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LETTER

Elevated atmospheric concentrations of carbon dioxide reduce monarch tolerance and increase parasite virulence by altering the medicinal properties of milkweeds

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Abstract

Hosts combat their parasites using mechanisms of resistance and tolerance, which together determine parasite virulence. Environmental factors, including diet, mediate the impact of parasites on hosts, with diet providing nutritional and medicinal properties. Here, we present the first evidence that ongoing environmental change decreases host tolerance and increases parasite virulence through a loss of dietary medicinal quality. Monarch butterflies use dietary toxins (cardenolides) to reduce the deleterious impacts of a protozoan parasite. We fed monarch larvae foliage from four milkweed species grown under either elevated or ambient CO_2, and measured changes in resistance, tolerance, and virulence. The most high-cardenolide milkweed species lost its medicinal properties under elevated CO_2; monarch tolerance to infection decreased, and parasite virulence increased. Declines in medicinal quality were associated with declines in foliar concentrations of lipophilic cardenolides. Our results emphasize that global environmental change may influence parasite–host interactions through changes in the medicinal properties of plants.

Keywords

Anthropogenic, *Asclepias*, cardenolides, *Danaus plexippus*, global environmental change, host–parasite interactions, monarch butterfly, *Ophryocystis elektroscirrha*.

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INTRODUCTION

When facing infection, hosts utilize two avenues of defense: resistance and tolerance (Råberg *et al*. 2007; Best *et al*. 2008). Resistance reduces the probability and degree of parasitic infection, and subsequent parasite replication (e.g. parasite fitness) (Best *et al*. 2008; Boots *et al*. 2009). In contrast, tolerance describes the ability of hosts to mitigate the negative fitness impacts of infection for a given pathogen load (Råberg *et al*. 2009; Kutzer & Armitage 2016a). While host resistance reduces parasite fitness by preventing infection or lowering parasite replication, host tolerance does not reduce parasite fitness. Therefore, the two defense strategies should engender different co-evolutionary outcomes for host–parasite dynamics (Roy & Kirchner 2000; Restif & Koella 2004).

Resistance and tolerance evolve because of the inherent damage (virulence) that parasites cause to their hosts. Virulence emerges from the interaction between parasite and host genotype (Lambrechts *et al*. 2006; Råberg *et al*. 2007) and varies with host ecology and condition (Thomas & Blanford 2003; Boots 2011; Howick & Lazzaro 2014). Together, host resistance and tolerance influence the rate at which parasites replicate and damage the host, governing the severity of virulence (de Roode *et al*. 2008a,b; Tao *et al*. 2015).

Understanding variation in resistance has long been a focus of disease ecology. Host genotype, physiology and environment all contribute to parasite resistance (Lambrechts *et al*. 2006; Wolinska & King 2009). In contrast, our understanding of host tolerance derives largely from the study of pests that attack plants (*reviewed in* Baucom & De Roode 2011). Recent work investigating host tolerance in animals has focused largely on host genotype under laboratory conditions (Råberg *et al*. 2007; Rohr *et al*. 2010; Jackson *et al*. 2014; Regoes *et al*. 2014). However, we miss important factors that contribute to variation in tolerance (Lefèvre *et al*. 2011) by isolating host–parasite interactions from the surrounding complex community of organisms and environmental conditions (Sternberg *et al*. 2012; Hayward *et al*. 2014; Tao *et al*. 2015; Clough *et al*. 2016; Kutzer & Armitage 2016b; Debes *et al*. 2017; Zeller & Koella 2017).

Global environmental change directly affects the ecology and evolution of host–parasite interactions (Tylianakis *et al*. 2008; Altizer *et al*. 2013; Becker *et al*. 2015). Generally, environmental change increases the distribution and prevalence of parasites in host populations (Garamszegi 2011; Zamora-Vilchis *et al*. 2012). Host resistance can increase or decrease in response to the direct effects of environmental change on parasite life cycle and host physiology (Bruno *et al*. 2003; Adamo & Lovett 2011; Paull & Johnson 2014). However, surprisingly little work has investigated how future environmental conditions may impact host tolerance of disease (Franke *et al*. 2017).

Moreover, indirect effects of environmental change on host–parasite interactions remain largely unexplored. Future environmental conditions will alter the composition and traits of the surrounding community (Tylianakis *et al*. 2008; Gunderson *et al*. 2017), which may lead to shifts in host resistance and tolerance, and parasite virulence. Phytophagous insect–parasite systems are excellent models with which to study the indirect effects of global change on host–parasite interactions in the context of their communities. Host–plant quality mediates the impacts of parasites on phytophagous insects (Cory & Hoover

2006; Shikano 2017), with significant medicinal effects of plant secondary metabolites (Felton & Duffey 1990; Hunter & Schultz 1993; Bernays & Singer 2005). Importantly, plant nutritional and defensive chemistry vary in response to environmental change (Bidart-Bouzat & Imeh-Nathaniel 2008). For instance, elevated concentrations of atmospheric CO_2 increase foliar carbohydrates, reduce nitrogen concentrations, and change secondary metabolite production (Hunter 2001; Robinson *et al*. 2012; Zavala *et al*. 2013). Such changes in host plant chemistry can alter herbivore performance against parasites (Cory & Hoover 2006; Shikano *et al*. 2010; Lampert 2012). In essence, environmental change alters plant quality, which can affect the interactions between herbivores and their parasites.

Here, we assess the impact of a pervasive driver of environmental change, elevated atmospheric CO_2 concentration (eCO_2), on the interaction between monarch butterflies, *Danaus plexippus,* and their protozoan parasite, *Ophryocystis elektroscirrha* (Mclaughlin *et al*. 1970). *Ophryocystis elektroscirrha* infection reduces adult monarch lifespan, fecundity, and flight ability (Bradley & Altizer 2005; de Roode *et al*. 2008b, 2009). Monarchs become infected by consuming dormant parasite spores on the surface of egg chorea and leaf tissue. During monarch development, parasites replicate within the insect and butterflies emerge covered in dormant parasite spores. As specialists, monarchs lay eggs on milkweed, *Asclepias,* host-plants (Malcolm & Brower 1989), thereby contaminating foliage and eggs with spores.

