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Fitness costs of animal medication: anti-parasitic plant chemicals reduce fitness of monarch butterfly hosts

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Running title: Costs of non-immunological defenses to parasites

Summary

1. The emerging field of ecological immunology demonstrates that allocation by hosts to immune defense against parasites is constrained by the costs of those defenses. However, the costs of non-immunological defenses, which are important alternatives to canonical immune systems, are less well characterized. Estimating such costs is essential for our understanding of

the ecology and evolution of alternative host defense strategies.

2. Many animals have evolved medication behaviors, whereby they use anti-parasitic compounds from their environment to protect themselves or their kin from parasitism. Documenting the costs of medication behaviors is complicated by natural variation in the medicinal components of diets and their covariance with other dietary components, such as macronutrients.

3. In the current study, we explore costs of the usage of anti-parasitic compounds in monarch butterflies (*Danaus plexippus*), using natural variation in concentrations of anti-parasitic compounds among plants. Upon infection by their specialist protozoan parasite *Ophryocystis elektroscirrha*, monarch butterflies can selectively oviposit on milkweed with high foliar concentrations of cardenolides, secondary chemicals that reduce parasite growth. Here, we show that these anti-parasitic cardenolides can also impose significant costs on both uninfected and infected butterflies.

4. Among eight milkweed species that vary substantially in their foliar cardenolide concentration and composition, we observed opposing effects of cardenolides on monarch fitness traits. While high foliar cardenolide concentrations increased the tolerance of monarch butterflies to infection, they reduced the survival rate of caterpillars to adulthood. Additionally, although nonpolar cardenolide compounds decreased the spore load of infected butterflies, they also reduced the life span of uninfected butterflies, resulting in a hump-shaped curve between cardenolide non-polarity and the life span of infected butterflies.

5. Overall, our results suggest that the use of anti-parasitic compounds carries substantial costs, which could constrain host investment in medication behaviors.

Key Words:

cardenolides, ecological immunology, host-parasite interactions, monarch butterfly, self-medication, tradeoffs

Introduction

Parasites can significantly reduce host fitness, such that hosts are under strong selection to evolve anti-parasitic defenses. In addition to canonical immunity, including cellular and humoral immune responses (Schmid-Hempel & Ebert 2003), many hosts have evolved alternative defenses, such as social immunity or self-medication (Cremer, Armitage & Schmid-Hempel 2007; Clayton *et al.* 2010; Parker *et al.* 2011; de Roode & Lefèvre 2012; de Roode, Lefèvre & Hunter 2013). Due to the parasite pressures that hosts face in their natural environments, an intuitive prediction is that hosts should maximize a diverse arsenal of defenses. However, the field of ecological immunology has suggested that this does not happen because immunity is costly (Sheldon & Verhulst 1996; Rolff & Siva-Jothy 2003). Indeed, many studies have demonstrated costs of canonical immunity in a wide range of organisms, including reductions in survival, competitive ability, sexual signaling and reproductive output (Moret & Schmid-Hempel 2000; Kraaijeveld, Limentani & Godfray 2001; Zuk & Stoehr 2002; Hanssen *et al.* 2004; Jacot, Scheuber & Brinkhof 2004; Baer, Armitage & Boomsma 2006; Duncan, Fellous & Kaltz 2011; Pompon & Levashina 2015). These costs may explain the reported lack of maximal investment in a wide variety of immune defenses, as well as the temporal and spatial variation in immunity that is often observed (Hawley & Altizer 2011).

Although there is now growing evidence of costs associated with canonical immune responses, costs of alternative defenses are still poorly understood. Some authors have suggested that non-immunological defenses may be less costly (Simone, Evans & Spivak 2009; Elliot & Hart 2010), but others have shown significant costs of behavioral immunity. For example, to avoid parasitism, water striders (*Aquarius paludum insularis*) tend to oviposit at deeper sites. However, such avoidance behavior can lead to lower hatching rates of the eggs (Hirayama & Kasuya 2010). Similarly, in the burying beetle (*Nicrophorus vespilloides*), the social immunity provided by the smearing of antibacterial substances on larval food resources by females reduces their survival and reproductive output (Cotter *et al.* 2010).

Animal medication is an important non-immunological defense, whereby animals use anti-parasitic compounds from their environment to protect themselves or their kin from parasitism (Lozano 1991; Clayton & Wolfe 1993; Huffman 2003; de Roode, Lefèvre & Hunter 2013; Abbott 2014). Some chemicals are used externally. For instance, primates and birds rub ants and millipedes on their fur or feathers to dose ecto-parasites with the pungent acids from ants (Valderrama *et al.* 2000; Clayton *et al.* 2010), and many organisms can fumigate their nests with plant materials that reduce parasite infection (Christe *et al.* 2003; Clayton *et al.* 2010). Other natural products are used internally as medicines. Upon parasite infection, ants, chimpanzees, moths and honeybees can preferentially choose food with anti-parasitic effects (Huffman *et al.* 1996; Singer, Mace & Bernays 2009; Gherman *et al.* 2014; Bos *et al.* 2015).

