Journal of Animal Ecology

British Ecological Society

Journal of Animal Ecology 2016, **85,** 1246–1254 doi: 10.1111/1365-2656.12558

Fitness costs of animal medication: antiparasitic plant chemicals reduce fitness of monarch butterfly hosts

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Summary

1. The emerging field of ecological immunology demonstrates that allocation by hosts to immune defence against parasites is constrained by the costs of those defences. However, the costs of non-immunological defences, 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 defence strategies.

2. Many animals have evolved medication behaviours, whereby they use antiparasitic compounds from their environment to protect themselves or their kin from parasitism. Documenting the costs of medication behaviours 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 the costs of the usage of antiparasitic compounds in monarch butterflies (*Danaus plexippus*), using natural variation in concentrations of antiparasitic 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 antiparasitic 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 the 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 non-polar 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 antiparasitic compounds carries substantial costs, which could constrain host investment in medication behaviours.

Key-words: cardenolides, ecological immunology, host–parasite interactions, monarch butterfly, self-medication, trade-offs

Introduction

Parasites can significantly reduce host fitness, such that hosts are under strong selection to evolve antiparasitic defences. In addition to canonical immunity, including cellular and humoral immune responses (Schmid-Hempel & Ebert 2003), many hosts have evolved alternative defences, 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 defences. 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

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ability, sexual signalling 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 defences, 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 defences are still poorly understood. Some authors have suggested that non-immunological defences may be less costly (Simone, Evans & Spivak 2009; Elliot & Hart 2010), but others have shown significant costs of behavioural immunity. For example, to avoid parasitism, water striders (*Aquarius paludum insularis*) tend to oviposit at deeper sites. However, such avoidance behaviour 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 defence, whereby animals use antiparasitic 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 ectoparasites 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 antiparasitic 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 antiparasitic 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, 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 behaviour that reduces parasite infection and virulence in their offspring (Lefèvre *et al*. 2010, 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 antiparasitic 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; Rasmann & Agrawal 2011; de Roode *et al*. 2011b; Agrawal *et al*. 2012). Because cardenolide concentration and polarity are not necessarily correlated, it is important to analyse 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: *Asclepias asperula*, *Asclepias curassavica*, *Asclepias incarnata incarnata*, *Asclepias incarnata pulchra*, *Asclepias linaria*, *Asclepias perennis*, *Asclepias physocarpa* and *Asclepias tuberosa*. *Asclepias physocarpa* is native to South Africa, while the other (sub)species are native to Central and North America. *Asclepias incarnata pulchra* seeds were purchased from Georgia Vines (Claxton, GA, USA), whole plants of *A. perennis* were purchased from Butterfly Plant Shop (Delray Beach, FL, USA), and seeds of all other species were purchased from Butterfly Encounters Inc. (San Ramon, CA, USA). Plants were grown in 10-cm-diameter pots under natural light conditions in a glasshouse where daily temperatures varied between 25–28 °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 the cardenolide concentration and polarity. Briefly, one leaf from the fourth leaf pair (counting from the top) on each plant was chosen, and six leaf discs (424 mm^2 in total) 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 the subsequent cardenolide analysis. Another six identical discs were taken from the opposite side of the same leaf to estimate the 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 five outcrossed lineages in a laboratory stock obtained from North American migratory monarchs, and randomly assigned them to treatments. We reared the newly hatched caterpillars on the remaining fourth leaf from their individual plant in a Petri dish for 2 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, 10 parasite spores from a single clone were deposited onto the leaf disc, which was then fed to its pre-assigned caterpillar; control caterpillars received leaf discs 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 on 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. Secondly, 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 inflicted during chemistry sampling likely had a minimal influence on subsequent milkweed cardenolides. In general, across different plant species, mechanical damage (such as punching holes) does not mimic the changes in plant chemistry induced by herbivores; rather, it is the chemical cues from herbivores that cause plants to increase the production of secondary chemicals (e.g. Pontoppidan *et al.* 2005). Current studies indicate that this is also the case in milkweeds: while it is known that caterpillar feeding can induce changes in cardenolides (Rasmann *et al.* 2009), studies on *A. curassavica*, a highly inducible milkweed (and one of the species used in this study), have shown that mechanical damage by way of hole punching 1, 3 or 7 days before parasite inoculation does not affect the parasite spore load or life span of infected butterflies (Z.R. Lynch and J.C. de Roode, unpublished results).