Certain milkweed species are strongly medicinal, reducing the probability of infection, growth rate, and virulence of *O. elektroscirrha* (de Roode *et al*. 2008a). The medicinal qualities of milkweed are related to cardenolides, toxic steroids produced in a majority of milkweed species (Gowler *et al*. 2015). Larvae that feed on plants with high cardenolide concentrations, or a high diversity of lipophilic cardenolides, suffer lower rates of infection, maintain higher fitness at a given parasite load, and produce fewer new parasites (de Roode *et al*. 2011b; Sternberg *et al*. 2012; Gowler *et al*. 2015). Additionally, high foliar nutrient concentrations can increase monarch tolerance to their parasites (Tao *et al*. 2015). Thus, foliar cardenolides and nutrients combine to mediate the resistance and tolerance of monarchs to their parasites. Recent work shows that eCO_2 causes decreases in cardenolide concentrations, changes in the composition of cardenolides, and declines in nutrient concentrations of milkweed (Vannette & Hunter 2011; Matiella 2012). Therefore, increasing atmospheric CO_2 concentrations may influence milkweed-mediated interactions between monarchs and their parasites.

Together these data motivate the overarching question of this study: Will monarch resistance and tolerance and mean *O. elektroscirrha* virulence change with milkweed phytochemistry under future atmospheric CO_2 concentrations? We performed a field mesocosm experiment to explore how eCO_2 alters the foliar chemistry of four milkweed species. We then measured the CO_2-mediated effects of altered food-plant chemistry on three aspects of monarch and parasite performance: (1) the spore load of infected monarchs; (2) the tolerance of monarchs, expressed as the rate of decline in lifespan with increasing spore load; and (3) the virulence of *O. elektroscirrha*, calculated as the decline in adult monarch lifespan due to infection. We hypothesised that the presence of lipophilic cardenolides, in conjunction with foliar nutrient quality, dictates the effects of eCO_2 on monarch–parasite interactions.

MATERIALS AND METHODS

We performed the experiment in two temporal blocks during 2014 and 2015. General experimental procedures were the same for both blocks, with minor differences noted below. The experiment was fully factorial, with milkweed species, parasite treatment (infected or uninfected) and CO_2 treatment (ambient or elevated) as main effects (Table S1).

Plant materials

We grew four species of milkweed under current (400 ppm, aCO_2) and future (760 ppm, eCO_2) concentrations of atmospheric CO_2 at the University of Michigan Biological Station (45.5587° N, 84.6776° W). Within this century, the concentration of atmospheric CO_2 will likely exceed 700 ppm (Solomon *et al*. 2009). Thus, following previous studies in this system (Vannette & Hunter 2011, 2014), we chose 760 ppm as our target future CO_2 concentration. Plants grew in a mesocosm array of 40 chambers, 20 maintained at aCO_2, and 20 at eCO_2 from dawn until dusk (Drake *et al*. 1989; Figure S1). We chose milkweed species that differed in their cardenolide concentrations, on a gradient of anti-parasitic effects from high to low: *Asclepias curassavica* (high), *A. speciosa, A. syriaca* (both medium) and *A. incarnata* (low) (Sternberg *et al*. 2012). All four milkweed species occur in sympatry in North America (Woodson 1954; Malcolm & Zalucki 1996). Seeds were obtained from commercial sources (Butterfly Encounters, CA in 2014 and Prairie Moon Nurseries, MN in 2015). After 6 weeks of cold stratification (for all but tropical *A. curassavica*), seeds were germinated and planted on 3 May 2014 and 5 May 2015 in deepots™ containing Metromix 360 and Osmocote 16:16:16 (N:P:K) controlled release fertiliser. Seedlings were watered daily and kept in a greenhouse for 2 weeks following germination to avoid frost damage. We transferred seedlings outside to their assigned chambers on 25 May 2014 and 23 May 2015.

Monarch caterpillars can consume three entire plants as larvae. Due to space limitations in 2014, only two plants of the assigned milkweed species and CO_2 treatment were grown for each larva. This made for 16 experimental plants in total in each chamber (4 milkweed species × 2 parasite treatments × 2 plants/monarch). Once larvae had consumed both assigned plants, they were fed cuttings from *A. tuberosa*, a milkweed with negligible cardenolides. Inoculation with the parasite took place 3 days after hatching (see below). Milkweed chemistry influences parasite infection success and severity just before and during consumption of parasite spores on plant material (de Roode *et al*. 2011a). Therefore, switching to an almost cardenolide-free host-plant just before pupation should not affect monarch–parasite interactions. In 2015, each chamber held enough plants to feed all larvae for their entire larval periods (4 milkweed species × 2 infection treatments × 3 plants/ monarch = 24 plants/chamber) with 20 aCO_2 and 20 eCO_2 chambers as before.

CO_2 concentrations were monitored during daylight hours in all eCO_2 chambers and one ambient chamber using a LI-COR 320 IRGA (LI-COR, Lincoln, NE, USA). The concentrations of CO_2 were adjusted throughout the day to maintain target values in each elevated chamber. Temperatures within the chambers were monitored using iButton dataloggers (IbuttonLink, Whitewater, WI, USA). Elevated CO_2 chambers averaged 21.03 ($\pm$ 0.03)°C, and aCO_2 chambers averaged 21.24 ($\pm$ 0.04)°C which were roughly 2°C higher than the outside average temperature of 18.93 ($\pm$ 0.04)°C and fell well within those temperatures experienced by monarchs in eastern North America. Plants were maintained in their chambers for 61 days in 2014 and 42 days in 2015 before experimental trials began.

Monarch sources and rearing methods

Monarchs were F_1 offspring of eight (2014) and seven (2015) crosses between eastern North American lineages. Monarch lineages are lab-bred full sib families, obtained by outcrossing wild-collected monarchs. We crossed 7 lineages in 2014 and 6 lineages in 2015 to produce our different families. Because we collected new wild monarchs each year (monarchs suffer from inbreeding depression and cannot be maintained as lab stocks between years), we did not make the same crosses in both years. Darkened monarch eggs (those about to hatch) were placed individually on milkweed cuttings taken from plants grown in the array. Only one larva from each treatment (4 milkweed species $\times$ 2 levels of parasite infection = 8 treatments) was reared on plants from each chamber (Table S1). We kept larvae individually in 0.64L plastic containers under aCO_2 on foliage transferred from the appropriate atmosphere to avoid any confounding direct effects of eCO_2 on insect performance (such effects are negligible (Bale *et al*. 2002)).