Similarly, parasitized woolly bear caterpillars are more likely to consume pyrrolizidine alkaloids (Singer, Mace & Bernays 2009). However, although some studies have demonstrated clear costs of self-medication (Singer, Mace & Bernays 2009; Bos *et al.* 2015), others have not (Huffman *et al.* 1997; Christe *et al.* 2003).

Here, we test for costs associated with the use of anti-parasitic host plants by monarch butterflies. Monarchs are commonly infected with the protozoan parasite *Ophryocystis elektroscirrha*, and use milkweeds (*Asclepias* spp.) as their host plants. Milkweeds contain cardenolides, toxic steroids that disrupt animal Na^+/K^+-ATPase (Agrawal *et al.* 2012), and monarchs that feed on milkweeds with higher concentrations of cardenolides experience lower parasite infection and growth (de Roode *et al.* 2008; de Roode *et al.* 2011b; Sternberg *et al.* 2012; Gowler *et al.* 2015). In addition, when given a choice between species with high and low concentrations of cardenolides, infected monarchs prefer to oviposit on the high-cardenolide milkweed, a behavior that reduces parasite infection and virulence in their offspring (Lefèvre *et al.* 2010; Lefèvre *et al.* 2012). Although monarchs have evolved considerable resistance to cardenolides, they are not fully resistant, and high concentrations of cardenolides have been shown to reduce larval performance (Zalucki, Brower & Malcolm 1990; Zalucki & Brower 1992;

Malcolm 1994). Thus, this system provides a useful way to compare the costs and benefits of consuming anti-parasitic plants.

Cardenolides have three components: a steroid backbone, a butenolide (lactone) ring and sugar moiety. Different cardenolides vary in their sugar moiety, the polarity of which determines their biological activity, with less polar molecules being more toxic (Scudder & Meredith 1982; de Roode *et al.* 2011b; Rasmann & Agrawal 2011; Agrawal *et al.* 2012). Because cardenolide concentration and polarity are not necessarily correlated, it is important to analyze the effects of both concentration and polarity on the fitness of hosts and parasites. In the current study, we capitalized on the large variation in cardenolide concentration and polarity of natural milkweed species to investigate the costs and benefits of using cardenolides as medication against parasites.

Materials and methods

Plants, butterflies and parasites

We used eight (sub)species of milkweeds that vary strongly in cardenolide concentration and polarity: *A. asperula*, *A. curassavica*, *A. incarnata incarnata*, *A. incarnata pulchra*, *A. linaria*, *A. perennis*, *A. physocarpa* and *A. tuberosa*. *A. physocarpa* is native to South Africa, while the other (sub)species are native to Central and North America. *A. incarnata pulchra* seeds were purchased from Georgia Vines (GA, USA), whole plants of *A. perennis* were purchased from Butterfly Plant Shop (FL, USA), and seeds of all other species were purchased from Butterfly Encounters Inc. (CA, USA). Plants were grown in 10cm diameter pots under natural light conditions in a greenhouse where daily temperatures varied between 25 °C - 28 °C. For each species, we grew 40 replicates, resulting in a total of 320 plants.

When the plants were around three months old, we obtained foliage samples to quantify cardenolide concentration and polarity. Briefly, one leaf from the fourth leaf pair (counting from the top) on each plant was chosen, and six leaf disks (total 424 mm^2) were taken with a paper hole punch from one side of the leaf, placed immediately into 1 mL of cold methanol and stored

at -20 °C for subsequent cardenolide analysis. Another six identical disks were taken from the opposite side of the same leaf to estimate sample dry mass. Immediately after chemical sampling, each plant was randomly assigned to one of two caterpillar treatments: infected (25 replicates per species) or control (15 replicates per species). Based on prior experience, we know that not all inoculated butterflies become infected (some can escape infection). Therefore, we increased replication in the infection treatment to obtain sample sizes that are large enough to accurately measure parasite spore load (Lefèvre, Williams & de Roode 2010).

We collected monarch eggs from 5 outcrossed lineages in a lab stock obtained from North-American migratory monarchs, and randomly assigned them to treatments. We reared the newly-hatched caterpillars on the remaining 4th leaf from their individual plant in a petri dish for two days, upon which the caterpillars became 2nd instar. On the third day, we took a hole punch from the third leaf pair (counting from the top) of each plant. For the parasite treatment, ten parasite spores from a single clone were deposited onto the leaf disk, which was then fed to its pre-assigned caterpillar; control caterpillars received leaf disks without spores. The parasite clone (E25) was generated from a single isolate taken from an infected, wild-caught Eastern North American adult monarch collected in 2010 (Sternberg *et al.* 2013).