After each caterpillar had fully consumed its leaf disc (usually within 48 h), both the caterpillar and its host plant were transferred into a clear plastic tube (7·62 cm in diameter, 30·48 cm in length; Visipak, MO, USA) with 20 venting holes in the lid, where they were allowed to completely consume their plant. Because plants were generally not big enough to support the complete larval development of caterpillars, they were then supplied with a separately grown batch of *A. incarnata* cuttings until pupation. This procedure is justified because the effects of milkweed chemistry on parasite infection, growth and virulence are not conferred during the larval development stage following the infection (de Roode *et al.* 2011a). We specifically chose *A. incarnata* as supplementary food because of its low cardenolide concentration. Importantly, by the time they had finished their experimental host plants, caterpillars were mostly 5th instars and had spent an average of 8·5 days on their individual plants, leaving an average of only 1·5 days of pre-pupal development on these new supplementary cuttings.

FITNESS MEASURES AND CHEMICAL ANALYSIS

We recorded the survival of caterpillars to adulthood. After emerging from their pupae, butterflies were placed in 8·9 × 8·9 cm glassine envelopes, stored in a 12 °C incubator and inspected daily until they died, upon which adult life span of each butterfly was recorded. This measurement combines both longevity and starvation resistance, both of which are highly correlated with the life span and lifetime fitness of monarchs under more natural conditions (de Roode *et al.* 2009). The difference in life span between infected and uninfected butterflies represents our index of parasite virulence (Sternberg *et al.* 2012). After death, the spore load of each butterfly was measured following described methods (de Roode *et al.* 2009). Specifically, they were quantified by vortexing monarch bodies in 5 mL w and estimating total spore loads using a haemocytometer slide. Spore load estimates the total number of spores on a butterfly, which is positively correlated with parasite transmission potential and negatively correlated with butterfly resistance and fitness (de Roode *et al.* 2008). In addition, we measured butterfly tolerance to infection by measuring the negative slope between log-transformed spore load and butterfly life span (Lefèvre, Williams & de Roode 2010).

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

STATISTICAL ANALYSIS

The primary goal of the study was to explore the potential costs of cardenolides on monarch fitness, using eight plant species that differ substantially in their cardenolide concentration and non-polarity to create large and biologically relevant variation. Because caterpillars were fed on individual plants, each with its own unique chemistry, we used individual butterfly and plant data as the level of replication in our analyses. In all the following models, we used mixed-effects models in which monarch lineage was included as a random factor. To analyse the species

differences in cardenolide concentration and non-polarity, we used analysis of variance in which cardenolide concentration (or non-polarity) was the dependent variable and plant species was the independent variable. To test whether plant species and parasite infection affect the survival of individual monarchs to adulthood, we used mixed-effects logistic regression models in which plant species identity, parasite treatment and their interactions were independent variables, while survivorship of individual monarchs (0 for failure to reach adulthood and 1 for successful development) was the dependent variable. To test whether plant species and parasite infection affect monarch life span, we used a mixed-effects linear model in which plant species identity, parasite treatment and their interactions were independent variables and life span (days) of individual monarchs was the dependent variable. To analyse whether plant species affects the spore loads of infected butterflies (an inverse measurement of antiparasite resistance), we used a mixed-effects linear model in which plant species was the independent variable and log-transformed spore load was the dependent variable. Additionally, using both uninfected and infected butterflies, we tested whether plant species affected the tolerance of butterflies to infection; we used a mixed-effects linear model in which log-transformed spore load and the interaction between plant species and log-transformed spore load were independent variables and the life span of butterflies was the dependent variable.