Each year, we infected monarchs with a single parasite family cultured from spores collected from an eastern North American butterfly. Hatchlings fed for three days on their assigned leaf tissue before inoculation with *O. elektroscirrha*. To infect larvae, 10 parasite spores were transferred to a 70.6 mm^2 leaf disk taken from each larva's assigned host plant. The leaf disk was placed in a petri dish containing moist filter paper and the assigned larva. Control larvae received spore-free leaf disks. Immediately after disks were punched from plants, foliage was collected for chemical analysis (below). Petri dishes containing disks and larvae were kept in an incubator held at 26°C with 16-h daylight. Once each larva had consumed its entire leaf disk (and therefore all spores) it was returned to its assigned container and fed foliage *ad libitum* from its designated plants until pupation.

Measures of monarch performance

Upon emergence, butterflies were transferred to individual glassine envelopes, stored at 14°C and inspected daily until death. Lifespan under these conditions correlates strongly with monarch lifetime reproduction (fitness) (de Roode *et al*. 2008b). Parasite virulence was measured as the magnitude reduction in lifespan of infected monarchs when compared to control monarchs. After death, infection success and spore loads were measured from adults following established methods (de Roode *et al*. 2008a, Table S2). Wings were removed and each monarch body was placed in a scintillation vial with 5 mL of deionised water. The mixture was vortexed for five minutes, and 10 μL aliquots were deposited into 4 wells in a hemocytometer for counting. Spore load represents the inverse of monarch resistance. Tolerance to *O. elektroscirrha* was measured as the slope of the relationship between spore load and lifespan, with a separate line (slope estimate) for each milkweed species by CO_2 treatment.

Plant defense measurements

On the same day as inoculations, we sampled milkweed cardenolide and nutrient concentrations using established methods (Zehnder & Hunter 2009; Tao & Hunter 2012). Six 424 mm^2 leaf disks were taken, deposited in 1 mL methanol and stored at −10°C for cardenolide analysis. Cardenolides were extracted, separated and quantified by reverse-phase high-performance liquid chromatography (HPLC) on a Waters Acquity UPLC with PDA detector (Waters Coperation, Milford, MA, USA) with 0.15 mg/mL digitoxin internal standard (Sigma Chemical Company, St. Louis, Missouri, USA). Peaks with symmetrical absorbance between 217–222 nm were identified as cardenolides. Another six disks were collected, weighed, dried, and reweighed to provide estimates of sample dry mass. Remaining foliage from the two punched leaves was collected, oven dried, and analysed using a TruMac CN Analyzer (Leco Corporation, St. Joseph, MI) to estimate foliar nitrogen (N) concentrations.

Statistical methods

Analyses were carried out in R (version 3.3.2). In all of the linear mixed effects models (lme4 package, LMMs) that follow, we included experimental year and chamber identity as random effects, to account for unintended temporal and spatial variation. We also included monarch family as a random effect in all models of monarch performance. For all models we visually inspected homogeneity of variance to confirm model best fit (Crawley 2012) and model comparisons were performed using likelihood ratio tests.

Monarch performance

Our analyses are limited to only those monarchs that survived to adulthood, including successfully infected monarchs, and uninoculated (control) monarchs (Table S1 & S2, Appendix S1) (Sternberg *et al*. 2012). Analyses of parasite burden were restricted to infected monarchs, but analyses of tolerance included both infected and uninfected monarchs.

To investigate effects of our treatments on monarch tolerance, we modelled adult lifespan (square-root-transformed) as a function of spore load (square-root-transformed) (Sternberg *et al*. 2012), including milkweed species and CO_2 treatment as fixed effects. To investigate the effects of CO_2 treatment and milkweed species on parasite virulence, we used monarch life span (square-root-transformed) as the dependent variable and parasite treatment as a fixed effect. Finally, to estimate monarch resistance, we included

monarch spore load (square-root-transformed) as the dependent variable, with milkweed species and CO_2 treatment as fixed effects. Analysis of monarch resistance was only conducted on infected individuals. Due to the nature of the parasite life cycle (Mclaughlin *et al.* 1970), we cannot measure the spore load of larval monarchs that died before adulthood because spores have not yet formed.

To test for a trade-off between host tolerance and resistance, we associated monarch tolerance with resistance to *O. elektroscirrha* using a linear regression (Råberg *et al.* 2009). We assessed any relationship between the 16 tolerance slope values of each treatment group in each year and the mean resistance values (1/spore load) of those treatment groups.

Milkweed chemistry and elevated CO_2

We explored the responses to our CO_2 treatments of foliar (1) cardenolide concentration (log-transformed), (2) cardenolide diversity, (3) cardenolide polarity, and (4) N concentration using LMMs. Cardenolide diversity was calculated using the Shannon diversity index: $H = -\text{sum}(P_i \log[P_i])$ where P_i is the relative amount of a cardenolide peak compared to the total amount of cardenolides in an individual plant (Rasmann & Agrawal 2011). We excluded *A. incarnata* plants from the LMM exploring effects of CO_2 treatments on cardenolide diversity because only 2 individuals produced more than one cardenolide peak. Following Rasmann & Agrawal (2011), we calculated an index of cardenolide polarity $P = \text{sum}(P_i\ RT_i)$, where RT_i is the retention time of the *i*th peak in the individual. The polarity index values that result range from 0 (highly polar) to 1 (highly lipophilic).

We also compared cardenolide composition among milkweed species and CO_2 treatments using permutational multivariate analysis of variance (PerMANOVA) (Anderson 2001) following Bray-Curtis ordination. The analysis was performed using the metaMDS function from the Adonis procedure of the Vegan package in R. We performed Nonmetric Multidimensional Scaling (NMDS) (McCune & Grace 2002) reducing the dimensions of the model with 999 permutations per model run and a maximum of 500 runs per dimension. We ultimately used a three-dimensional model in subsequent analyses (model stress = 0.119) (McCune & Grace 2002).

