



The Effects of Milkweed Induced Defense on Parasite Resistance in Monarch Butterflies, *Danaus plexippus*

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Received: 5 June 2018 / Revised: 5 August 2018 / Accepted: 10 August 2018 / Published online: 20 August 2018
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Abstract

Many plants express induced defenses against herbivores through increasing the production of toxic secondary chemicals following damage. Phytochemical induction can directly or indirectly affect other organisms within the community. In tri-trophic systems, increased concentrations of plant toxins could be detrimental to plants if herbivores can sequester these toxins as protective chemicals for themselves. Thus, through trophic interactions, induction can lead to either positive or negative effects on plant fitness. We examined the effects of milkweed (*Asclepias* spp.) induced defenses on the resistance of monarch caterpillars (*Danaus plexippus*) to a protozoan parasite (*Ophryocystis elektroscirrha*). Milkweeds contain toxic secondary chemicals called cardenolides, higher concentrations of which are associated with reduced parasite growth. Previous work showed that declines in foliar cardenolides caused by aphid attack render monarch caterpillars more susceptible to infection. Here, we ask whether cardenolide induction by monarchs increases monarch resistance to disease. We subjected the high-cardenolide milkweed *A. curassavica* and the low-cardenolide *A. syriaca* to caterpillar grazing, and reared infected and uninfected caterpillars on these plants. As expected, monarchs suffered less parasite growth and disease when reared on *A. curassavica* than on *A. syriaca*. We also found that herbivory increased cardenolide concentrations in *A. curassavica*, but not *A. syriaca*. However, cardenolide induction in *A. curassavica* was insufficient to influence monarch resistance to the parasite. Our results suggest that interspecific variation in cardenolide concentration is a more important driver of parasite defense than plasticity via induced defenses in this tri-trophic system.

Keywords Induction · Herbivory · Trophic interactions · *Asclepias* · *Ophryocystis elektroscirrha*

Introduction

Plants have evolved multiple forms of defense against herbivores, including the production of secondary chemicals that are toxic to animals. In addition to producing constitutive levels of toxic compounds, some plants increase their production of secondary chemicals upon herbivory, a phenomenon known as phytochemical induction (Karban and Baldwin 1997). This increase in toxic phytochemicals can have direct

or indirect effects on other trophic levels, with either net positive or net negative effects on plant fitness (Cory and Hoover 2006). Specifically, if plant induced defenses have negative effects on parasites or pathogens of herbivores, they indirectly lower plant fitness through trophic interactions (Hunter and Schultz 1993). When specialist herbivores sequester plant toxins and use them for their own defense, induced defenses present a problem for plants as they confer benefits to their herbivores (Foord 2001).

In this study, we examined the effects of phytochemical induction on higher-trophic interactions, specifically herbivore-parasite interactions, with a herbivore that sequesters plant toxins as protective chemicals. We studied milkweeds (*Asclepias* spp.), monarch butterflies (*Danaus plexippus*), and a specialist protozoan parasite (*Ophryocystis elektroscirrha*). Milkweeds contain toxic cardenolides that disrupt animal Na^+/K^+ -ATPase (Agrawal et al. 2012b), and monarch caterpillars sequester cardenolides as protective chemicals against bird predators (Brower et al. 1972). *O.*

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elektroscirra spores are ingested by monarch caterpillars as they feed on milkweed and can be transmitted vertically from females to their offspring during oviposition. Parasite infection reduces host fitness, by lowering pre-adult survival, mating ability, and adult lifespan (de Roode et al. 2008). A body of work has shown that the consumption of milkweed species with higher concentrations of cardenolides also increases monarch resistance to infection with the parasite (e.g. Sternberg et al. 2012). Moreover, declines in foliar cardenolide concentrations resulting from aphid attack render monarch caterpillars more susceptible to the parasite (de Roode et al. 2011b). In contrast with aphid-associated reductions in cardenolides, some milkweed species increase their concentrations of cardenolides following herbivory by monarchs (Agrawal et al. 2012a, 2014). Thus, we hypothesized that the induced upregulation of cardenolides by herbivory could enhance monarch resistance to the parasite.

