monarchs received clean discs (De Roode et al. 2007, 2008a). The parasite spores used for inoculation came from a clonal line (denoted C1C10-P2–3), originally isolated from a monarch collected in California, USA.

After consuming their leaf discs, larvae were placed in individual plastic containers with florist tubes holding cuttings from their assigned plants. These containers were kept in a climate-controlled room at 26°C on a 16L:8D light cycle, and checked daily until pupation. Fresh cuttings of each larva's assigned plant were provided as needed. If the individual plant was not big enough to feed the monarch until pupation, randomly selected cuttings of the same species were used. Although some monarchs consumed foliage from multiple individuals, previous studies have shown that the milkweed fed after infection has no effect on adult monarch longevity or parasite burden (De Roode et al. 2011a).

Monarchs were transferred to a new room (also held at 26°C, 16L:8D) 6 days after pupation to prevent parasite contamination of the larval rearing room by emerging infected adults. When the monarchs eclosed, they were sexed, then placed in individual glassine envelopes, held at 12°C and checked daily for death. The difference in days between eclosion and death under these conditions provides a combined measure of longevity and starvation resistance (referred to as adult longevity). Previous experiments have shown that adult longevity is an important component of monarch fitness and the effects of infection and parasite burden on monarch longevity under starvation conditions are similar to the effects under more natural, nonstarvation, conditions (De Roode et al. 2008b, 2009).

After the monarchs died, we quantified their parasite burden (referred to as spore load) by vortexing their bodies for 5 min in 5 mL of water to shake off the parasite spores, and then counting the spores using a hemocytometer (De Roode et al. 2007, 2008b).

COLLECTING AND MEASURING CARDENOLIDES

To assess effects of plant chemistry on parasite infections, we quantified the foliar cardenolides of the plants assigned to infected monarchs. When leaves were collected for inoculations, we also obtained samples for chemical analysis. Six leaf discs were collected into methanol and stored at −80°C until analysis, as described previously by Vannette and Hunter (2011). Six additional leaf discs were oven-dried overnight to estimate sample dry weights. The cardenolides were analyzed using reverse-phase high-performance liquid chromatography (HPLC). Digitoxin was used as an internal standard, and absorbance spectra were recorded from 200 to 300 nm. Peaks were detected by diode array at 218 nm and those with symmetrical absorbance maxima between 217 and 222 nm were considered to be cardenolides (Malcolm and Zalucki 1996). The concentration of each peak was calculated relative to the internal standard and the total cardenolide concentration of each plant was the sum of the peaks.

In addition to assessing total cardenolide concentration, we calculated two additional measures of the chemical community present in the milkweed: diversity and relative polarity. Diversity was quantified by adapting the Shannon–Wiener index H, taken from the biodiversity literature (as described by Rasmann and Agrawal 2011). This index measures the number of different cardenolides present in a plant as well as the evenness of their distribution, and it is calculated as—sum($P_i$log[P_i]), where P_i is the relative amount of a given cardenolide in a plant. Polarity was calculated using sum(P_iRT_i), where RT_i is the retention time of a given peak, weighted by the relative amount of the peak (P_i) (Rasmann and Agrawal 2011). Under reverse-phase HPLC, cardenolide retention time increases as polarity decreases; therefore, our polarity index increases with the presence of more nonpolar cardenolides. More nonpolar cardenolides are thought to be an important mediator of food plant effects on other species due to their increased toxicity (e.g., Fordyce and Malcolm 2000; Zehnder and Hunter 2007).

STATISTICAL ANALYSIS

Logistic regression by generalized linear model (GLM with binomial error distribution, logit link) was used to assess effects of food plant species on monarch survival to adulthood and infection probability. GLMs with normal error distributions were used to examine effects of food plant species on the $\log_{10}$-transformed parasite spore load of infected monarchs, the effects of food plant species on the longevity of all monarchs, and the effects of parasite spore load on the longevity of infected monarchs. Tolerance was measured as the slope of a regression line between square-root-transformed spore load (a measure of parasite burden) and monarch longevity (a measure of host fitness) in infected and control monarchs (Mauricio et al. 1997; Simms 2000; Råberg et al. 2007; Blanchet et al. 2010; Lefèvre et al. 2011). We included the interaction between spore load (square-root-transformed) and food plant species in our model, to investigate whether tolerance varied in monarchs reared on different food plant species. We also included a quadratic term for square-root-transformed spore load (i.e., untransformed spore load) in our model to investigate the possibility of a nonlinear relationship between spore load and host fitness (Tiffin 2000; Råberg et al. 2007; Blanchet et al. 2010;

Lefèvre et al. 2011). Linear regression was used to test for a relationship between tolerance and resistance (measured as the inverse of mean spore load) (Råberg et al. 2007).