Within the cardenolides monarchs ingest, two specific cardenolides (RT585 and RT650) have been associated with the medicinal efficacy of milkweed species against *O. elektroscirrha* (de Roode *et al.* 2011b). In our experiment, we found these two cardenolides only in the foliage of *A. curassavica,* with the exception of one *A. syriaca* plant grown under aCO_2. Milkweed species are known to vary substantially in the types of cardenolides they produce (Sternberg *et al.* 2012). Given the established importance of these two compounds, and the losses that we observed in the medicinal activity of *A. curassavica* under elevated CO_2 (see Results, below), we ran separate LMMs with each of these cardenolides as dependent variables and CO_2 treatment as a fixed effect.

Milkweed chemistry and monarch performance

We observed significant effects of elevated CO_2 on monarch tolerance and parasite virulence on only one milkweed species, *A. curassavica* (see Results). We did not analyse resistance further because it did not vary with any of our treatments. We used LMMs to assess any individual and interactive effects of *A. curassavica* traits on monarch tolerance and parasite virulence. All analyses were restricted to those *A. curassavica* plants for which we had measures of cardenolide diversity and corresponding N data (N = 77). We performed a PerMANOVA on the cardenolide communities produced in *A. curassavica* alone. Additionally, we created a PCA of the center-log-ratios of cardenolide concentrations and used PCA axes 1 and 2 in separate LMMs to gauge the explanatory strength of the CO_2 treatment in determining monarch tolerance.

We used Akaike's information criterion (AIC) scores to select chemical traits that were associated with virulence or tolerance. We planned to add additional traits (and interactions) to each model only if the AIC scores improved by two points (Burnham & Anderson 2002, Table S6). However, in no case was more than one independent variable included in any model.

(1) *Chemistry and Tolerance*. Using the procedure described above, we assessed associations between *A. curassavica* traits and monarch tolerance (slope of fitness decline) by investigating effects of spore load and plant traits on lifespan (square-root-transformed).
(2) *Chemistry and Virulence*. Likewise, we measured associations between individual A. *curassavica* traits and parasite virulence by investigating the effects of infection treatment and plant traits on lifespan (square-root-transformed).

RESULTS

Monarch tolerance and parasite virulence

Monarch tolerance to *O. elektroscirrha* declined by 77% under eCO_2 for individuals reared on the medicinal milkweed, *A. curassavica* (Fig. 1). The tolerance of monarchs feeding on less medicinal milkweed species remained unchanged under eCO_2 (spore load *species*CO_2: $F_{3,315} = 4.50$, $P = 0.00415$, Fig. 1, Table S3). These results suggest that eCO_2 reduced the protective properties of *A. curassavica* to similar strength as *A. incarnata* under eCO_2 (Fig. 1).

Consistent with effects on tolerance, the virulence of *O. elektroscirrha* increased under eCO_2 in those monarchs reared on *A. curassavica* and remained unchanged on the lower cardenolide milkweed species (infection*species*CO_2: $F_{3,308} = 4.44$, $P = 0.0045$, Fig. 2a). Essentially, eCO_2 made *A. curassavica* non-medicinal, magnifying the reduction in fitness caused by infection to values similar to those of monarchs feeding on the other three milkweed species. The magnitude of the reduction in lifespan between control and infected monarchs feeding on *A. curassavica* increased from 1.8 to 7.2 days under eCO_2 (Fig. 2b). As expected, all infected monarchs had shorter lifespans than uninfected monarchs (infection: $F_{1,314} = 263.55$, $P < 0.0001$, Fig. 2b).

In contrast to their effects on monarch tolerance and parasite virulence, we found no effects of eCO_2 ($F_{1,129} = 1.71$, $P = 0.1931$), host plant species ($F_{3,137} = 1.28$, $P = 0.2845$), or their interaction ($F_{3,138} = 1.35$, $P = 0.2596$) on monarch

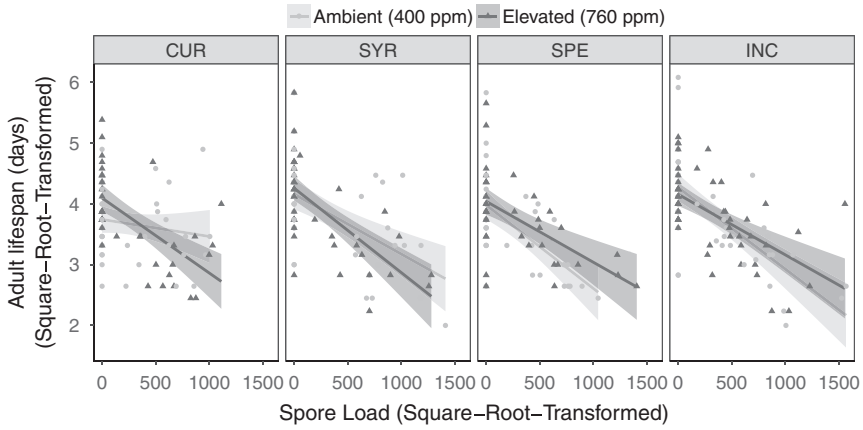

Figure 1 Monarch tolerance to *Ophryocystis elektroscirrha* infection as a function of milkweed species and CO_2 treatment. Light grey lines and points correspond to tolerance slopes of monarchs reared on plants grown under ambient CO_2 (400 ppm) and dark grey lines and points correspond to tolerance slopes of monarchs reared on plants grown under elevated CO_2 (760 ppm). Tolerance slopes are presented by milkweed species: CUR = *Asclepias curassavica*, SYR=*A. syriaca*, SPE=*A. speciosa*, INC=*A. incarnata.*