In our experiment, we chose to measure cardenolides on day 1 (when commencing larval rearing) rather than day 3 (when inoculating caterpillars with parasites) for two reasons. First, the costs of cardenolides on monarch caterpillars are mostly expressed in neonates, rather than larger instars (Zalucki *et al.* 2001). Therefore, the measured cardenolides on day 1 reflect the cardenolides to which monarch caterpillars are exposed during their most susceptible life stage. Second, our previous work (de Roode *et al.* 2011a) has shown that the cardenolide chemistry of plants fed to caterpillars during the days prior to inoculation has the same effects on parasite growth as the chemistry of the plant fed to caterpillars during inoculation. Therefore, the cardenolide chemistry measured on day 1 should accurately reflect the chemistry that reduces parasite infection and growth. It is also important to point out that the mechanical damage we

156 inflicted during chemistry sampling likely had minimal influence on subsequent milkweed
157 cardenolides. In general, across different plant species, mechanical damage (such as punching
158 holes) does not mimic the changes in plant chemistry induced by herbivores; rather it is the
159 chemical cues from herbivores that cause plants to increase production of secondary chemicals
160 (e.g. Pontoppidan et al. 2005). Current studies indicate that this is also the case in milkweeds:
161 while it is known that caterpillar feeding can induce changes in cardenolides (Rasmann *et al.* 2009),
162 studies on *A. curassavica*, a highly inducible milkweed (and one of the species used in this study),
163 have shown that mechanical damage by way of hole punching 1, 3 or 7 days before parasite
164 inoculation does not affect parasite spore load or lifespan of infected butterflies (Lefèvre et al,
165 unpublished results).
166 After each caterpillar had fully consumed its leaf disk (usually within 48 hrs), both the
167 caterpillar and its host plant were transferred into a clear plastic tube (7.62cm in diameter, 30.48
168 cm in length; Visipak, MO, USA) with 20 venting holes in the lid, where they were allowed to
169 completely consume their plant. Because plants were generally not big enough to support
170 complete larval development of caterpillars, they were then supplied with a separately grown
171 batch of *A. incarnata* cuttings until pupation. This procedure is justified because the effects of
172 milkweed chemistry on parasite infection, growth and virulence are not conferred during the
173 larval development stage following infection (de Roode *et al.* 2011a). We specifically chose *A.*
174 *incarnata* as supplementary food because of its low cardenolide concentration. Importantly, by
175 the time they had finished their experimental host plants, caterpillars were mostly 5^{th} instars and
176 had spent an average of 8.5 days on their individual plants, leaving an average of only 1.5 days
177 of pre-pupal development on these new supplementary cuttings.
178
179 Fitness measures and chemical analysis
180 We recorded the survival of caterpillars to adulthood. After emerging from their pupae,
181 butterflies were placed in 8.9 × 8.9 cm glassine envelopes, stored in a 12°C incubator, and

182 inspected daily until they died, upon which adult lifespan of each butterfly was recorded. This
183 measurement combines both longevity and starvation resistance, both of which are highly
184 correlated with the lifespan and life-time fitness of monarchs under more natural conditions (de
185 Roode *et al*. 2009). The difference in lifespan between infected and uninfected butterflies
186 represents our index of parasite virulence (Sternberg *et al*. 2012). After death, the spore load of
187 each butterfly was measured following described methods (de Roode *et al.* 2009). Specifically,
188 they were quantified by vortexing monarch bodies in 5 ml H_2O and estimating total spore loads
189 using a haemocytometer slide. Spore load estimates the total number of spores on a butterfly,
190 which is positively correlated with parasite transmission potential and negatively correlated with
191 butterfly resistance and fitness (De Roode *et al.* 2008). In addition, we measured butterfly
192 tolerance to infection by measuring the negative slope between log transformed spore load and
193 butterfly lifespan (Lefèvre, Williams & de Roode 2010).

194 Analysis of foliar cardenolides followed Tao & Hunter (2012) using reverse phase
195 ultra-performance liquid chromatography (UPLC, Waters Inc., Milford, MA, USA). Peaks were
196 detected by absorption at 218 nm using a diode array detector, and absorbance spectra were
197 recorded from 200 to 300 nm. Peaks with symmetrical absorption maxima between 216 and 222
198 nm were recorded as cardenolides (Zehnder & Hunter 2007). Total cardenolide concentration
199 was calculated as the sum of all separated cardenolide peaks, corrected by the concentration of
200 the internal standard (digitoxin) and the estimated sample mass. An index of cardenolide
201 non-polarity for each plant was calculated following Rassman & Agrawal (2011) and Sternberg
202 et al. (2012), where the relative concentration of each peak in a sample was multiplied by its
203 relative retention time (relative to digitoxin), and summed. Resulting values were from 0-1 for
204 each plant, with values close to 1 indicating high non-polarity and a value of 0 corresponding
205 with high polarity. Note that we specifically created an index of non-polarity instead of an index
206 of polarity, so that higher values correspond with greater toxicity. Individual plants that contained
207 no cardenolides were excluded from the analysis of non-polarity.

