

# Phytochemical changes in milkweed induced by elevated CO<sub>2</sub> alter wing morphology but not toxin sequestration in monarch butterflies

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## Funding information

Division of Environmental Biology, Grant/Award Number: 1256115 and 1257160

Handling Editor: Julia Koricheva

## Abstract

1. Environmental change has the potential to influence trophic interactions by altering the defensive phenotype of prey.
2. Here, we examine the effects of a pervasive environmental change driver, elevated atmospheric concentrations of CO<sub>2</sub> (eCO<sub>2</sub>), on toxin sequestration and flight morphology of a specialist herbivore.
3. We fed monarch butterfly larvae, *Danaus plexippus*, foliage from four milkweed, *Asclepias*, species of varying chemical defence profiles grown under either ambient or eCO<sub>2</sub>. We also infected a subset of these herbivores with a protozoan parasite, *Ophryocystis elektroscirrha*, to understand how infection and environmental change combine to alter herbivore defences. We measured changes in phytochemistry induced by eCO<sub>2</sub> and assessed cardenolide, toxic steroid, sequestration and wing morphology of butterflies.
4. Monarchs compensated for lower plant cardenolide concentrations under eCO<sub>2</sub> by increasing cardenolide sequestration rate, maintaining similar cardenolide composition and concentrations in their wings under both CO<sub>2</sub> treatments. We suggest that these increases in sequestration rate are a by-product of compensatory feeding aimed at maintaining a nutritional target in response to declining dietary quality under eCO<sub>2</sub>.
5. Monarch wings were more suitable for sustained flight (more elongated) when reared on plants grown under eCO<sub>2</sub> or when reared on *Asclepias syriaca* or *Asclepias incarnata* rather than on *Asclepias curassavica* or *Asclepias speciosa*. Parasite infection engendered wings less suitable for sustained flight (wings became rounder) on three of four milkweed species. Wing loading (associated with powered flight) was higher on *A. syriaca* than on other milkweeds, whereas wing density was lower on *A. curassavica*. Monarchs that fed on high cardenolide milkweed developed rounder, thinner wings, which are less efficient at gliding flight.
6. Ingesting foliage from milkweed high in cardenolides may provide protection from enemies through sequestration yet come at a cost to monarchs manifested as lower quality flight phenotypes: rounder, thinner wings with lower wing loading values.

7. Small changes in morphology may have important consequences for enemy evasion and migration success in many animals. Energetic costs associated with alterations in defence and morphology may, therefore, have important consequences for trophic interactions in a changing world.

#### KEYWORDS

*Asclepias*, cardenolides, *Danaus plexippus*, environmental change, *Ophryocystis elektroscirrha*, plant secondary metabolites, predator-prey interactions

## 1 | INTRODUCTION

Environmental change influences trophic interactions through multiple mechanisms (Gilman, Urban, Tewksbury, Gilchrist, & Holt, 2010; Tylanakis, Didham, Bascompte, & Wardle, 2008). For example, elevated atmospheric carbon dioxide (eCO<sub>2</sub>) has both direct and indirect effects on organismal physiology and behaviour. Plants grown under eCO<sub>2</sub> produce tissues with lower nitrogen concentrations (Drake, Gonzalez-Meler, & Long, 1997; Robinson, Ryan, & Newman, 2012), causing herbivores to increase the amount of foliage they consume (Docherty et al., 1996; Johnson, Lopaticki, & Hartley, 2014). Moreover, eCO<sub>2</sub> also changes the composition and concentration of plant secondary metabolites (PSMs) (Klaiber, Dorn, & Najar-Rodriguez, 2013; Ryan, Rasmussen, & Newman, 2010). Because catabolizing PSMs is energetically costly, changes in these compounds affect the ecology of herbivores (Hunter, 2016). In general, herbivore growth, fecundity and survival decline under eCO<sub>2</sub> (Jamieson et al., 2017; Robinson et al., 2012). Higher trophic levels are also affected by CO<sub>2</sub>-induced shifts in plant quality (Facey, Ellsworth, Staley, Wright, & Johnson, 2014; Ode & Crompton, 2013; Ode, Johnson, & Moore, 2014), often mediated by shifts in prey nutrition and growth (Chen, Ge, & Parajulee, 2005; Klaiber, Najar-Rodriguez, Dialer, & Dorn, 2013). For example, eCO<sub>2</sub> reduces alfalfa nutritional quality, which increases armyworm larval development times, resulting in asynchrony between larvae and parasitoid wasps, ultimately reducing parasitoid fitness (Dyer, Richards, Short, & Dodson, 2013). Elevated CO<sub>2</sub> may also benefit higher trophic levels by inhibiting herbivore defence and escape (Hentley, Vanbergen, Hails, Jones, & Johnson, 2014).