To assess effects of total cardenolide concentration, diversity, and nonpolarity on the longevity of infected monarchs, we used GLMs with normal error distributions. We included a quadratic term in all models to test for a nonlinear relationship between the measures of cardenolide chemistry and the longevity of infected monarchs. Again using GLMs, we assessed the effects of our measures of cardenolide chemistry on monarch tolerance to parasitism by associating tolerance with the log-transformed mean cardenolide concentration of each milkweed species.

We also compared cardenolide composition among milkweed species using permutational multivariate analysis of variance (PerMANOVA) (Anderson 2001) following Bray–Curtis ordination. Analysis was conducted using the Adonis procedure of the Vegan package in R version 2.7.1. We used metaMDS in Vegan for Nonmetric Multidimensional Scaling (NMDS) (McCune and Grace 2002), stepping down from a six-dimensional model to a one-dimensional model, with 999 permutations per model run and a maximum of 20 runs per dimension. Inspection of the scree plot illustrated that model stress declined rapidly from a one-dimensional to two-dimensional model, declining only slightly thereafter. We therefore used a two-dimensional model in subsequent analysis (model stress = 13.60, well within the range of 10–20 that is typical of ecological data (McCune and Grace 2002). We used both NMDS axes as independent variables in GLMs (normal error distribution) to associate milkweed cardenolide composition with monarch longevity.

Throughout our analyses, variables were transformed as necessary to ensure compliance with model assumptions and Fligner–Killeen tests were used to confirm homogeneity of variance (Crawley 2007). Minimal models were derived by removing terms, followed by model comparisons. Terms were retained in the model if their removal significantly ($P < 0.05$) reduced the explanatory power of the model (Crawley 2007). All analyses were carried out in R version 2.7.1.

Results

HOST FITNESS, PARASITE REPLICATION, AND FOOD PLANT SPECIES

A total of 463 out of 520 (89%) monarchs survived to adulthood, with 366 out of 409 (89%) inoculated monarchs surviving and 97 out of 111 (87%) control monarchs surviving. Inoculation with the parasite had no effect on the probability of larvae surviving to adulthood (GLM with binomial error distribution, likelihood ratio χ^2; $\chi^2 = 1.45$, df = 1, $P = 0.23$) whereas food plant species did ($\chi^2 = 26.1$, df = 11, $P = 0.01$); larval survival ranged from 80% on *A. purpurascens* to 100% on *A. verticillata*. The total number of surviving monarchs per plant ranged from 24 (inoculated = 20, control = 4) out of 30 larvae on *A. purpurascens*, to 49 (inoculated = 39, control = 10) out of 50 larvae on *A. physocarpa*. All subsequent analyses were restricted to monarchs that survived to adulthood. Analyses of parasite burden were restricted to infected monarchs, but analyses of tolerance included both infected and uninfected monarchs (Råberg et al. 2009; Svensson and Råberg 2010; Baucom and de Roode 2011).

Parasite infection did significantly reduce adult longevity in monarchs that survived to adulthood (Fig. 1A; $F_{1,407} = 867$, $P < 0.001$). Adult longevity also varied among milkweed species (Fig. 1A; $F_{11,407} = 4.48$, $P < 0.001$) for both infected and uninfected monarchs. Moreover, the effect of plant species on longevity differed between infected and uninfected monarchs (interaction between infection and plant species $F_{11,407} = 5.86$, $P < 0.001$). This interaction between infection status and plant species is clearly illustrated by comparing monarchs reared on *A. incarnata* and *A. curassavica* (Fig. 1A). Uninfected monarchs reared on *A. incarnata* lived longer as adults than those reared on *A. curassavica*; in contrast, infected monarchs had longer adult life spans when reared on *A. curassavica*, indicating that *A. curassavica* mitigates the reduction in monarch fitness due to parasitism.