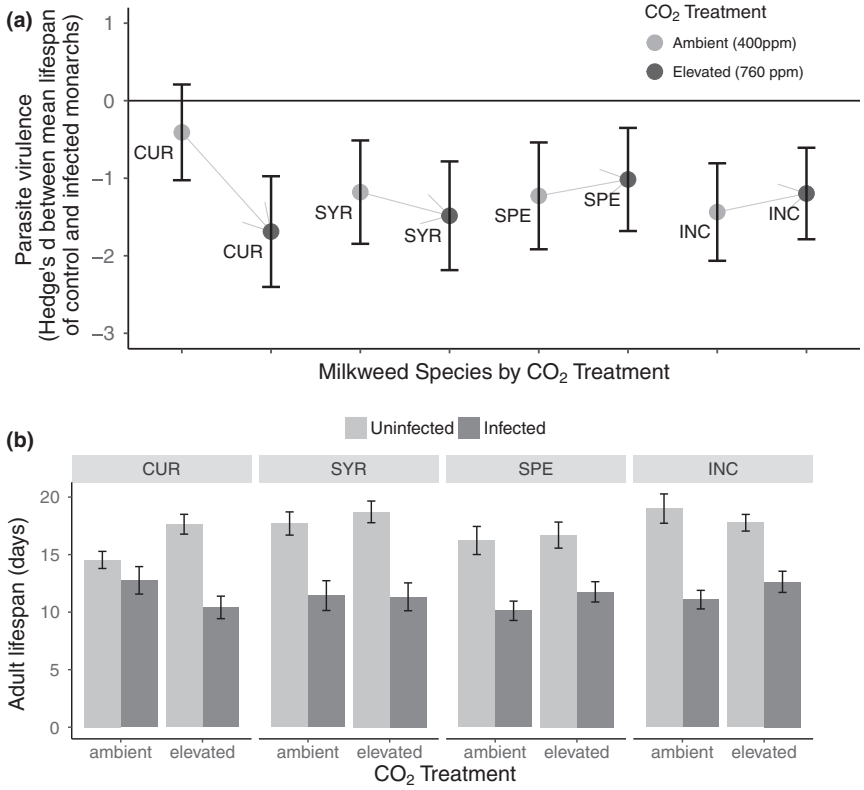

Figure 2 The virulence of *Ophryocystis elektroscirrha* parasites increases under elevated CO_2 when monarch larvae feed on *Asclepias curassavica*. Virulence is measured as the magnitude of the reduction in host fitness resulting from infection. In (a), points represent the standardised difference (Hedge's d ± 95% CI) in mean lifespan between uninfected and infected monarchs fed different species of milkweed under ambient CO_2 (400 ppm, light grey) and elevated CO_2 (760 ppm, dark grey). In (b), we show mean lifespan of parasite-infected (dark grey bars) and uninfected (light grey bars) monarchs (± 1 SE) used to calculate the Hedge's d values shown in (a). Lifespans were transformed to approximate normality of errors before statistical analyses but are presented here as untransformed values for ease of interpretation. Milkweed species codes match those presented above.

resistance to the parasite as measured by spore load. Additionally, we found no tradeoff between monarch tolerance and resistance to *O. elektroscirrha* ($F_{1,14} = 0.91$, $P = 0.3559$).

Milkweed chemistry and elevated CO_2

Foliar cardenolide concentrations were twelve times higher in *A. curassavica* than in *A. syriaca* (milkweed species:

$F_{3,166} = 192.31$, $P < 0.0001$, Fig. 3a). Cardenolide concentrations declined under eCO_2 across all milkweed species (CO_2: $F_{1,166} = 5.77$, $P = 0.0174$, Fig. 3a), and there was no interaction between milkweed species and CO_2 treatment ($F_{1,166} = 0.48$, $P = 0.6963$). The diversity of cardenolide molecular forms was four times higher in *A. curassavica* than in the other milkweed species (milkweed species: $F_{3,109} = 47.11$, $P < 0.0001$, Fig. 3b) and declined under eCO_2 in all milkweed species but *A. incarnata*, a species which rarely produces more than one cardenolide (CO_2: $F_{1,33} = 5.63$, $P = 0.02362$). There was no interaction between milkweed species and CO_2 treatment on cardenolide diversity (milkweed species*CO_2: $F_{2,141} = 0.54$, $P = 0.58274$). The average polarity of *A. curassavica* and *A. speciosa* cardenolides was reduced by eCO_2 treatments, while the average polarity of cardenolides increased in *A. syriaca* (species*CO_2: $F_{3,153} = 2.99$, $P = 0.03281$, Fig. 3c).

Across all four species, foliar N concentrations (plant nutritional quality estimate) declined under eCO_2 (CO_2: $F_{1,48} = 12.33$, $P = 0.00098$, Fig. 3d). Milkweed species also varied in their foliar N concentrations from 1.42% in *A. curassavica* to 1.14% in *A. syriaca* ($F_{1,137} = 4.43$, $P = 0.0052$, Fig. 3d).

Beyond simple measures of cardenolide polarity and diversity, milkweed species differed in the composition of foliar cardenolides (PerMANOVA; species: $F_{3,171} = 20.02$, $P = 0.001$, $R^2 = 0.26$, Fig. S2). The effects of CO_2 treatment on cardenolide community composition varied among milkweed species (PerMANOVA; species*CO_2: $F_{1,171} = 2.12$, $P = 0.001$, $R^2 = 0.027$, Fig. S2). In *A. curassavica* alone, the communities of cardenolides produced by individual plants differed between CO_2 treatments (CO_2: $F_{1,76} = 2.80$, $P = 0.03$, $R^2 = 0.036$, Fig. 4a). NMDS1 was associated with declines in the concentrations of lipophilic cardenolides (decline in polarity index) ($P < 0.0001$, $R^2 = 0.47$, Fig. 4b).

Concentrations of both RT585 and RT650, the two cardenolides with established medicinal activity (de Roode *et al.* 2011b), declined in *A. curassavica* under eCO_2. Concentrations of RT585 declined by 25% under eCO_2 ($F_{1,61} = 5.36$, $P = 0.02401$, Fig. 5a). Far fewer *A. curassavica* individuals produced RT650 (N = 6). Nonetheless, we detected a marginally significant 65% decline in RT650 under eCO_2 ($F_{1,4} = 5.92$, $P = 0.0717$, Fig. 5b).