Methods and Materials

Plants, Butterflies, and Parasites Three species of milkweed were used for the experiment: *A. curassavica*, *A. syriaca*, and *A. incarnata*. Milkweed seeds were obtained from Prairie Moon Nursery (Winona, MN, USA). All milkweeds used for this study were about 3 months old, grown in a greenhouse under natural light conditions with weekly fertilization. Monarchs were obtained from five lab-reared outcrossed lineages generated from wild-caught migratory monarchs collected in St. Marks, Florida, USA. The parasite clone used was generated from an infected, wild-caught monarch from the same population.

Experimental Design and Procedures Our experiment represented a $2 \times 2 \times 2$ fully factorial design, with plant species, induction, and parasite infection as main factors. Two milkweed species, *A. curassavica* and *A. syriaca*, were used for the induction treatments. *A. curassavica* leaves contain higher cardenolide concentrations than do those of *A. syriaca*, and monarchs reared on *A. curassavica* experience reduced parasite growth and disease symptoms (e.g. Sternberg et al. 2012). For each species, plants were divided between induced and control treatments; within each induction/control treatment, plants were further divided between infected and uninfected treatments. Individual plants in the induced treatment were treated with one uninfected second instar larva, which was allowed to feed for 5 days, a timeframe that is sufficient to trigger induced defenses in milkweed (Agrawal et al. 2014). The herbivory treatment caused about 20–50% of leaf damage by visual assessment. Control uninduced plants did not receive grazing caterpillars. After 5 days of induction treatment,

an 8-mm diameter leaf disk was taken from each experimental plant for parasite inoculation. Second instar monarch larvae were inoculated by adding 10 parasite spores to the leaf disk taken from their pre-assigned plant (de Roode et al. 2011b; Sternberg et al. 2012). Uninfected controls received disks without spores. Before inoculation, all larvae had been reared on *A. incarnata*, a low cardenolide milkweed species, to homogenize their dietary experience prior to treatment. After larvae consumed their entire leaf disk, and therefore parasite dose, larvae were transferred to individual rearing cups (473 mL) and fed with leaf cuttings from the same individual plant that was used for parasite inoculation. Thus, in our experiment, phytochemical induction occurred on live plants, which were then subsampled to feed experimental caterpillars. Previous work (de Roode et al. 2011a) has shown that the effects of cardenolides on parasite infection are mediated during the time of infection, and that the milkweed fed to caterpillars following infection has no further effects on parasitism. Thus, feeding caterpillars with leaf disks from induced and uninduced plants, followed by the feeding of leaf cuttings, provides a relevant approach to study the effects of cardenolide induction on monarch susceptibility to parasites. Sample sizes ranged from 13 to 25 per treatment group. After pupation, pupae were placed in a laboratory room maintained at 25 °C under 14/10 h L/D cycle. After eclosion, adults were placed in 8.9 × 8.9 cm glassine envelopes without a food source at 12 °C under 14/10 h L/D cycle. Previous studies have demonstrated that the effects of parasite infection on adult longevity are similar between starvation conditions and more natural, non-starvation conditions (de Roode et al. 2009). Adults were inspected daily until death to measure lifespan. Parasite load was quantified using a vortexing protocol described in de Roode et al. (2011b).

Chemical Analyses Plant chemical samples were collected on the same day as parasite inoculation, which was after 5 days of experimental herbivory. One leaf from the fourth pair on each plant was chosen, and six leaf disks (424 mm² total) were taken with a paper hole punch from one side of the leaf and placed immediately into a 1 mL collection tube with cold methanol. Another six identical leaf disks were taken from the opposite side of the same leaf to measure sample dry mass. Total cardenolide concentrations were analyzed using reverse-phase ultra-performance liquid chromatography (UPLC; Waters Inc., Milford, MA, USA) following established methods (de Roode et al. 2011b; Tao et al. 2015).

Statistical Analyses The main goal of our analyses was to test for the effects of plant species and induction on foliar cardenolide concentration, parasite spore load, and adult lifespan. We tested the main effects of plant species, induction,

and/or infection as fixed effects using linear models. Normality and variance homogeneity were checked with the Shapiro-Wilk normality test and Fligner-Killeen test. Cardenolide concentration was analyzed separately by plant species using a Mann-Whitney test with Bonferroni corrections for multiple comparisons due to violation of assumptions of normality and variance homogeneity. Parasite spore load data were Box-Cox transformed and analyzed using linear models with weighted least squares to meet model assumptions, with parasite load as dependent variable and plant species, induction, and their interaction as independent variables. Lifespan data were Box-Cox transformed to meet assumptions and analyzed separated by species using linear models with Bonferroni corrections for multiple comparisons with lifespan as dependent variable and infection, induction, and their interaction as independent variables. Post hoc Tukey pairwise comparisons were performed when the interaction term in a linear model was significant. All analyses were performed using R version 3.4.1. Box-Cox transformation was performed with the package *car* 2.1–5. Post hoc Tukey pairwise comparisons were performed with the package *multcomp* 1.4–7.