208

209 Statistical analysis

210 The primary goal of the study was to explore potential costs of cardenolides on monarch fitness,
211 using eight plant species that differ substantially in their cardenolide concentration and
212 non-polarity to create large and biologically relevant variation. Because caterpillars were fed on
213 individual plants, each with its own unique chemistry, we used individual butterfly and plant data
214 as the level of replication in our analyses. In all following models, we used mixed effect models
215 in which monarch lineage was included as a random factor. To analyze species differences in
216 cardenolide concentration and non-polarity, we used analysis of variance in which cardenolide
217 concentration (or non-polarity) was the dependent variable and plant species was the independent
218 variable. To test if plant species and parasite infection affect survival of individual monarchs to
219 adulthood, we used mixed effects logistic regression in which plant species identity, parasite
220 treatment and their interactions were independent variables, while survivorship of individual
221 monarchs (0 for failure to reach adulthood and 1 for successful development) was the dependent
222 variable. To test if plant species and parasite infection affect monarch lifespan, we used a mixed
223 effects linear model in which plant species identity, parasite treatment and their interactions were
224 independent variables, and lifespan (days) of individual monarchs was the dependent variable.
225 To analyze if plant species affects the spore loads of infected butterflies (an inverse measurement
226 of anti-parasite resistance), we used a mixed effects linear model in which plant species was the
227 independent variable, and log transformed spore load was the dependent variable. Additionally,
228 using both uninfected and infected butterflies, we tested if plant species affected the tolerance of
229 butterflies to infection; we used a mixed effects linear model in which log transformed spore load,
230 and the interaction between plant species and log transformed spore load were independent
231 variables, and the lifespan of butterflies was the dependent variable.

232 To investigate if plant cardenolide concentration and non-polarity affected caterpillar
233 survival to adulthood, we used mixed effects logistic regressions (as above) in which cardenolide

concentration (or non-polarity) were independent variables and caterpillar survival was the dependent variable. To examine the effects of cardenolide concentration and non-polarity on spore loads, we used mixed effects linear regression in which cardenolide concentration or non-polarity were independent variables and the spore load of infected butterflies was the dependent variable. We used the lifespan of both uninfected and infected butterflies to test if cardenolide concentration and non-polarity affect butterfly tolerance to parasites. Specifically, we used mixed effects general linear models in which butterfly spore load (log transformed), and the interaction term between spore load (log transformed) and cardenolide concentration (or non-polarity) were independent variables and butterfly lifespan was the dependent variable. Finally, we examined effects of cardenolide concentration and non-polarity on the lifespan of uninfected and infected butterflies in mixed effects general linear models. In the model on cardenolide concentration, cardenolide concentration, parasite treatment and their interactions were independent variables and lifespan was the dependent variable. For non-polarity, to capture the non-linear relationships that we observed (see Results), we included non-polarity, the quadratic term of non-polarity, parasite treatment and their interactions as independent variables (Sternberg *et al.* 2012). The non-linear model is an explicit test of the hypothesis of an increasing net cost of self-medication at high foliar cardenolide non-polarities. Additionally, we performed separate analyses for uninfected and infected butterflies to explore the effects in more detail. Specifically, for both treatments, we included non-polarity and the quadratic term of non-polarity to explore any non-linear relationships.

Prior to conducting the above analyses, plant cardenolide concentrations and butterfly spore loads were log-transformed (natural log). For all regression models, homogeneity of variance of dependent variables was confirmed by Levene's test, and normality of errors was confirmed by the Shapiro-Wilk normality test. All statistical tests were performed using R 2.15.3 (R Development Core Team 2012); mixed effects logistic regression models were performed by package lme4 1.1-11 (Bates *et al.* 2014) and mixed effects linear models were performed by

package nlme (Pinheiro *et al.* 2007).

Results

The eight milkweed species varied substantially in their foliar cardenolide concentrations (Fig. 1a ; $F_{7, 308} = 244.49$, $p < 0.001$). Post-hoc comparisons showed that *A. asperula* (13.32 ± 1.68 mg/g), *A. linaria* (14.65 ± 1.18 mg/g) and *A. perennis* (12.02 ± 0.88 mg/g) had similarly high cardenolide concentrations, followed by *A. curassavica* (2.09 ± 0.25 mg/g) and *A. physocarpa* (2.43 ± 0.42 mg/g). *A. incarnata incarnata* (0.14 ± 0.04 mg/g), *A. incarnata pulchra* (0 mg/g) and *A. tuberosa* (0 mg/g) had very low to undetectable foliar cardenolide concentrations.

Similarly, milkweed species varied in their foliar cardenolide non-polarity (Fig. 1b; $F_{5, 202} = 159.07$, $p < 0.001$). *A. curassavica* (0.74 ± 0.01), *A. physocarpa* (0.79 ± 0.02) and *A. incarnata incarnata* (0.77 ± 0.03) had indistinguishably high non-polarity, followed by *A. linaria* (0.64 ± 0.01) and *A. asperula* (0.56 ± 0.02). Although foliar concentrations of cardenolides in *A. perennis* were very high, their cardenolides had the lowest non-polarity (0.31 ± 0.01).