Many specialist herbivores have evolved mechanisms to co-opt PSMs for their own defence (Dyer & Deane Bowers, 1996; Opitz & Müller, 2009; Petschenka & Agrawal, 2016). Sequestration by insect herbivores involves the modification, transfer and storage of toxic compounds at high metabolic costs, potentially reducing immune responses to other enemies (Greeney, Dyer, & Smilanich, 2012; Smilanich, Dyer, Chambers, & Bowers, 2009). Monarch butterflies, *Danaus plexippus*, store toxic steroids (cardenolides) derived from the foliage of their milkweed, *Asclepias*, host plants (Reichstein, Euw, Parsons, & Rothschild, 1968). Cardenolides disrupt the function of Na<sup>+</sup>/K<sup>+</sup> channels in animal cells (Agrawal, Petschenka, Bingham, Weber, & Rasmann, 2012) and render monarchs well-defended prey (Malcolm & Brower, 1989). The concentration and composition of

cardenolides sequestered by monarchs depend on milkweed species, the amount of tissue consumed and sequestration efficiency (Agrawal, Ali, Rasmann, & Fishbein, 2015; Bowers & Collinge, 1992; Camara, 1997; Malcolm, 1990, 1994). Environmental factors that alter phytochemistry and consumption rates could also influence sequestration and affect vulnerability to enemies. Despite a growing body of work illustrating the effects of environmental change on milkweed chemistry (Tao, Berns, & Hunter, 2014; Vannette & Hunter, 2011), little is known about how monarch sequestration will respond to future environmental conditions (but see Tao & Hunter, 2015).

In addition to sequestration, the seasonal migration of monarchs may also reduce mortality imposed by natural enemies. In eastern North America, monarchs migrate up to 4,500 km from their summer breeding grounds to overwintering sites in Mexico every fall (Urquhart & Urquhart 1978; Brower & Malcolm, 1991; Flockhart et al., 2017). For many flying animals, foraging, escape and migration are strongly influenced by wing size, shape and wing loading, the ratio between body mass and wing area (Berwaerts, Van Dyck, & Aerts, 2002; Dudley, 2002). Subtle changes in wing size and shape can affect drag, lift and flight behaviour (Srygley & Thomas, 2002). To maximize energy use efficiency, flying animals both glide and actively propel (Kovac, Vogt, Ithier, Smith, & Wood, 2012; Park, Bae, Lee, Jeon, & Choi, 2010). Larger, more elongated wing shapes, with high wing length-to-width ratios (aspect ratio), allow for optimal gliding flight (Kerlinger, 1989). Migratory eastern N. American monarchs have larger and more elongated wings (higher aspect ratios) than non-migratory conspecifics (Altizer & Davis, 2010; Li, Pierce, & de Roode, 2016). Migratory monarchs also have higher wing loading values, correlated with larger energy reserves for stronger powered flight (Dudley & Srygley, 2008). However, despite an extensive body of literature detailing the importance of dietary chemistry for insect fitness (Awmack & Leather, 2002), few studies have explored the effects of diet on wing morphology and flight ability (Boggs & Freeman, 2005; Johnson, Solensky, Satterfield, & Davis, 2014; Pellegrons, Van Dongen, Van Dyck, & Lens, 2009). Food restriction reduces monarch wing size (Johnson, Solensky et al., 2014), but no study to date has examined the effects of changing phytochemistry on monarch wing morphology.