Overall infection probability was high in all monarchs exposed to parasites, ranging from 23 out of 25 monarchs infected (92%) on *A. verticillata* to 100% on *A. eriocarpa* (35 monarchs), *A. physocarpa* (36 monarchs), *A. purpurascens* (19 monarchs), *A.sullivantii* (20 monarchs), and *A. tuberosa* (25 monarchs). We found no significant effect of plant species on the probability of infection ($\chi^2 = 0.447$, df = 11, $P = 0.95$). In monarchs that became infected, however, there was a significant effect of food plant on parasite spore load (Fig. 1B; $F_{11,312} = 3.27$, $P < 0.001$), as well as an effect of monarch sex ($F_{1,312} = 5.70$, $P = 0.02$). Some plant species (e.g., *A. curassavica* and *A. erosa*) exhibited antiparasitic effects such that monarchs reared on these species had a lower mean spore load than did monarchs reared on less antiparasitic plant species (e.g., *A. incarnata* and *A. tuberosa*). These results indicate that milkweed species can confer resistance (i.e., a reduction of parasite growth) to monarch butterflies. Because of a significant negative effect of spore load on infected adult longevity ($F_{1,312} = 3.82$, $P < 0.001$), the mean longevity of infected monarchs was negatively correlated with mean parasite burden across all food plant species (Fig 1B; $F_{1,10} = 12.92$; $R^2 = 0.56$, $P = 0.005$).

Critically, in addition to effects on disease resistance, we also observed effects of plant species on monarch tolerance to parasite infection. Specifically, the negative relationship between monarch longevity and parasite spore load varied significantly among plant species (Fig. 1C; plant species by spore load interaction $F_{11,387} = 2.66$, $P = 0.003$). This variation in slopes indicates