Caterpillar survival to adulthood varied among plant species (Fig. 2a; $\chi^2_7 = 124.0$, $p < 0.001$). However, neither parasite infection, nor interactions between plant species and parasite infection affected survival rate ($\chi^2_1 = 0.02$, $p = 0.88$; $\chi^2_7 = 9.66$, $p = 0.21$, respectively). This is expected, because *O. elektroscirrha* has not been found to reduce larval survival in previous studies. Lifespan of butterflies varied substantially among plant species ($F_{6, 182} = 6.00$, $p < 0.001$), with infection status ($F_{1, 182} = 105.42$, $p < 0.001$) and with their interaction (Fig. 2b; $F_{6, 182} = 2.74$, $p = 0.01$), the latter of which demonstrates that plant species affected parasite virulence. In addition, plant species affected monarch resistance to the parasite as measured by spore load of butterflies (Fig. 2c; $F_{6, 104} = 6.42$, $p < 0.001$). The tolerance of butterflies to infection also varied among plant species (Fig. 2d; $F_{6, 188} = 3.90$, $p = 0.001$). These results are consistent with previous studies (Lefèvre *et al.* 2010; Sternberg *et al.* 2012).

Overall, foliar cardenolide concentration exhibited a strong negative relationship with caterpillar survival to adulthood (Fig. 3a; $\chi^2_1 = 38.78$, $p < 0.001$). The average survival rate of caterpillars was 80% on species excluding *A. asperula* and *A linaria*. However, when feeding on *A. asperula* and *A. linaria*, the two species with the highest cardenolide concentrations, caterpillar survival rates were only 42.5% and 2.5%, respectively. When analyzing adult lifespan of uninfected and infected monarchs in the same model, we found that lifespan was unrelated to foliar cardenolide concentrations ($F_{1, 192} = 2.14$, $p = 0.14$; cardenolide concentration × infection: $F_{1, 192} = 0.96$, $p = 0.33$). Although cardenolide concentration was unrelated to the spore load of infected butterflies (Fig. 3c; $F_{1, 109} = 0.15$, $p = 0.70$), it increased the tolerance of butterflies to infection (spore load × cardenolide concentration, $F_{1, 194} = 5.93$, $p = 0.02$).

Cardenolide non-polarity was unrelated to survival rate (Fig. 3e; $\chi^2_1 = 0.008$, $p = 0.93$). When analyzing the effects of cardenolide non-polarity on lifespan of uninfected and infected monarchs in the same model, we found a significant interaction between the quadratic term of non-polarity and parasite treatment ($F_{1, 107} = 7.19$, $p = 0.009$), indicating that cardenolide non-polarity affected lifespan of uninfected and infected butterflies differently. Specifically, higher cardenolide non-polarity was associated with lower adult lifespan of uninfected butterflies (Fig. 3f; linear term $F_{1, 42} = 4.23$, $p = 0.046$; quadratic term $F_{1, 42} = 0.46$, $p = 0.50$), whereas in infected monarchs, there was a quadratic relationship between non-polarity and lifespan (Fig. 3h; quadratic term $F_{1, 61} = 9.38$, $p = 0.003$). This non-linear relationship exists because high non-polarity was associated with reduced parasite spore load (Fig. 3g; $F_{1, 62} = 17.92$, $p < 0.001$). Because the adult lifespan of infected butterflies was strongly negatively associated with parasite spore load ($F_{1, 109} = 33$, $p < 0.001$), these contrasting associations with cardenolide non-polarity resulted in a hump-shaped relationship. This quadratic relationship indicates a trade-off between the costs (innate toxicity to the monarch) and benefits (anti-parasitic resistance) of non-polar cardenolides.

Discussion

Many animals use environmentally derived secondary chemicals to combat disease (de Roode *et al*. 2013). Documenting the costs associated with using these secondary chemicals in natural systems is important for our understanding of the ecology and evolution of behavioral defenses. Upon infection by *O. elektroscirrha*, female monarch butterflies preferentially lay their eggs on *A. curassavica*, a milkweed with high cardenolide concentrations, when compared to *A. incarnata*, a species with low cardenolide concentrations, because cardenolides can confer anti-parasitic effects to monarch butterflies (Lefèvre *et al*. 2010). In the current study, we unveiled the costs of using cardenolides as medicine. These costs derive from two different mechanisms: (i) although high foliar cardenolide concentrations increase the tolerance of infected butterflies, they decrease the survival rate of caterpillars to pupation; (ii) although cardenolides of high non-polarity decrease parasite spore load, they also reduce the adult lifespan of uninfected butterflies. This apparent tradeoff in the use of non-polar cardenolides results in a non-linear relationship between cardenolide non-polarity and the lifespan of infected butterflies. Overall, our results suggest that medication behaviors can incur substantial fitness costs, which are mediated by both the concentration and composition of biologically active secondary metabolites.

Our results are somewhat in contrast with a recent study (Petschenka & Agrawal 2015) that found limited costs of high cardenolide concentrations on monarch butterflies. However, that study focused on the growth rate of caterpillars during their first five days of development, whereas we found that high cardenolide concentration significantly reduced caterpillar survival to adulthood, and that high cardenolide non-polarity significantly reduced adult butterfly lifespan. Therefore, while negative effects of cardenolides may be hard to detect in the short term (but see Zalucki *et al.* 2001), their costs are more prominent when caterpillars are subjected to them throughout their larval period.