To investigate whether plant cardenolide concentration and non-polarity affected caterpillar survival to adulthood, we used mixed-effects logistic regression models in which cardenolide concentration (or non-polarity) was the independent variable 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 models in which cardenolide concentration or non-polarity was the independent variable and the spore load of infected butterflies was the dependent variable. We used the life span of both uninfected and infected butterflies to test whether 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 life span was the dependent variable. Finally, we examined the effects of cardenolide concentration and non-polarity on the life span 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 life span was the dependent variable. For non-polarity, to capture the nonlinear 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 nonlinear 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 nonlinear 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 the normality of errors was confirmed by 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 with the package lme4 1.1-11 (Bates *et al.* 2014), and mixed-effects linear models were performed with the package nlme (Pinheiro *et al.* 2009).

Results

The eight milkweed species varied substantially in their foliar cardenolide concentrations (Fig. 1a; $F_{7,\ 308} = 244{\cdot}49$, $P < 0{\cdot}001$). Post hoc comparisons showed that *A. asperula* ($13{\cdot}32 \pm 1{\cdot}68$ mg g^{-1}), *A. linaria* ($14{\cdot}65 \pm 1{\cdot}18$ mg g^{-1}) and *A. perennis* ($12{\cdot}02 \pm 0{\cdot}88$ mg g^{-1}) had similarly high cardenolide concentrations, followed by *A. curassavica* ($2{\cdot}09 \pm 0{\cdot}25$ mg g^{-1}) and *A. physocarpa* ($2{\cdot}43 \pm 0{\cdot}42$ mg g^{-1}). *Asclepias incarnata incarnata* ($0{\cdot}14 \pm 0{\cdot}04$ mg g^{-1}), *A. incarnata pulchra* (0 mg g^{-1}) and *A. tuberosa* (0 mg g^{-1}) had very low to undetectable foliar cardenolide concentrations.

Similarly, milkweed species varied in their foliar cardenolide non-polarity (Fig. 1b; $F_{5,\ 202} = 159{\cdot}07$, $P < 0{\cdot}001$). *Asclepias curassavica* ($0{\cdot}74 \pm 0{\cdot}01$), *A. physocarpa* ($0{\cdot}79 \pm 0{\cdot}02$) and *A. incarnata incarnata* ($0{\cdot}77 \pm 0{\cdot}03$) had indistinguishably high non-polarity, followed by *A. linaria* ($0{\cdot}64 \pm 0{\cdot}01$) and *A. asperula* ($0{\cdot}56 \pm 0{\cdot}02$). Although

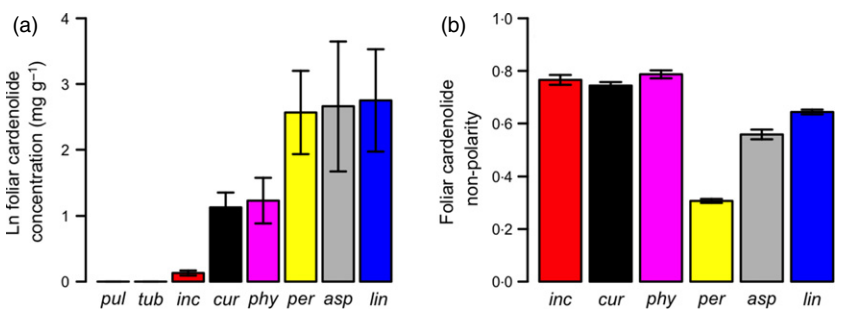


Fig. 1. Milkweed species differ in their foliar cardenolide (a) concentration and (b) non-polarity. Cardenolide concentrations in *Asclepias incarnata pulchra* and *Asclepias tuberosa* were 0; therefore, no non-polarity can be calculated for these two species. Data represent mean ± 1 SEM. Species abbreviations: *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*.