Natural enemies may also influence toxin sequestration and morphology of herbivores. While sequestration can reduce insect immunity to parasites (reviewed in Greeney et al., 2012), it is

unclear how parasite infection alters sequestration. Throughout their range, monarchs suffer infection by a debilitating, protozoan parasite, *Ophryocystis elektroscirrha*, that reduces monarch life span, fecundity and flight ability (Bradley & Altizer, 2005; de Roode, Chi, Rarick, & Altizer, 2009). Heavily infected butterflies are more likely to die during migration, termed “migratory culling” (Altizer, Hobson, Davis, De Roode, & Wassenaar, 2015). Thus, monarchs that do arrive at overwintering grounds support lower pathogen loads (Altizer, Bartel, & Han, 2011). For moderately infected monarchs, there is no clear effect of infection on wing morphology (Bradley & Altizer, 2005). *Ophryocystis elektroscirrha* likely depletes the energy reserves necessary for flight (Altizer et al., 2015). However, additional stressors, such as reductions in diet quality induced by eCO<sub>2</sub>, may influence the impact of infection on flight ability.

Here, we investigate the effects of eCO<sub>2</sub> on the defensive phenotype of monarch butterflies. We fed larvae foliage from four milkweed species with varying phytochemistry grown under either ambient CO<sub>2</sub> (aCO<sub>2</sub>) or eCO<sub>2</sub>. We also infected a subset of monarchs with *O. elektroscirrha*, to understand how infection and environmental change combine to alter monarch defence, including wing traits associated with migration. We measured changes in phytochemistry induced by eCO<sub>2</sub> and assessed sequestration and morphology of butterflies. We expected sequestration profiles to mirror changes in plant chemistry induced by eCO<sub>2</sub> because factors that alter phytochemistry and consumption rates should also influence the types and amounts of PSMs monarchs sequester. We also predicted that changes in cardenolides and reductions in the nutritional quality of larval host plants grown under eCO<sub>2</sub> (Decker, de Roode, & Hunter, 2018; Robinson et al., 2012) would cause declines in the quality of the insect flight phenotype: smaller, thinner and rounder wings with lower wing loading values. Feeding on lower quality food with different types and amounts of cardenolides may engender a metabolic cost inflicting stress upon the insect and inducing morphological changes. Finally, we hypothesized that the metabolic costs of infection would exacerbate any deleterious effects of eCO<sub>2</sub> on cardenolide sequestration or wing morphology.

## 2 | MATERIALS AND METHODS

### 2.1 | Milkweed and monarch source materials

We analysed the wings of monarchs reared on milkweeds grown under ambient (400 ppm) or elevated (760 ppm) CO<sub>2</sub> at the University of Michigan Biological Station (UMBS). We provide details of the UMBS CO<sub>2</sub> array in Decker, de Roode, and Hunter 2018. In 2015, we grew four milkweed species in a 40 chamber array (Drake, Leadley, Arp, Nassiry, & Curtis, 1989), with 20 chambers maintained at aCO<sub>2</sub> and 20 at eCO<sub>2</sub>. Within those chambers, we grew milkweed that varied in cardenolide concentrations, ranging from high to low: *A. curasavica*, *A. syriaca*, *A. speciosa* and *A. incarnata*. Seeds were planted in the UMBS glasshouse, and 18 days later, seedlings were transferred outside into the chamber array for the remainder of the experiment.

Each chamber contained 24 milkweeds (four species × six plants per species).