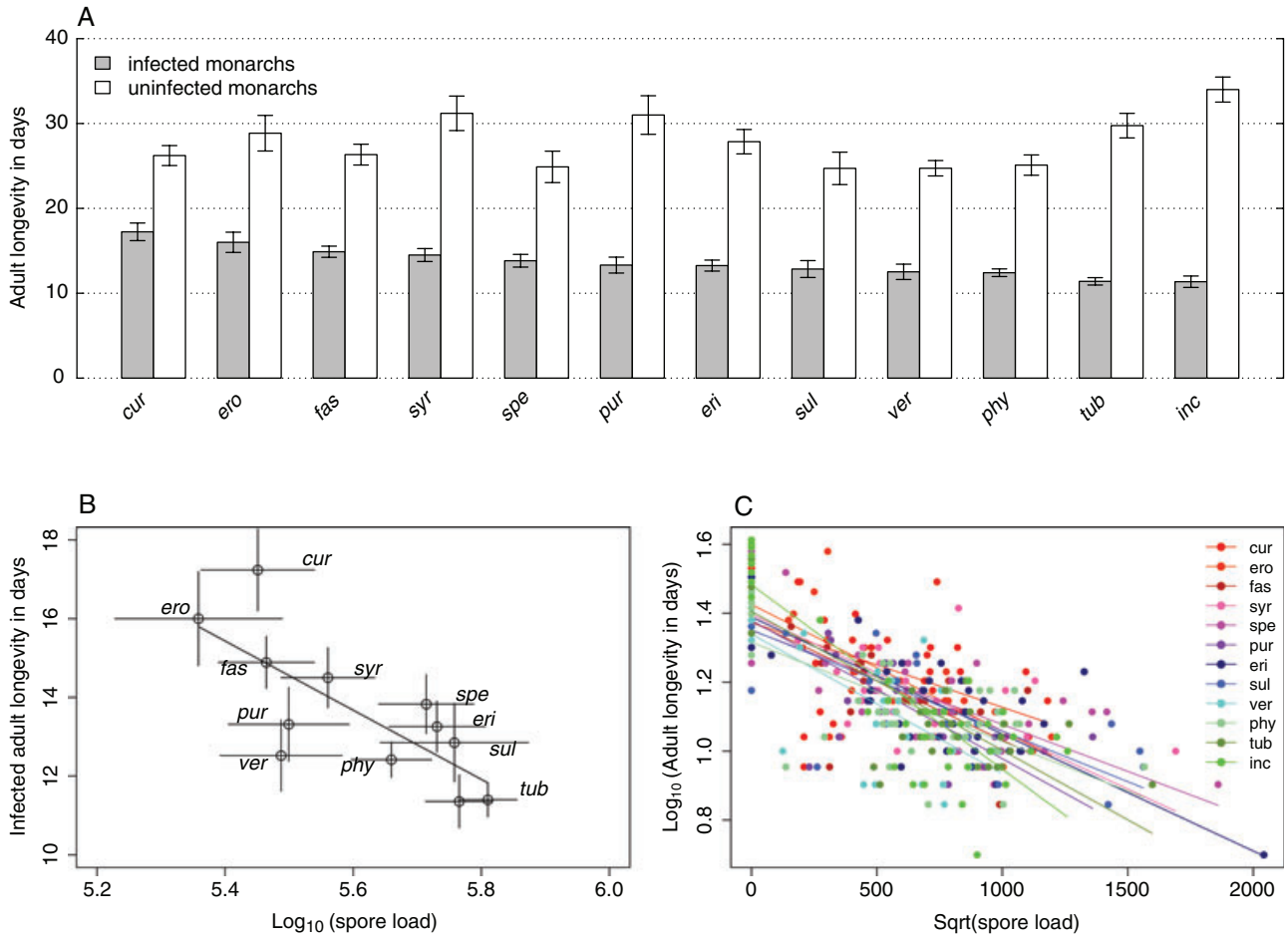

Figure 1. **Effects of milkweed food plant species on parasite spore load and monarch adult longevity. (A) Adult longevity of infected (gray) and uninfected monarchs (white) reared on the 12 milkweed species. Bars show mean longevity ± 1 SE. (B) Adult longevity and spore load of infected monarchs on the 12 milkweed species. The *x*-axis indicates parasite burden (i.e., the inverse of resistance) for each group of monarchs. Points indicate means for each plant species ± 1 SE; line indicates regression line. (C) Monarch adult longevity as a function of parasite spore load. Lines indicate species-specific regression lines. The differences in slopes of these lines indicate variation in tolerance. Data points indicate individual monarchs. (Three letter abbreviations used for plant species names: cur = *A. curassavica*; ero = *A. erosa*; fas = *A. fascicularis*; syr = *A. syriaca*; spe = *A. speciosa*; pur = *A. purpurascens*; eri = *A. eriocarpa*; sul = *A. sullivantii*; ver = *A. verticillata*; phy = *A. physocarpa*; tub = *A. tuberosa*; inc = *A. incarnata*.)**

that monarchs reared on different milkweed species vary in their ability to maintain fitness with increasing parasite loads, and thus indicates variation in tolerance. We also found a significant effect of the quadratic term for spore load ($F_{1,387} = 56.6$, $P < 0.001$), suggesting a nonlinear relationship between spore load and host fitness. We did not find evidence of an association, either negative or positive, between milkweed-conferred tolerance (measured as the slope of the regression of adult longevity and spore load) and resistance (measured as the inverse of spore load) ($F_{1,10} = 0.05$; $R^2 = 0.005$, $P = 0.8$). In combination with the observed effect of plant species on the longevity of uninfected monarchs and on parasite spore load, these results indicate that food plant species are crucial in determining host and parasite fitness via effects on tolerance and resistance.

FOOD PLANT CHEMISTRY AND HOST FITNESS

We began our analyses of plant chemistry with total cardenolide concentration as a straightforward measure of individual plant chemistry and we found no simple linear ($F_{1,315} = 0.03$, $P = 0.870$) or quadratic ($F_{1,315} = 2.48$, $P = 0.116$) relationship between the total concentration of cardenolides present in the plant and the longevity of infected monarchs reared on the plant. However, we noted that the average cardenolide concentration in *A. physocarpa* was over twofold higher than that in any other *Asclepias* species (Fig. 2A). The principle of hormesis predicts that plant toxins can have conflicting effects so that a smaller dose of toxins increases herbivore fitness while a larger dose decreases fitness (Kaiser 2003; Forbey and Foley 2009). There is some preliminary evidence for hormesis in our results, based on the