The combined importance of foliar cardenolide concentration and non-polarity in

monarch-parasite interactions is best illustrated by comparing *A. asperula*, *A. perennis* and *A. linaria*. These species had the highest – and comparable – foliar concentrations of cardenolides, yet varied substantially in their cardenolide non-polarity and effects on monarch fitness. In particular, *A. perennis* cardenolides had low non-polarity; as a result, this plant species did not reduce parasite growth, but it also did not incur fitness costs on the monarch host. In contrast, *A. asperula*, with cardenolides of intermediate non-polarity, substantially reduced parasite spore load; however, it also reduced monarch survival and the adult lifespan of uninfected monarchs. Finally, *A. linaria* foliage, which had high cardenolide concentration combined with high non-polarity, resulted in very low caterpillar survival. Similarly, a recent study found that caterpillars that fed on *A. linaria* had lower growth rates than those fed on other species (Petschenka & Agrawal 2015). As a result, the most ideal medicinal plant species for monarch butterflies is one with cardenolides that are moderately high in concentration and intermediate to high in non-polarity. An example of such a species is *A. curassavica*, on which monarchs did experience reduced parasite spore loads, but did not suffer reduced survival. While previous studies of monarch medication behavior have focused on choices between plants with different cardenolide concentrations (Lefèvre *et al.* 2010; Lefèvre *et al.* 2012), we currently do not know if female butterflies are able to medicate by choosing among plants of different cardenolide non-polarity. Additionally, although monarchs display medication behavior when they are infected, we do not currently know if infected butterflies are able to avoid those plants on which the costs of medication become too high.

Petschenka and Agrawal (2015) recently found that monarchs have evolved much greater ability to sequester cardenolides compared to other danaine specialist herbivores of milkweeds. Nevertheless, high levels of cardenolides can still reduce activity of Na^+/K^+-ATPase in monarchs, targets of cardenolides, consistent with our findings here. Non-polar cardenolides are especially toxic, because the lipophilic R group can bind tightly with Na^+/K^+-ATPase, reducing its activity to a greater extent. As a result, sequestration is a highly selective process where cardenolides with

intermediate polarity are preferentially stored (Tao & Hunter 2015). How this sequestration relates to parasite infection requires further study. Previous work has shown that high-cardenolide milkweed reduces parasite infection and growth when fed to caterpillars before and during infection, but not when fed after infection (de Roode *et al.* 2011a), suggesting that cardenolide sequestration regulates fitness costs more than the anti-parasitic benefits of these chemicals.

As an important non-immunological defense, many animals have evolved the ability to utilize chemicals from the natural environment against parasites and pathogens, which can significantly reduce parasite growth and improve host fitness (Lozano 1991; Clayton & Wolfe 1993; Huffman 2003; de Roode, Lefèvre & Hunter 2013). Documenting the costs of such medication using natural variation in anti-parasitic substances is not always straightforward (but see Singer *et al.* 2009; Bos *et al*. 2015). Sometimes, natural levels of variation in the medicinal components of diets are unknown, while at other times they co-vary with other dietary components. For example, while nectar alkaloids can reduce parasite load in bumblebees, there are other secondary metabolites in nectar that also have anti-parasitic properties, making explicit tests of costs using natural diet difficult (Manson, Otterstatter & Thomson 2010; Gherman *et al.* 2014; Richardson *et al.* 2015). Our results, on the other hand, demonstrate clearly that anti-parasitic substances are toxic for hosts in the absence of parasites. Such costs may explain spatial and temporal variation in medication behaviors. Additionally, the magnitude of costs may determine whether medication behavior will be prophylactic (preventive) or therapeutic (Choisy & de Roode 2014). For example, self-medication by swallowing whole leaves in chimpanzees is most frequent during the rainy season when the risk of gastrointestinal nematode infection is the highest (Huffman *et al.* 1997). Likewise, baboons only consume berries that are toxic to *Schistosoma* in areas of high risk of infection (Phillips-Conroy 1986).

In the monarch system, while infected monarchs preferentially choose *A. curassavica*, a species with high cardenolide concentrations compared to *A. incarnata* (Lefèvre *et al.* 2010; Lefèvre *et al.* 2012), effects of cardenolides on performance and oviposition behavior of

uninfected monarchs appear highly variable (Cohen & Brower 1982; Oyeyele & Zalucki 1990; Zalucki, Brower & Malcolm 1990; Ladner & Altizer 2005; Petschenka & Agrawal 2015). While some studies have found that cardenolide concentrations do not affect oviposition choice (Cohen & Brower 1982), others have found that females preferentially lay their eggs on plants with intermediate concentrations of cardenolide (Oyeyele & Zalucki 1990; Zalucki, Brower & Malcolm 1990). If cardenolides can confer protection against parasite infection and predation without costs, females should always preferentially lay their eggs on plants with high cardenolide concentrations. In contrast, our results suggest that costs associated with high cardenolide concentrations and high cardenolide non-polarity should favor therapeutic medication behaviors, allowing hosts to benefit from these plant toxins when infected, but avoiding the costs when uninfected. Additionally, nutritional content has been shown to affect oviposition choices in other insects (e.g. Jauset *et al.* 1998), suggesting that ultimately, monarch oviposition may be based on a variety of factors, including defensive and nutrional milkweed chemistry. More generally, our results demonstrate that the assessment of costs and benefits of anti-parasitic compounds requires an understanding of the chemical composition in addition to the overall concentration of such chemicals.