The monarchs used in this study were the F<sub>1</sub> offspring of seven full-sib crosses between monarch lineages from eastern N. America (St Marks, FL). Monarch larvae were assigned to one of the 16 treatments (two parasite treatments × four host plant species × two levels of CO<sub>2</sub> × 20 replicate chambers) making for 320 monarchs reared in total. Not all monarchs survived to adulthood, and some inoculated monarchs resisted infection, inflating the sample size of uninfected monarchs. Final sample sizes varied for each species by CO<sub>2</sub> treatment between  $N = 19\text{--}27$  for uninfected and  $N = 5\text{--}11$  for infected monarchs (Supporting Information Table S1). Each individual monarch larva was assigned three plants of the same treatment grown in the same chamber on which to complete development.

Monarch larvae were inoculated with *O. elektroscirrha* on cuttings from one of their three assigned plants. After 42 days of growth in the array, we placed plant cuttings in individual containers kept under aCO<sub>2</sub>. A darkened monarch egg (darkening indicates eggs ready to hatch) was attached to a leaf on each cutting to ensure that neonates consumed their assigned plant before parasite inoculation. Three days after hatching, larvae were inoculated with *O. elektroscirrha* following the methods of de Roode, Pedersen, Hunter, and Altizer (2008). Parasites originated from one lineage collected from an eastern N. American, wild-caught butterfly. Control larvae were fed leaf discs of the same size with no spores. Foliar chemistry samples were taken from each plant at the same time as inoculations (see below) and assumed to adequately reflect the chemistry of the other two assigned plants the larvae consumed in later instars.

Larvae were fed cuttings from their assigned host plants ad libitum until pupation. Adult butterflies were sexed, weighed and kept in 5.75 × 9.5 cm glassine envelopes at 15°C for the remainder of their adult lives (de Roode, Gold, & Altizer, 2007). Three weeks after death, we removed and stored the monarch wings at -20°C, and estimated parasite spore load from bodies using established methods following de Roode et al. (2008). We scanned the left forewing of each monarch on an HP ScanJet 6300C (Hewlett-Packard, Palo Alto, USA), weighed the wing and stored it in 1-ml centrifuge tubes for cardenolide analyses.

### 2.2 | Cardenolide chemical analysis

We quantified foliar cardenolide concentrations (Vannette & Hunter, 2011; Zehnder & Hunter, 2009) from the first milkweed that monarchs consumed and in left forewings, because wing cardenolides correlate tightly with body cardenolide concentrations and wings are typically the first tissues tasted by bird predators upon attack (Fink & Brower 1981). We detail our chemical analysis in Supporting Information Appendix S1.

### 2.3 | Wing morphometrics

We measured forewing morphometrics because monarchs position forewings to cover their hindwings during soaring flight (Altizer &

Davis, 2010), allowing forewing size and shape to have the largest influence on flight ability. Additionally, preliminary work established that milkweed chemistry only affects forewing morphology (Berns, Zelditch, & Hunter, 2014).

We scanned the left forewing of each specimen with a ruler for scale and calibrated Adobe Photoshop (Adobe, San Jose, USA) to calculate distance measures based on a pixel-to-millimetre ratio. We took four basic measures of forewing morphology: first, length of the butterfly wing from wing apex to thorax insertion (mm); second, width of the forewing at the longest axis perpendicular to the length measurement (mm); third, total forewing area (mm<sup>2</sup>); and fourth, wing perimeter (mm, Supporting Information Figure S1). For slightly damaged wings, we estimated wing edges to create a complete outline. Butterflies with substantial wing damage were discarded from all analyses.

We calculated two metrics of forewing shape: wing aspect ratio (length divided by width) and roundness (area-to-perimeter ratio:  $4\pi \text{ area/perimeter}^2$ ) (Altizer & Davis, 2010). We also calculated wing loading (body mass/wing area), a common aeronautical measure indicative of manoeuvrability and flight performance. Finally, we examined butterfly wing density, which we termed specific wing area (wing area/wing mass).