A. curassavica chemistry and monarch performance

Tolerance

A significant interaction between spore load and a given plant trait on monarch lifespan indicates a correlation between that trait and tolerance to *O. elektroscirrha*. Monarch tolerance varied with the expression of lipophilic cardenolides in leaves (spore load* polarity: $F_{1,70} = 4.10$, $P = 0.04678$, Fig. 6a). Interestingly, the model containing

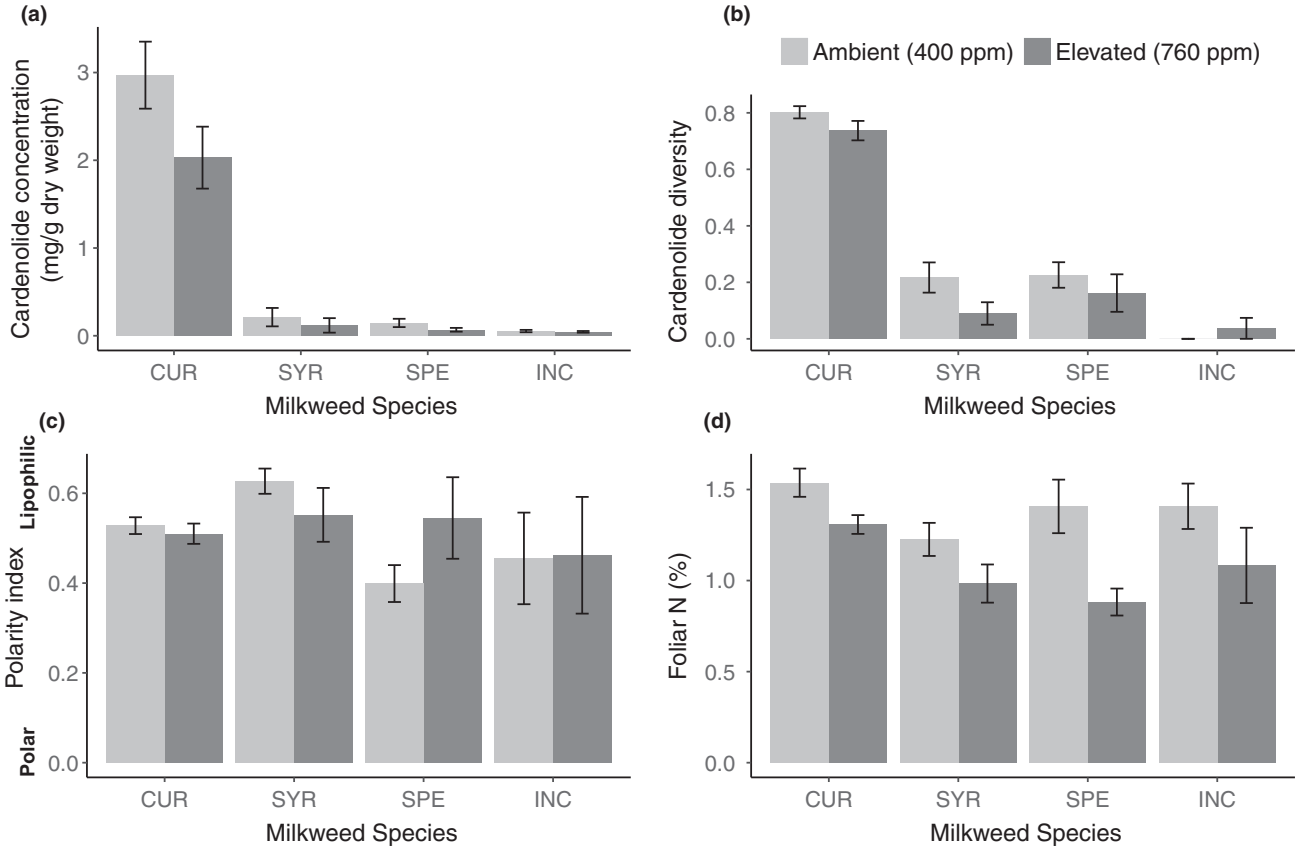

Figure 3 Effects of elevated CO_2 on foliar cardenolide concentrations (mg/g dry mass, a), cardenolide diversity (b), cardenolide polarity index (c), and foliar nitrogen concentration (%N) (d) of four milkweed species. Trait values were transformed to approximate normality of errors before analyses but are presented here in their untransformed values for ease of interpretation. Light grey bars represent plants grown under ambient CO_2 and dark grey bars are those from elevated CO_2 (± 1 SE). Milkweed species codes match those presented above.

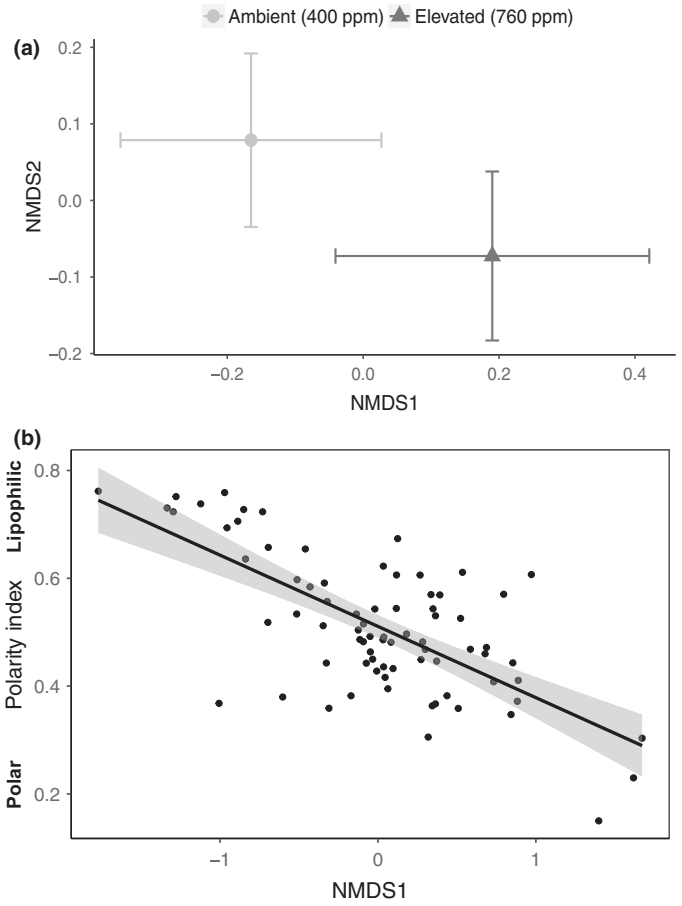

Figure 4 *Asclepias curassavica* plants differed in the composition of cardenolides that they produced under the different CO_2 treatments. In (a) light grey points represent plants grown under ambient CO_2 (400 ppm) and dark grey points represent plants grown under elevated CO_2 (760 ppm). In (b), we illustrate the negative association between NMDS axis 1 and the occurrence of lipophilic cardenolides.