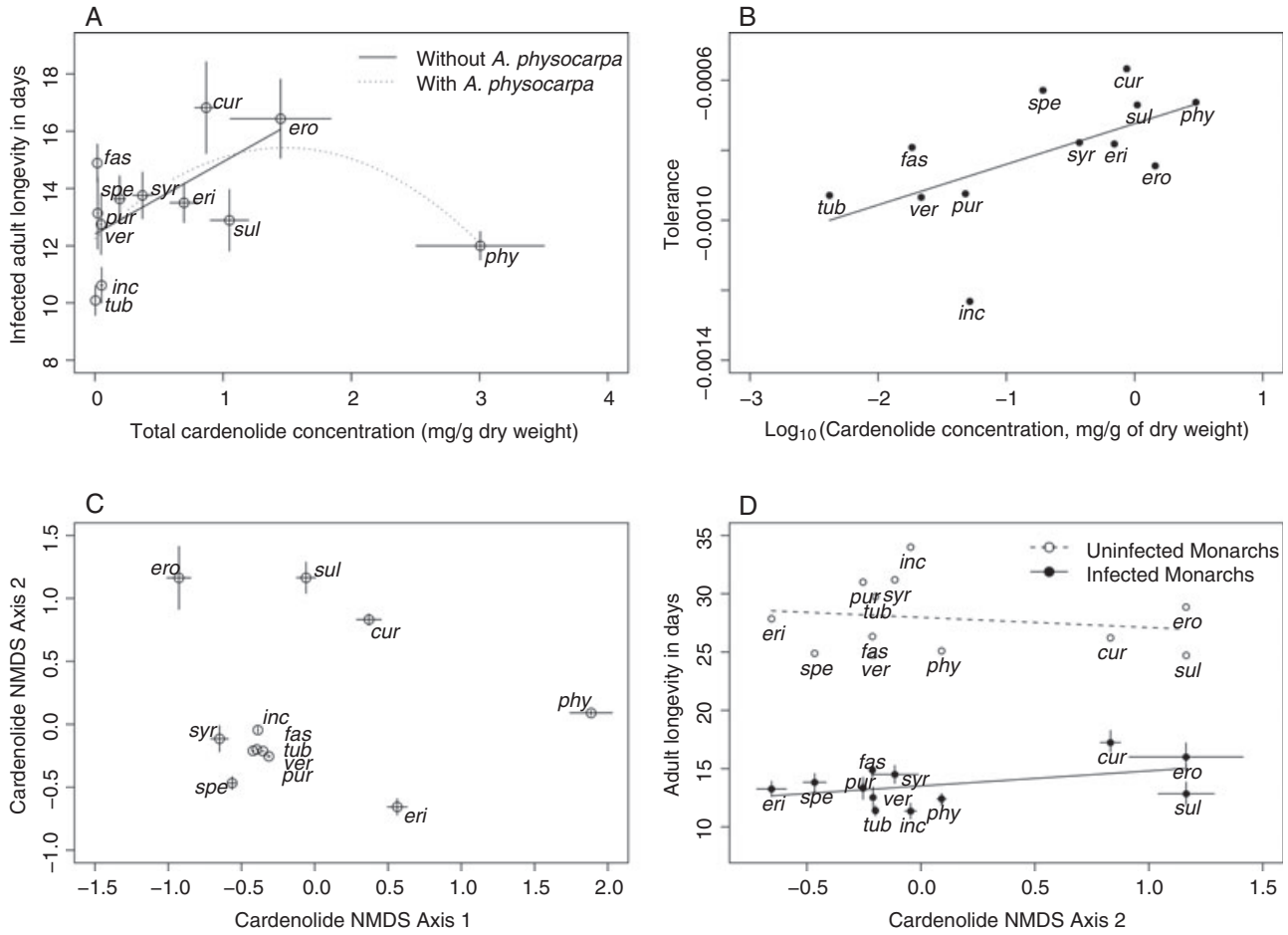

Figure 2. Associations between milkweed cardenolide chemistry and the fitness of infected monarch butterflies. (A) Average total cardenolide concentrations related linearly with monarch adult longevity (a fitness measure) when the outlier *A. physocarpa* was excluded, and nonlinearly when *A. physocarpa* was included. (B) The tolerance of monarchs to parasites was associated with foliar cardenolide concentration. Datapoints indicate milkweed species means, bars indicate ± SE; lines indicate least-squares regression lines. (C) Milkweed species differed in the composition of cardenolides that they contained, separating in two-dimensional NMDS analysis. (D) NMDS axis 2 tended to associate positively with longevity of infected monarchs and negatively with the longevity of uninfected monarchs. This association was significant across all infected monarchs, but not significant for the mean longevities of infected and uninfected monarchs.