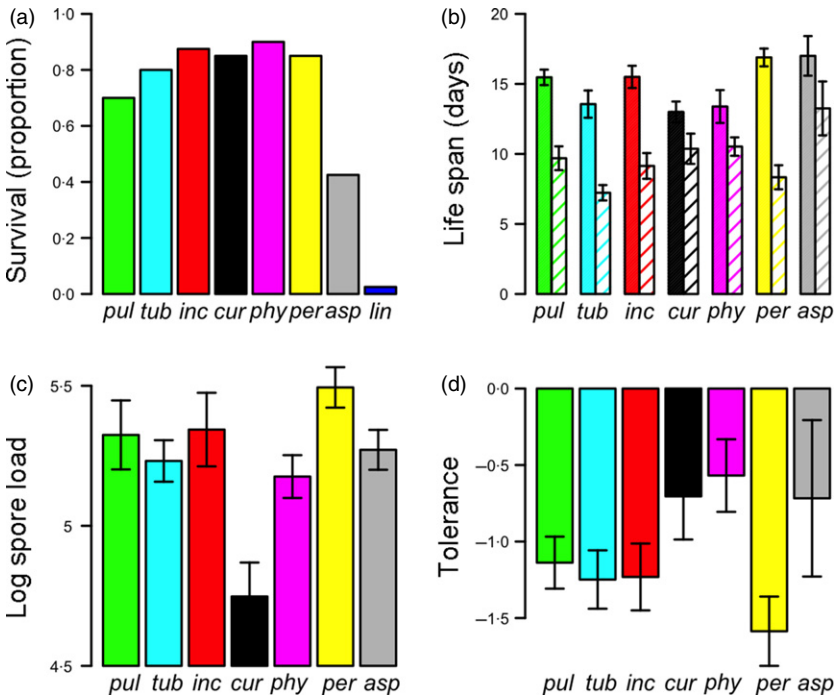


Fig. 2. Effects of milkweed species on (a) monarch caterpillar survival to adulthood, (b) life span of uninfected (solid bars) and infected monarch butterflies (hashed bars), (c) spore loads of infected monarch butterflies and (d) tolerance of monarch butterflies to parasite infection. Data represent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

foliar concentrations of cardenolides in *A. perennis* were very high, their cardenolides had the lowest non-polarity (0·31 ± 0·01).