## 2.4 | Analyses

We used linear mixed models (LMMs; R version 3.3.2.; package: lme4) to assess effects of our treatments on phytochemistry, toxin sequestration and wing morphology. Chamber identity was included as a random effect in all models (Littell, Stroup, & Freund, 2002; Vannette & Hunter, 2011). For models with monarch traits, we also included monarch lineage as a random effect because it was not specifically manipulated and had uninformative factor levels. We transformed all (dependent and independent) variables when necessary to achieve homogeneity of variance, simplified models when possible and visually inspected model residuals to confirm best fit (Crawley, 2012). We present model structures, results and random effects in Supporting Information Table S3.

To determine the effects of CO<sub>2</sub> and milkweed species on foliar cardenolide concentration, we ran LMMs with foliar cardenolide concentration (square-root-transformed) as a response variable and CO<sub>2</sub> treatment and milkweed species as fixed effects. Because there was a significant interaction between milkweed species and CO<sub>2</sub>, we analysed each plant species separately to determine which species drove the interaction. We used similar LMMs to test for effects of CO<sub>2</sub>, host plant species and infection on monarch wing cardenolides (square-root-transformed). Additionally, we tested whether CO<sub>2</sub> treatment or infection altered the relationship between foliar and wing cardenolides by including these factors (CO<sub>2</sub> treatment and infection) as fixed effects in an LMM with foliar cardenolide concentration (square-root-transformed) as an independent variable and wing cardenolide concentration (square-root-transformed) as the dependent variable. A significant interaction between foliar cardenolide concentration and either of the factors (CO<sub>2</sub> treatment or

infection) indicates a change in the slope of the relationship between plant and butterfly cardenolides dependent on that factor.

Some monarchs exposed to *O. elektroscirra* resist infection and are spore-free as adults. We compared the wing cardenolides of control monarchs (never exposed to the parasite) with those of monarchs that were exposed to the parasite but had no spores. We ran an LMM with monarch cardenolide concentration (square-root-transformed) as the dependent variable and parasite exposure (control and exposed but uninfected) as a fixed effect. There were no significant differences between resistant monarchs and control monarchs in the sequestration of total cardenolides ( $F_{1,190} = 0.90$ ,  $p = 0.345$ ). Therefore, in all analyses of sequestration, we grouped these two monarch treatments (control and exposed but uninfected) into one “uninfected” status. We followed a similar procedure to determine whether or not monarch sex influenced sequestration chemistry and found no effect of monarch sex on sequestered cardenolide concentrations ( $F_{1,250} = 0.24$ ,  $p = 0.624$ ). Therefore, monarch sex was not included in models that explored treatment effects on toxin sequestration.

We used permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) to compare the effects of CO<sub>2</sub> treatment, milkweed species and, for butterfly cardenolides, infection status on the assemblage of cardenolide compounds produced in milkweed and sequestered by monarchs. For plant cardenolide composition, we performed a PERMANOVA with CO<sub>2</sub> treatment, milkweed species and their interaction as independent variables, and Bray–Curtis distance of percentage weight of each foliar cardenolide peak as dependent variables. We chose Bray–Curtis as our ordination because it includes relative abundance information and accounts for peak identity. To determine which factors altered wing cardenolide composition, we performed a PERMANOVA with CO<sub>2</sub> treatment, milkweed species, infection status and their interactions as independent variables, and the Bray–Curtis distance of percentage weight of each sequestered wing cardenolide peak as dependent variables.