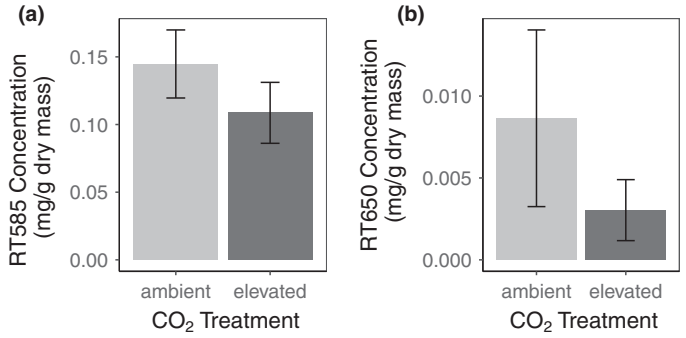

Figure 5 Effects of elevated CO_2 on the concentration of two medicinal cardenolides: (a) RT585 and (b) RT650 in *Asclepias curassavica*. Light grey bars represent foliar samples taken from plants grown under ambient CO_2 and dark grey bars are those from elevated CO_2 (± 1 SE).

cardenolide polarity fit the tolerance data better than did a model containing CO_2 treatment alone. Further, neither models containing the PCA axes of center-log-ratio-transformed cardenolide concentrations fit the data better than a model with just CO_2 treatment (Table S5), supporting our findings that cardenolide traits are more important in determining tolerance than concentration.

Virulence

A significant interaction between a plant trait and parasite treatment on monarch lifespan indicates a relationship between that trait and parasite virulence. As with tolerance, there was a positive relationship between the expression of lipophilic cardenolides and the lifespan of infected individuals (declining virulence) but it was marginally significant (parasite treatment*polarity: $F_{1,70} = 3.26$, $P = 0.0753$, Fig. 6b).

DISCUSSION

Monarch butterflies benefit from the medicinal properties of milkweeds when combating their parasites (de Roode *et al.* 2008a, 2011a; Sternberg *et al.* 2012; Gowler *et al.* 2015), and infected females can choose the most medicinal milkweeds for oviposition in laboratory choice tests (Lefèvre *et al.* 2010, 2012). Here, we show that a medicinal milkweed species, *A. curassavica*, loses its protective abilities under eCO_2. Our results suggest that rising CO_2 will reduce the tolerance of monarch butterflies to their common parasite, *Ophryocystis elektroscirrha*, and will increase parasite virulence. Ongoing changes in water availability (Andrews 2015), temperature (Couture *et al.* 2015) and soil nutrient loading (Zehnder & Hunter 2009; Tao *et al.* 2014) have already been shown to influence the cardenolide chemistry of milkweeds, with consequences for parasite–monarch interactions. Here, we add eCO_2 to the list of drivers that may alter monarch parasite–host interactions in a changing world.

We observed the lowest tolerance values in those monarchs feeding on *A. syriaca* grown under eCO_2 and the highest tolerance values in those monarchs feeding on *A. curassavica* grown under aCO_2. However, monarchs feeding on the same species of milkweed that once conveyed a tolerance advantage under aCO_2 (*A. curassavica*), experienced a 77% reduction in tolerance under eCO_2. Parasites caused the most virulence when monarchs fed on *A. incarnata* under aCO_2, reducing host lifespan by nearly 8 days (see Fig. 2b). Parasites caused the least virulence in those monarchs feeding on *A. curassavica* grown under aCO_2, reducing mean lifespan by only 2 days. Importantly, monarchs feeding on this same species, *A. curassavica*, under eCO_2 experienced virulence of comparable values to non-medicinal species like *A. incarnata*, suffering a reduction in lifespan of 7 days due to infection. These results are the first to show effects of environmental change on host tolerance to parasites and parasite virulence resulting from indirect effects mediated by community members.

A growing number of studies stress the importance of understanding the indirect mechanisms by which disease will respond to changing environmental conditions (Tylianakis *et al.* 2008; Altizer *et al.* 2013; Gunderson *et al.* 2017). Indirect effects of environmental change on host–parasite interactions emerge from additional members of ecological communities (Keesing *et al.* 2006; Wolinska & King 2009; Vuong *et al.* 2017). Associated predators, competitors and symbionts are all subject to the effects of environmental