Data Accessibility

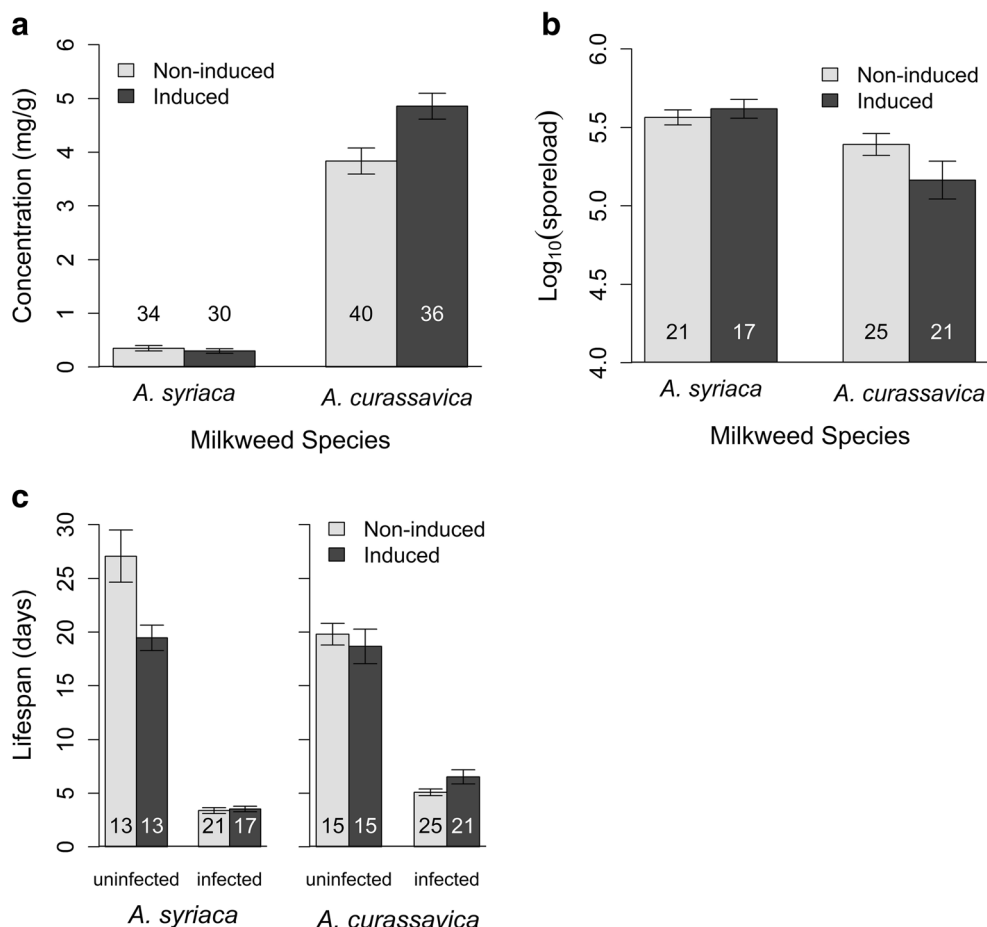
All data are available at the Dryad Digital Repository (doi:<https://doi.org/10.5061/dryad.3pf346v>).

Results

Herbivory by monarch caterpillars induced significantly higher cardenolide concentrations in *A. curassavica* foliage (Fig. 1a; $W = 398$, $P < 0.001$), but not in *A. syriaca* foliage (Fig. 1a; $W = 514$, $P = 0.96$). *A. curassavica* foliage also had higher constitutive cardenolide concentrations (Fig. 1a). Monarchs reared on *A. curassavica* had significantly lower spore loads than monarchs reared on *A. syriaca*, but induction did not significantly affect spore load (Fig. 1b; plant species: $F_{1, 80} = 8.55$, $P < 0.01$; induction: $F_{1, 80} = 0.09$, $P = 0.76$; plant species \times induction: $F_{1, 80} = 1.55$, $P = 0.22$).