We followed Altizer and Davis (2010) and used principal component analysis (PCA) to reduce butterfly morphology measures into one PCA axis explaining forewing size (PCA-size) and another PCA axis explaining forewing shape (PCA-shape). Forewing length, width, area and perimeter were used to create the PCA-size axis that explained 99.6% of the total variance, while forewing area and roundness were used to create the PCA-shape axis that explained 95.2% of the total variance. Higher values of PCA-size represent larger wings, and higher values of PCA-shape represent more elongated wings. We first ran LMMs similar to those used above for sequestration results, to test whether there was a difference between control monarchs and those that did not become infected in the size and shape of their wings. We ran two LMMs with either PCA-shape or PCA-size as the dependent variables and parasite exposure (control or cleared) as a fixed effect to examine the possibility that resisting infection could result in morphological differences. There were no significant differences between resistant monarchs and control monarchs in the size ( $F_{1,166} = 0.35$ ,  $p = 0.552$ ) and shape of wings

( $F_{1,180} = 1.90, p = 0.170$ ). We then ran models with these PCA axes as response variables, and CO<sub>2</sub> treatment, milkweed species, infection and monarch sex as fixed effects. Due to sample size limitations (see Supporting Information Table S1), we could not include the four-way interaction in any of our full models, but we did initially include all other interaction terms between CO<sub>2</sub> treatment, milkweed species, infection and monarch sex.

To examine effects of the treatments on monarch wing loading and specific wing area, we ran LMMs with wing loading and specific wing area (log-transformed) as response variables, with identical fixed effects as above. We ran two LMMs with either wing loading or specific wing area as response variables and CO<sub>2</sub> treatment, milkweed species, infection and monarch sex as fixed effects.

Finally, we tested whether cardenolide sequestration affected wing shape, loading and density. We ran three LMMs with either PCA-shape, wing loading or wing density (log-transformed) as dependent variables, and monarch cardenolide concentration (square-root-transformed), milkweed species and their interaction as fixed effects.

### 3 | RESULTS

#### 3.1 | Milkweed host plant chemistry

Of the 252 milkweeds that supported surviving monarchs (see Supporting Information Table S1), 114 produced measurable cardenolides. To address the large occurrence of zeros in our chemistry dataset, we followed methods detailed in Appendix S1. There was no effect of CO<sub>2</sub> treatment on the proportion of plants that produced detectable foliar cardenolides (CO<sub>2</sub>:  $\chi^2 = 0.82, p = 0.366$ ). *Asclepias incarnata* and *A. syriaca* were significantly more likely to express zero cardenolides (species:  $\chi^2 = 19.52, p = 0.0002$ ). The interaction term between CO<sub>2</sub> and species was not significant (CO<sub>2</sub>\*species:  $\chi^2 = 1.49, p = 0.684$ ). We therefore chose to exclude the plants that did not produce cardenolides from further analyses of phytochemistry.

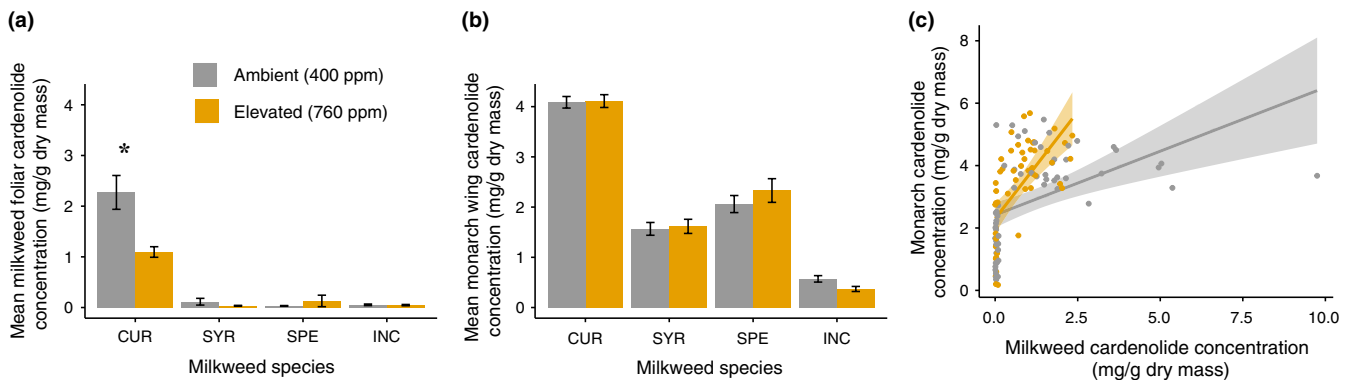
The 114 plants with detectable cardenolides included 65 *A. curassavica*, 19 *A. syriaca*, 18 *A. speciosa* and 12 *A. incarnata*. Elevated