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Data accessibility

Data available from the Dryad Digitial Repository http://dx.doi.org/10.5061/dryad.69bj8 (Tao et al. 2016).

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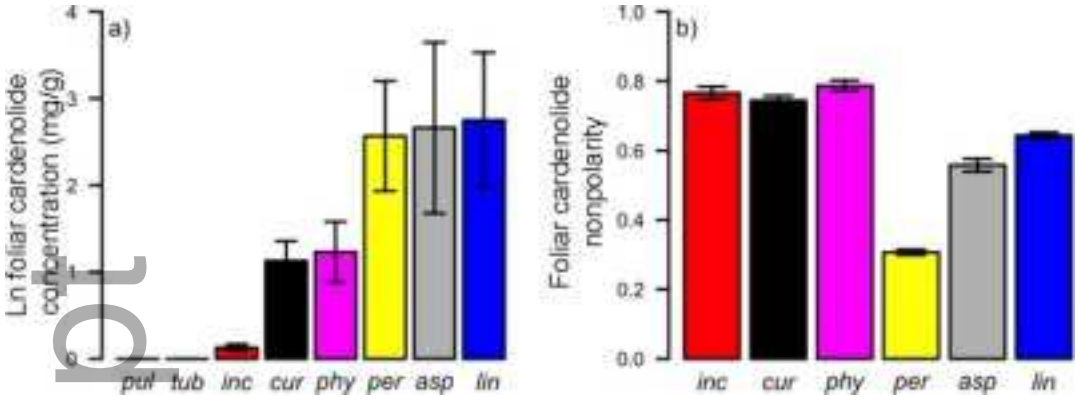
Figure 1. Milkweed species differ in their foliar cardenolide (a) concentration and (b) non-polarity. Cardenolide concentrations in *Asclepias incarnata pulchra* and *A. tuberosa* were 0, therefore no non-polarity can be calcualted for these two species. Data represent mean ± 1 SEM. Species abbreviations: *pul = A. incarnata pulchra*; *tub = A. tuberosa*; *inc = A. incarnata*; *cur = A. curassavica*; *phy = A. physocarpa*; *per = A. perennis*; *asp = A. asperula*; *lin = A. linaria*.

Figure 2. Effects of milkweed species on (a) monarch caterpillar survival to adulthood, (b) lifespan of uninfected (solid bars) and infected monarch butterflies (hashed bars), (c) spore loads of infected monarch butterflies, and (d) tolerance of monarch butterflies to parasite infection. Data represent mean ± 1 SEM. Because only one infected individual that fed on *A. linaria* survived to adulthood, this species' effects on adult butterfly lifespan, parasite spore load and tolerance cannot be shown. Species abbreviations are as in Figure 1.

Figure 3. Effects of milkweed foliar cardenolide concentration (a-d) and non-polarity (e-h) on monarch caterpillar survival to adulthood (a, e), adult lifespan of uninfected monarch butterflies (b, f), parasite spore load of infected monarch butterflies (c, g), and adult lifespan of infected monarch butterflies (d, h). Regression lines indicate significant relationships. Color coding for

598 different milkweed species follows Figures 1 and 2.