observation that monarchs fed on plants with intermediate levels of cardenolides exhibited increased longevity compared to monarchs that received either very small or large doses of cardenolides. We found a significant quadratic relationship between the mean longevity of infected monarchs and the mean cardenolide concentrations of their milkweed food (Fig. 2A; $F_{2,9} = 3.01$, linear term: $P = 0.037$; quadratic term: $P = 0.041, R^2 = 0.40$); the relationship is linear when *A. physocarpa* is removed ($F_{1,9} = 5.50, P = 0.044$, $R^2 = 0.38$). We also found a significant association between monarch disease tolerance (i.e., the slope of the regression of adult longevity and spore load) and average milkweed cardenolide concentration (Fig. 2B; $F_{1,10} = 2.25, P = 0.047, R^2 = 0.34$). Tolerance was also associated with cardenolide diversity ($F_{1,10} = 2.98$, $P = 0.014$, $R^2 = 0.47$) but not with cardenolide polarity ($F_{1,10} = 1.46$, $P = 0.175$, $R^2 = 0.176$). Neither diversity nor polarity was retained in a model that accounted first for the effect of cardenolide concentration ($P = 0.169$ and $P = 0.540$, respectively).

In addition to our analyses using the concentrations of cardenolides, we found that milkweed species differed dramatically in their cardenolide compositions (PerMANOVA; $F_{11,306} = 67.81$, $P < 0.001, R^2 = 0.71$). These differences were plotted using an ordination technique, nonmetric multidimensional scaling (NMDS) (McCune and Grace 2002), with a two-dimensional model separating most milkweed species by their cardenolide compositions. The exceptions were a cluster of four milkweed species with extremely low cardenolide concentration (Fig. 2C). NMDS axis 2 was positively associated with the longevity of infected monarchs

across all milkweed plants (Fig. 2D; $F_{1,315} = 5.60, P = 0.02$). Because we did not measure the cardenolide chemistry of plants upon which uninfected monarchs were reared, we used the mean NMDS scores of each host plant species to compare mean responses in longevity of infected and uninfected monarchs. There was a nonsignificant trend with the longevity of infected monarchs increasing and the longevity of uninfected monarchs decreasing with increases in NMDS axis 2 (Fig. 2D; $F_{1,20} = 3.29, P = 0.085$). These results support the hypothesis that the interaction between monarchs and certain foliar cardenolides is contingent upon whether the monarchs are infected with *O. elektroscirrha*.

Lastly, we examined cardenolide diversity (a composite index of the number and relative abundance of cardenolides present) and relative nonpolarity (a measure that is inversely proportional to the average polarity of cardenolides), in relation to the longevity of infected monarchs. Unlike total cardenolide concentrations, the relationship between mean longevity of infected monarchs and mean diversity was not significant, and neither was the relationship between mean longevity and mean nonpolarity. For both measurements, analyses on individual monarchs found significant linear (Fig. 3A and B; $F_{1,315} = 8.00$, $P = 0.005$ and $F_{1,315} = 15.2$, $P < 0.001$, respectively) and quadratic terms ($F_{1,315} = 11.3$, $P < 0.001$ and $F_{1,315} = 16.4$, $P < 0.001$, respectively). This suggests that the polarity and diversity of cardenolides present in milkweeds may be important for understanding the effects of milkweed chemistry on infection, but additional species will be necessary to determine whether the relationship holds true among milkweed species. The observation that infected monarchs experience the highest longevity on plant species with intermediate levels of cardenolide diversity and polarity is again consistent with a trade-off between the antiparasitic effect of cardenolides and the physiological cost to monarchs from the cardenolides (e.g., Fig. 2C). However, of the 12 species in this experiment, *A. physocarpa* appears to be the only one beyond the threshold where the physiological cost of cardenolides outweighs the antiparasitic effect.

Discussion

Our results show that milkweed species can affect relative levels of resistance and tolerance to parasite infection in monarch butterflies. Across the 12 species of milkweed that we tested, monarch butterflies experienced highest resistance (i.e., lowest parasite spore loads) on *A. erosa* and lowest resistance on *A. tuberosa* (Fig. 1B). Highest tolerance (i.e., smallest reduction in adult longevity with increasing parasite spore load) was observed in monarchs reared on *A. curassavica* and lowest tolerance in monarchs on *A. incarnata* (Fig. 1C). We found no significant relationship between milkweed-conferred resistance and tolerance, suggesting that milkweed species do not simultaneously confer greater resistance and tolerance to monarchs and that there is no trade-off between milkweed-conferred resistance and tolerance. Because our experiment used only a single parasite genotype for infection, follow-up studies will be necessary to examine the effect of food plants on tolerance across parasite genotypes. However, the effect of food plants on host resistance has previously been confirmed using multiple parasite genotypes (De Roode et al. 2008a; Lefèvre et al. 2010). Our results are an important addition to the growing number of studies indicating that environmental factors are important modulators of host–parasite interactions (reviewed in Wolinska and King 2009). Until now, these studies have focused primarily on resistance, but as illustrated by our results, environmental factors—including interacting species—can also significantly affect tolerance.