CO<sub>2</sub> changed the concentration of foliar cardenolides in a species-specific manner (species\*CO<sub>2</sub>:  $F_{3,106} = 3.05, p = 0.032$ , Figure 1a). Under eCO<sub>2</sub>, there was a 52% decline in the foliar cardenolide concentrations of *A. curassavica* ( $F_{1,36} = 13.43, p = 0.0008$ , Figure 1a). Cardenolide concentrations in *A. syriaca* ( $F_{1,13} = 1.0847, p = 0.32$ ), *A. speciosa* ( $F_{1,13} = 0.76, p = 0.399$ ) and *A. incarnata* ( $F_{1,11} = 0.01, p = 0.910$ ) remained unaffected by eCO<sub>2</sub>. Across CO<sub>2</sub> treatments, *A. curassavica* produced the highest cardenolide concentrations, while *A. incarnata* produced the lowest (species:  $F_{3,106} = 71.72, p < 0.0001$ , Figure 1a). Milkweed species varied in the assemblage of cardenolides that they produced (PERMANOVA, species:  $F_{3,110} = 24.16, R^2 = 0.39, p = 0.001$ ). In addition, the effect of CO<sub>2</sub> treatment on cardenolide composition varied among milkweed species (PERMANOVA, CO<sub>2</sub>\*species:  $F_{3,110} = 2.26, R^2 = 0.037, p = 0.004$ ).

#### 3.2 | Monarch wing chemistry

Monarchs maintained constant wing cardenolide concentrations between CO<sub>2</sub> treatments (CO<sub>2</sub>\*species:  $F_{3,214} = 1.60, p = 0.191$ , Figure 1b) despite the decline in foliar cardenolide concentration in *A. curassavica* induced by eCO<sub>2</sub> (Figure 1a). Critically, monarchs feeding on milkweed foliage grown under eCO<sub>2</sub> sequestered more cardenolides per unit cardenolide available in host plants (plant cardenolides\*CO<sub>2</sub>:  $F_{1,109} = 5.54, p = 0.020$ , Figure 1c). When feeding on *A. syriaca*, monarchs infected with parasites sequestered 20% less cardenolide in their wings than did uninfected monarchs (infection\*species:  $F_{3,228} = 2.71, p = 0.0462$ , Supporting Information Figure S2). CO<sub>2</sub> treatment, milkweed species and infection did not interact to influence the concentration of cardenolides sequestered by monarchs (CO<sub>2</sub>\*species\*infection:  $F_{3,225} = 0.83, p = 0.4803$ ).

Despite the interactive effects of CO<sub>2</sub> treatment and milkweed species on the composition of foliar cardenolides, the cardenolide profiles sequestered by monarchs were influenced only by milkweed species (PERMANOVA, species:  $F_{3,247} = 157.00, R^2 = 0.65, p = 0.001$ ). Neither eCO<sub>2</sub> alone (PERMANOVA, CO<sub>2</sub>:  $F_{1,250} = 2.37, R^2 = 0.003, p = 0.073$ ) nor its interaction with plant species