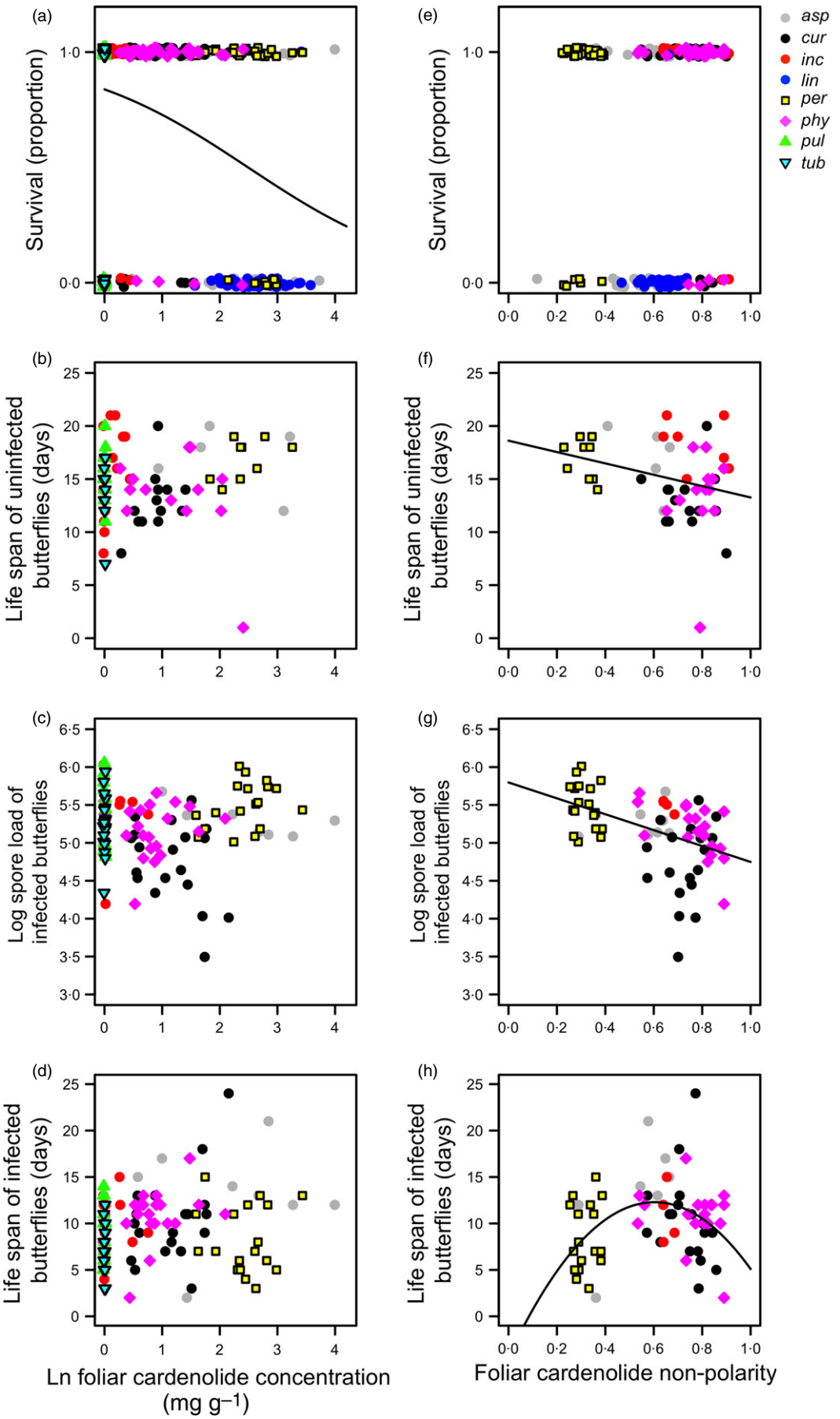
Caterpillar survival to adulthood varied among plant species (Fig. 2a; $\chi^2_7 = 124{\cdot}0$, $P < 0{\cdot}001$). However, neither parasite infection nor interactions between plant species and parasite infection affected the survival rate ($\chi^2_1 = 0{\cdot}02$, $P = 0{\cdot}88$; $\chi^2_7 = 9{\cdot}66$, $P = 0{\cdot}21$, respectively). This is expected, because *O. elektroscirrha* has not been found to reduce larval survival in previous studies. Life span of butterflies varied substantially among plant species ($F_{6,182} = 6{\cdot}00$, $P < 0{\cdot}001$), with infection status ($F_{1,182} = 105{\cdot}42$, $P < 0{\cdot}001$) and with their interaction (Fig. 2b; $F_{6,182} = 2{\cdot}74$, $P = 0{\cdot}01$), 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; $F_{6,104} = 6{\cdot}42$, $P < 0{\cdot}001$). The tolerance of butterflies to infection also varied among plant species (Fig. 2d; $F_{6,188} = 3{\cdot}90$, $P = 0{\cdot}001$). 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; $\chi^2_1 = 38{\cdot}78$, $P < 0{\cdot}001$). 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 analysing adult life span of uninfected and infected monarchs in the same model, we found that life span was unrelated to foliar cardenolide concentrations ($F_{1,192} = 2{\cdot}14$, $P = 0{\cdot}14$; cardenolide concentration × infection: $F_{1,192} = 0{\cdot}96$, $P = 0{\cdot}33$). Although cardenolide concentration was unrelated to the spore load of infected butterflies (Fig. 3c; $F_{1,109} = 0{\cdot}15$, $P = 0{\cdot}70$), it increased the tolerance of butterflies to infection (spore load × cardenolide concentration, $F_{1,194} = 5{\cdot}93$, $P = 0{\cdot}02$).