Although induction did not affect parasite spore load, it reduced the lifespan of some monarchs. Specifically, uninfected larvae reared on induced *A. syriaca* lived shorter lives than did uninfected larvae reared on control *A. syriaca* (Fig. 1c;

Fig. 1 (a) The effect of induction by monarch caterpillars on foliar cardenolide concentrations in two milkweed species; (b) The effect of milkweed species and induction on parasite spore load in infected monarchs; (c) The effect of milkweed species, induction, and parasite infection on the lifespan of monarchs. Data represent mean \pm 1 SEM. Sample sizes are reported on each bar



infection: $F_{1,60} = 571.50$, $P < 0.01$; infection x induction: $F_{1,60} = 6.67$, $P = 0.01$). Induction had no such effects on monarchs reared on *A. curassavica*, although infection reduced lifespan as expected (Fig. 1c; infection: $F_{1,72} = 246.45$, $P < 0.01$; infection x induction: $F_{1,72} = 3.64$, $P = 0.06$). Overall, our results suggest that induced cardenolide defenses of milkweeds have weak effects on monarch defenses against the parasite.

Discussion

In this monarch-milkweed-parasite system, foliar cardenolides increase the resistance of monarchs to parasite infection (Sternberg et al. 2012). Aphid-induced declines in foliar cardenolides render monarchs more susceptible to parasites (de Roode et al. 2011b). Here, we show that herbivory by monarch caterpillars induces a significant increase in foliar cardenolide concentrations in *A. curassavica* but not in *A. syriaca*. However, induction of cardenolides in *A. curassavica* did not translate into a statistically significant increase in monarch resistance to parasite infection. Previous work has shown that milkweed species vary strongly in cardenolide concentrations and in their effects on parasite resistance (e.g. Sternberg et al. 2012); our current study suggests that these interspecific differences outweigh within-species variation due to monarch induction. Thus, while induction caused a 1.3-fold increase in cardenolide concentration in *A. curassavica*, the difference between the two species, which resulted in a significant difference in parasite resistance, was 11-fold (Fig. 1a). Other studies have also reported modest increases in cardenolide concentrations in response to monarch caterpillar grazing, and demonstrated that environmental factors such as light can alter the expression and magnitude of milkweed induced defenses (Agrawal et al. 2012a, 2014). In addition, the age of plants could be a factor influencing the strength of phytochemical induction: some studies that observed cardenolide induction in *A. syriaca* used one-month old plants (Mooney et al. 2008; Agrawal et al. 2014), while we used three-month old plants. The relatively small magnitude of cardenolide induction by monarchs in older plants could be the reason for the small effect sizes on parasite resistance observed in this study. It is important to realize that one-month old plants cannot support the entire larval development of a monarch caterpillar; as such, our results using large three-month old plants are highly relevant for our understanding of cardenolide induction on parasite resistance in monarchs.

In addition to inducing cardenolides, herbivory by monarch caterpillars may also alter other forms of milkweed defense, including latex exudation and cysteine protease production, and/or the nutritional properties of foliage such as the carbon to nitrogen ratio (Rasmann et al. 2009). Nutritional properties such as phosphorus concentration also affect monarch lifespan

under parasite infection (Tao et al. 2015). We observed reductions in the lifespan of monarchs reared on induced *A. syriaca*, even though cardenolide concentrations were not induced. This result suggests that induction altered other milkweed qualities aside from cardenolides, and that these changes affected monarch fitness. This result confirms that an array of defense mechanisms and nutritional properties respond to herbivore damage (Karban and Baldwin 1997), and may influence multi-trophic interactions (Hunter and Schultz 1993).

Overall, our study illustrates that monarch-induced increases in cardenolides, a class of anti-parasitic chemicals, are not large enough to increase monarch resistance to their parasites. These results suggest that in this milkweed-monarch-parasite tri-trophic system, interspecific variation in cardenolide concentration is a more important driver of parasite defenses than is plasticity via inducible defense.

Acknowledgements We thank M. Tsai, A. Ahmad, and Y. Li for help with the experiments, H. Streit for help with the chemical analyses, H. Salem, Y. Yang, and two anonymous reviewers for providing constructive comments on the manuscript. This work was supported by National Science Foundation grant IOS-1557724 to J.C.d.R and M.D.H.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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