Demonstrating that tolerance can be environmentally determined has important implications for the study of host–parasite systems. It suggests that, when hosts are removed from their natural environments, researchers may erroneously conclude that hosts have not evolved tolerance. As a case in point, our own previous study of tolerance in monarch butterflies revealed no genetic variation and concluded that monarch butterflies either had not evolved tolerance or that it had become fixed at a maximum level (Lefèvre et al. 2011). However, in that study, we reared monarchs on a single species of milkweed, thus excluding the possibility of measuring tolerance conferred by milkweed species. In our current experiment, we have tested multiple species of milkweeds, most of which (11 out of 12) are found in sympatry with the monarch population represented in our experiment (Woodson 1954; Malcolm and Zalucki 1996). This includes milkweed species with overlapping distributions and as our results indicate, monarchs can obtain tolerance to infection by using particular species of milkweed. This could also impact the oviposition preference of female monarchs. As we have previously shown, parasite-infected monarchs preferentially lay eggs on *A. curassavica* over *A. incarnata* in two-species choice tests (Lefèvre et al. 2010, 2012) and this preference could provide the monarchs' offspring not only with greater effective resistance, but also greater effective tolerance (Fig. 1C). The ability to obtain tolerance to parasitism through such interactions would be missed in experiments that do not incorporate environmental variability.

Environmental variation is one potential mechanism for the maintenance of polymorphism in host resistance (Lazzaro and Little 2009; Wolinska and King 2009), and this may be true for tolerance as well. Although the majority of theoretical models have predicted a lack of genetic variation in tolerance (but see Best et al. 2008), many empirical studies have found such variation, both in plants and animals (reviewed in Baucom and de Roode 2011). Authors have mostly attributed this variation to fitness costs associated with tolerance (Simms and Triplett 1994; Tiffin and Rausher 1999; Koskela et al. 2002) and trade-offs between

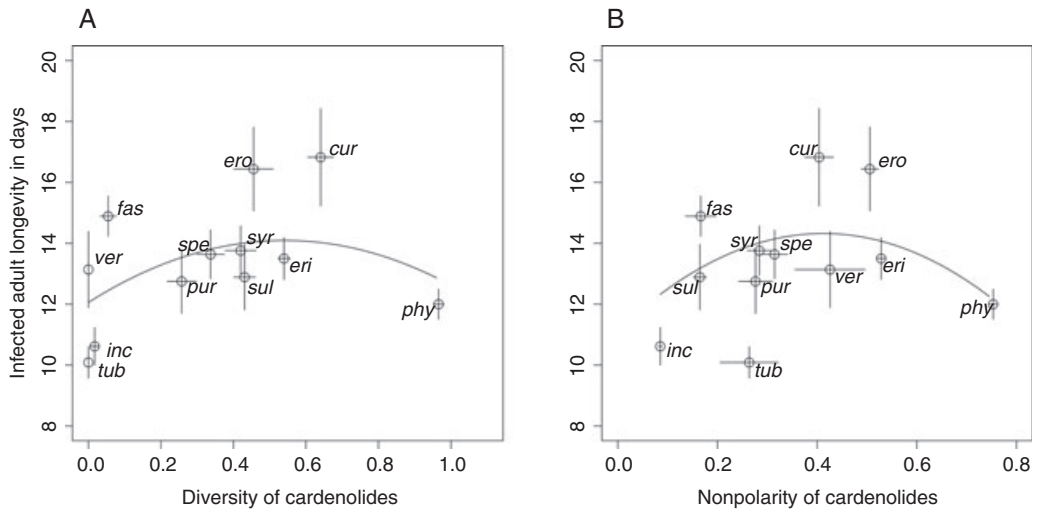

Figure 3. Associations between milkweed cardenolide composition and the performance of infected monarch butterflies. Both (A) the diversity and (B) the average nonpolarity of cardenolides in milkweeds were associated with adult longevity of infected monarchs, with monarchs experiencing greatest longevity when reared on milkweed species with intermediate cardenolide diversity and polarity.