Cardenolide non-polarity was unrelated to survival rate (Fig. 3e; $\chi^2_1 = 0{\cdot}008$, $P = 0{\cdot}93$). When analysing the effects of cardenolide non-polarity on life span of uninfected and infected monarchs in the same model, we found a significant interaction between the quadratic term of non-polarity and parasite treatment ($F_{1,107} = 7{\cdot}19$, $P = 0{\cdot}009$), indicating that cardenolide non-polarity affected the life span of uninfected and infected butterflies differently. Specifically, higher cardenolide non-polarity was associated with lower adult life span of uninfected butterflies (Fig. 3f; linear term $F_{1,42} = 4{\cdot}23$, $P = 0{\cdot}046$; quadratic term $F_{1,42} = 0{\cdot}46$, $P = 0{\cdot}50$), whereas in infected monarchs, there was a quadratic relationship between non-polarity and life span (Fig. 3h; quadratic term $F_{1,61} = 9{\cdot}38$, $P = 0{\cdot}003$). This nonlinear relationship exists because high non-polarity was associated with reduced parasite spore load (Fig. 3g; $F_{1,62} = 17{\cdot}92$, $P < 0{\cdot}001$). Because the adult life span of infected butterflies was strongly negatively associated with parasite spore load ($F_{1,109} = 33$, $P < 0{\cdot}001$), 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 (antiparasitic resistance) of non-polar cardenolides.

Discussion

Many animals use environmentally derived secondary chemicals to combat disease (de Roode, Lefèvre & Hunter


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Survival (proportion)
Life span of uninfected butterflies (days)
Log spore load of infected butterflies
Life span of infected butterflies (days)
Ln foliar cardenolide concentration (mg g−1)
Foliar cardenolide non-polarity
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2013). Documenting the costs associated with using these secondary chemicals in natural systems is important for our understanding of the ecology and evolution of behavioural defences. 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 antiparasitic 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 the parasite spore load, they also reduce the adult life span of uninfected butterflies. This apparent trade-off in the use of non-polar cardenolides results in a nonlinear relationship between cardenolide non-polarity and the life span of infected butterflies. Overall, our results suggest that medication behaviours 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 life span. 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 life span 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 behaviour have focused on choices between plants with different cardenolide concentrations (Lefèvre *et al.* 2010, 2012), we currently do not know whether female butterflies are able to medicate by choosing among plants of different cardenolide non-polarity. Additionally, although monarchs display medication behaviour when they are infected, we do not currently know whether infected butterflies are able to avoid those plants on which the costs of medication become too high.

Petschenka & 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 the 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 antiparasitic benefits of these chemicals.

As an important non-immunological defence, 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 antiparasitic substances is not always straightforward (but see Singer, Mace & Bernays 2009; Bos *et al.* 2015). Sometimes, natural levels of variation in the medicinal components of diets are unknown, while at other times they covary with other dietary components. For example, while nectar alkaloids can reduce the parasite load in bumblebees, there are other secondary metabolites in nectar that also have antiparasitic properties, making

Fig. 3. Effects of milkweed foliar cardenolide concentration (a–d) and non-polarity (e–h) on monarch caterpillar survival to adulthood (a, e), adult life span of uninfected monarch butterflies (b, f), parasite spore load of infected monarch butterflies (c, g) and adult life span of infected monarch butterflies (d, h). Regression lines indicate significant relationships. Colour coding for different milkweed species follows Figs 1 and 2.

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 antiparasitic substances are toxic for hosts in the absence of parasites. Such costs may explain the spatial and temporal variation in medication behaviours. Additionally, the magnitude of costs may determine whether medication behaviour 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 tract 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, 2012), the effects of cardenolides on performance and oviposition behaviour 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 favour therapeutic medication behaviours, 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 nutritional milkweed chemistry. More generally, our results demonstrate that the assessment of costs and benefits of antiparasitic compounds requires an understanding of the chemical composition in addition to the overall concentration of such chemicals.

Acknowledgements

We thank C.D. Gowler for greenhouse maintenance and H. Streit for chemical analyses. The work was supported by National Science Foundation grants DEB-1257160 and DEB-1256115 to J.C.dR and M.D.H., respectively. We thank three anonymous reviewers for their constructive comments on earlier versions of the paper.

Data accessibility

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

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Received 8 December 2015; accepted 25 May 2016
Handling Editor: Sheena Cotter