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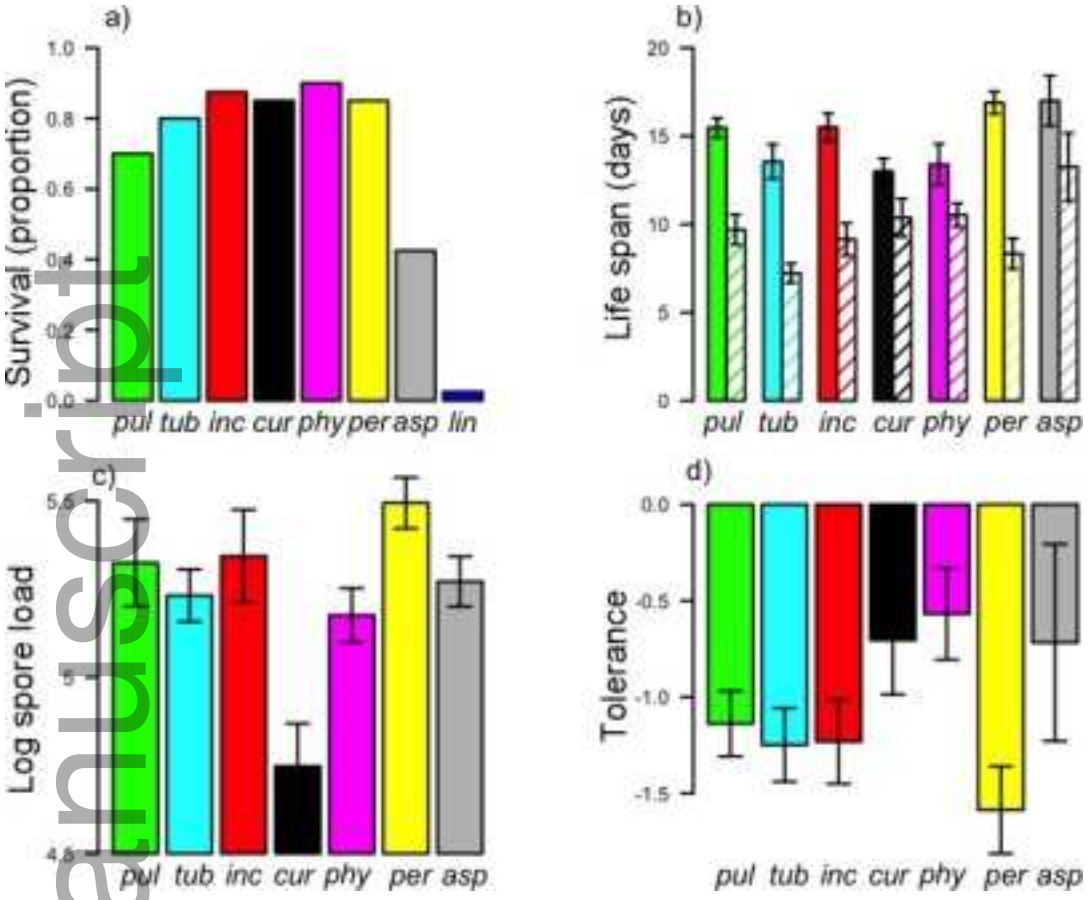

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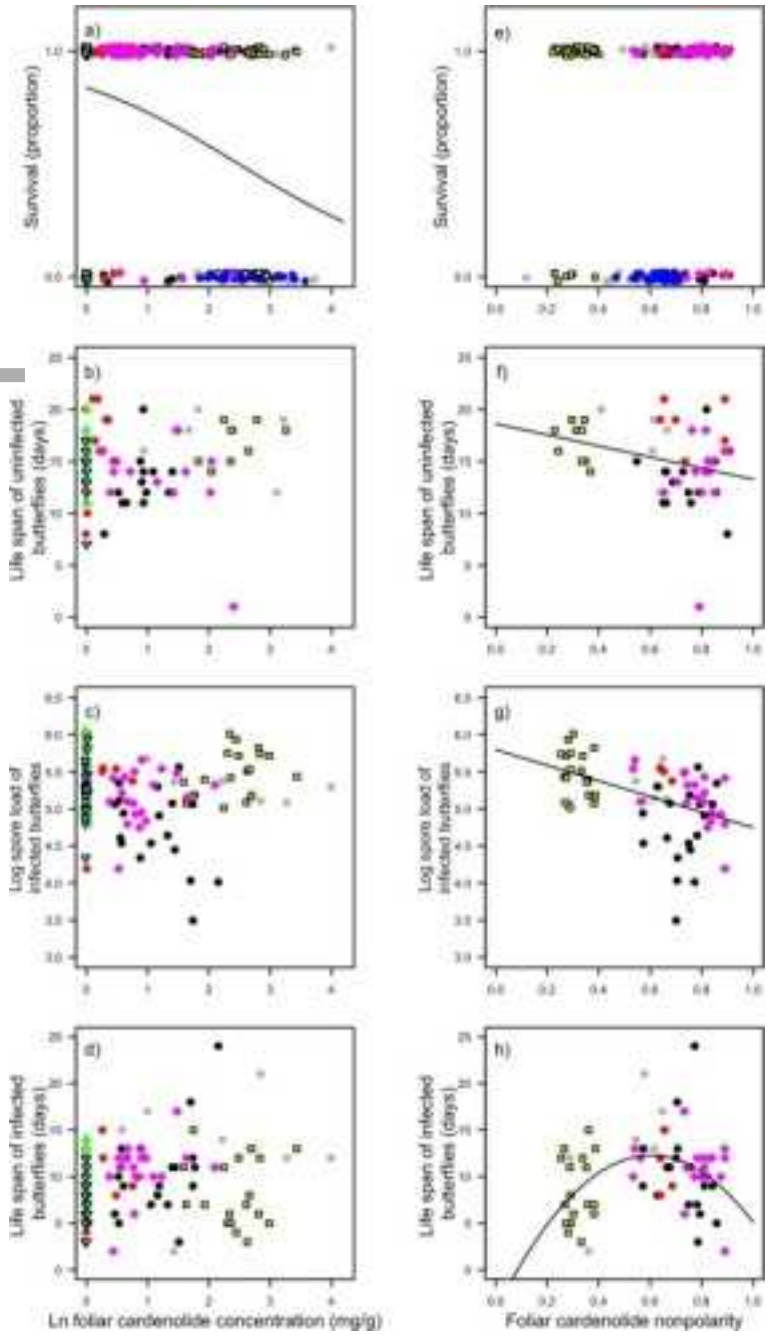

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