resistance and tolerance (Fineblum and Rausher 1995; Carr et al. 2006; Råberg et al. 2007). In some cases, however, environmental effects may explain the observed variation in tolerance when measured under natural conditions. For example, recent work on ectoparasites in fish reported a significant interaction between sampling site and parasite burden, suggesting that the environment is influencing tolerance (Blanchet et al. 2010). Conversely, environmental factors may negate genetic variation in tolerance observed under standard laboratory conditions. For example, if different host genotypes are subject to different environmental factors in the wild, and if those factors affect tolerance, it is possible that the observed variation in the laboratory is not actually expressed in nature.

In addition to contributing to our understanding of resistance and tolerance, our findings add to a growing body of evidence that food plants are major determinants of fitness in phytophagous hosts and their parasites (reviewed in Cory and Hoover 2006). With this type of tritrophic interaction, understanding the role of plant chemistry, including nutrient content (Lee et al. 2006) and defensive or allelopathic chemicals (Felton and Duffy 1990; Keating et al. 1990), is essential for predicting how plants will influence infection. Diet quality can have profound effects on the immune system (Bhaskaram 2002; Wintergerst et al. 2007; Ponton et al. 2011) and this may contribute to the dietary-based tolerance that we observed in our experiment. However, plant chemistry can impose conflicting effects on hosts (e.g., Singer et al. 2004; Haviola et al. 2007), likely resulting in the interactions between infection status and plant chemistry in our study (Figs. 1A and 2D). This interaction is illustrated by *A. curassavica* which, relative to other milkweed species, depresses adult longevity in the absence of the parasite and promotes adult longevity in the presence of the parasite. We also found a significant, curvilinear relationship between the mean concentration of cardenolides present in milkweed species and the mean longevity of infected adults, and between the proportion of nonpolar cardenolides in individual milkweed plants and the longevity of the infected adult monarchs reared on these plants. A curvilinear relationship is consistent with the general predictions of a pharmacological approach to plant–herbivore interactions, wherein herbivores are expected to respond to plant chemical variation in a dose-dependent fashion (Forbey and Foley 2009). The curvilinear relationships are also consistent with the specific biology of this system, where nonpolar cardenolides are thought to be more toxic than polar cardenolides (Fordyce and Malcolm 2000; De Roode et al. 2011b). This is apparent in the adult longevity of infected monarchs reared on *A. physocarpa*, a milkweed species with over 40 distinct cardenolides, including many highly nonpolar cardenolides present at high concentrations. Because adult longevity is a measure of the combined effect of the plant on the parasite and on the monarch, the cardenolides present in *A. physocarpa* may have direct negative effects on monarch health that outweigh any negative effect on the parasite (Fig. 2A). Given the complexity of plant chemistry and the capacity for direct and indirect effects on monarch health, we emphasize that there are no universally beneficial milkweeds or cardenolides. Rather, the effects of food plants on monarchs depend on multiple aspects of plant chemistry and the prevalence of parasites.

It is clear that environmental factors vary within and among natural populations, both spatially and temporally, and the idea that environmental variability can affect selection has been present in the literature for over half a century (Haldane 1946; Falconer 1952). It is only recently, however, that this concept has been extended specifically to infectious diseases (Lazzaro

and Little 2009; Wolinska and King 2009). As our results show, environmental factors—such as interacting species in a food web—can have an important effect on host tolerance to infection. This suggests that environmental factors need to be investigated to obtain a complete picture of host–parasite coevolution. Moreover, by identifying the chemical and physiological mechanisms that provide hosts with tolerance, studies on environmentally induced tolerance may aid in the development of disease therapies that are less likely to be circumvented by parasite evolution than are therapies based on resistance (Roy and Kirchner 2000; Rausher 2001; Schneider and Ayres 2008).

ACKNOWLEDGMENTS

We thank R. Rarick for helping with the experiment, P. Johnson and L. Waller for statistical advice, and members of the Invertebrate Host-Parasite journal club at Emory University, E. Svensson and three anonymous reviewers for helpful comments on the manuscript. This work was supported by National Science Foundation grant DEB-1019746 to JdR and MDH.

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Associate Editor: E. Svensson