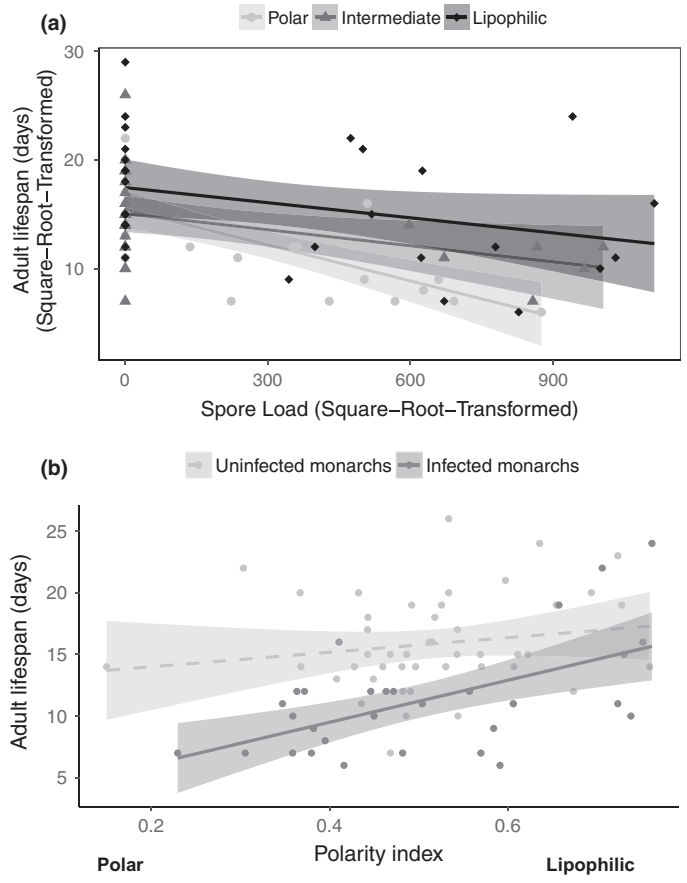

Figure 6 The effects of cardenolide polarity on (a) monarch tolerance to infection by *Ophryocystis elektroscirrha* and (b) the lifespan of infected and uninfected monarchs. A high polarity index reflects greater expression of lipophilic cardenolides. The slopes of the lines in (a) indicate monarch tolerance to infection, with steeper slopes representing lower tolerance. For visual simplicity, we have binned butterflies in (a) by the polarity of the cardenolides that they consumed as larvae. However, the analysis was performed with un-binned polarity data, and binning was used purely as a simplified alternative to a 3D graph. We present the square-root-transformed variables for ease of comparison with Fig. 1. In (b), light grey points and lines indicate uninfected (Control) monarchs, and dark grey points and lines indicate infected monarchs.

change, which may alter their interactions with host–parasite pairs (Ritchie 2006; Gherlenda *et al*. 2015). Here, we discover a previously unrecognised indirect mechanism by which environmental change can act on disease: the loss of medicinal compounds in host diet, contributing to reductions in host tolerance and increases in parasite virulence.

Changes in host tolerance and parasite virulence under future environmental conditions have important evolutionary implications. Theory predicts that reductions in resistance will lesson antagonistic coevolution between host and parasite (Roy & Kirchner 2000; Råberg *et al*. 2009; Rohr *et al*. 2010). However, we are less certain what changes in host tolerance could mean for host-parasite dynamics (Best *et al*. 2008; Schneider & Ayres 2008). Because tolerance helps to maintain host fitness when infected, less tolerant hosts should suffer shorter infections due to increased mortality, thereby potentially decreasing transmission and the prevalence of parasites in the host population (Miller *et al*. 2006). In our study, reduced tolerance was also accompanied by increased parasite virulence. In some cases, increased in virulence may lead to local extinction (Kutzer & Armitage 2016a; Wilber *et al*. 2017). We expect parasites that cause higher virulence to be selected against when host tolerance is also reduced because the risk of premature host mortality is higher. Early host death reduces parasite fitness and thus, induces selection on parasite virulence to decrease to a new optimum (Little *et al*. 2010). Given the reductions in host tolerance and increases in early monarch death, we predict that future environmental conditions may select for intrinsically less virulent parasites. Moreover, it is important to note that even when parasites evolve lower levels of intrinsic virulence, the actual virulence experienced by infected monarchs is likely to increase due to their reduced plant-derived tolerance. Additionally, our study only investigated the response of two parasite families (one in each year) to eCO_2 and milkweed species. To make better inferences about the evolutionary trajectories of parasite virulence and host tolerance under future conditions, further studies should examine the importance of parasite families under eCO_2.

Our results add to a substantial body of work that emphasises the role of environmental factors in phytophagous host–parasite interactions (Cory & Hoover 2006; Myers & Cory 2016; Shikano 2017). The largest declines in tolerance and increases in virulence occurred in monarchs feeding on *A. curassavica*, a species in which cardenolide production declined by nearly 25% when grown under eCO_2. However, total cardenolide concentrations did not correlate with changes in tolerance. Rather, reductions in cardenolide concentration under eCO_2 occurred in concert with changes in cardenolide community composition and declines in the expression of lipophilic cardenolides (Figs 4 and 5). Because the polarity of cardenolides partially determines their biological activity (Agrawal *et al*. 2012), the loss of lipophilic cardenolides under eCO_2 compromises the anti-parasitic properties of milkweed foliage. Infected monarchs that consume lipophilic cardenolides live longer than do those infected monarchs consuming polar cardenolides (Sternberg *et al*. 2012; Tao *et al*. 2016a). Previous work has shown that declines in the concentrations of two key lipophilic cardenolides, RT585 and RT650, increase parasite virulence (de Roode *et al*. 2011b). We also observed reductions in these two cardenolides in our populations of *A. curassavica* that were exposed to eCO_2, which likely led to the observed increases in parasite virulence. Interestingly, *A. syriaca* and *A. speciosa* did not produce these compounds and were also not protective in our study. Perhaps this is because different milkweed populations and individuals vary substantially in the concentration and composition of cardenolides produced within species (Vannette & Hunter 2011). *Asclepias syriaca,* specifically, is known to exhibit striking variation in cardenolide concentrations among populations (Andrews 2015). It is also important to note that the lifespan of uninfected monarchs feeding on *A. curassavica* increased under eCO_2. This increase in the fitness of uninfected monarchs may illustrate the cost of consuming toxic, lipophilic cardenolides (Sternberg *et al*. 2012; Tao *et al*. 2016b) because the frequency of those potent compounds declined under eCO_2 as monarch lifespan increased.

By demonstrating that tolerance to parasite infection can be altered by environmental change, we reinforce the idea that tolerance is not solely determined by intrinsic host factors but relies additionally on environmental conditions including interactions with other community members. As the environmental factors that mediate disease change, further empirical studies are sorely needed to explore the interplay between multiple global change drivers and host–parasite interactions embedded within diverse ecological communities.

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STATEMENT OF AUTHORSHIP

LED, MDH & JCdR designed the experiment. JCdR provided butterfly and parasite materials along with disease protocols. LED & MDH collected and analysed the data with suggestions from JCdR. LED wrote the manuscript and all authors contributed substantially to revisions.

DATA ACCESSIBILITY STATEMENT

Data are available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.d68kg81

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