**FIGURE 1** Effects of eCO<sub>2</sub> on (a) foliar cardenolide concentrations (mg/g dry mass), (b) monarch wing cardenolide concentrations (mg/g dry mass) and (c) the relationship between foliar and wing cardenolide concentrations. Bars represent mean values  $\pm 1$  SE. Traits were transformed before analyses but are presented as untransformed values for ease of interpretation. Grey bars represent plants grown under aCO<sub>2</sub>, and orange bars are those from eCO<sub>2</sub> or the monarchs that fed on those plants. Milkweed species codes: CUR = *Asclepias curassavica*, SYR = *Asclepias syriaca*, SPE = *Asclepias speciosa*, INC = *Asclepias incarnata*

(PERMANOVA,  $\text{CO}_2$ \*species:  $F_{3,247} = 1.41$ ,  $R^2 = 0.006$ ,  $p = 0.149$ ) influenced the cardenolide profiles sequestered by monarchs.

### 3.3 | Monarch wing morphology

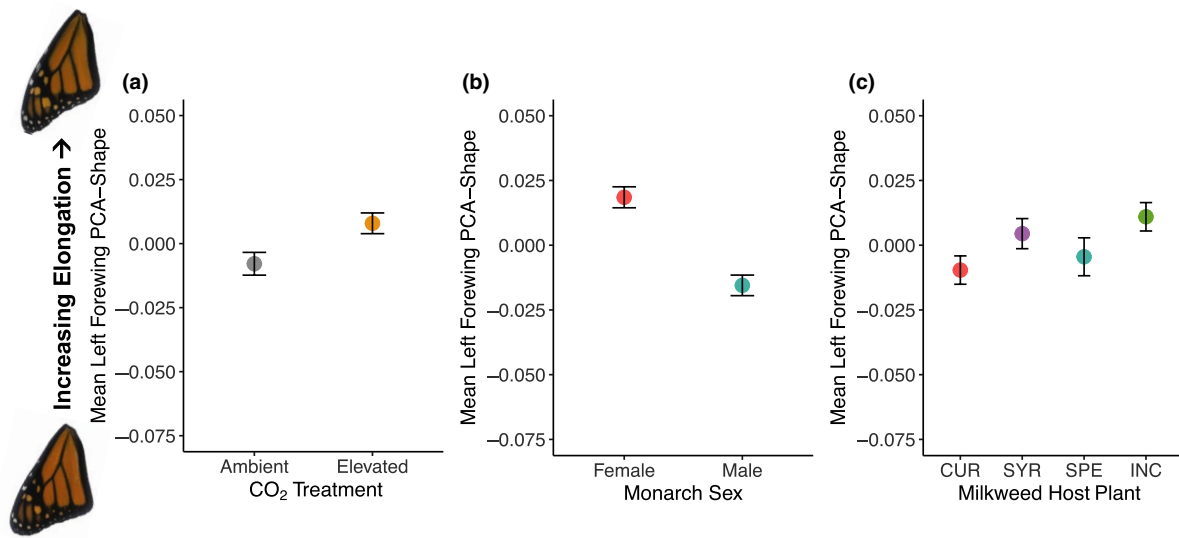
Monarch wings were more elongated (higher values of PCA-shape) when larvae fed on milkweed grown under e $\text{CO}_2$  ( $\text{CO}_2$ :  $F_{1,214} = 15.82$ ,  $p < 0.0001$ , Figure 2a) or when larvae consumed *A. syriaca* or *A. incarnata* (species:  $F_{3,212} = 3.78$ ,  $p = 0.011$ , Figure 2c). Additionally, the wings of female butterflies were more elongated than those of males (sex:  $F_{1,213} = 15.50$ ,  $p = 0.0001$ , Figure 2b).

While *O. elektroscirra* infection had no independent effect on forewing shape (infection:  $F_{1,212} = 0.90$ ,  $p = 0.3550$ ), infected monarchs from a $\text{CO}_2$  plants had rounder wings than butterflies from e $\text{CO}_2$  infection treatments (infection\* $\text{CO}_2$ :  $F_{1,212} = 9.46$ ,  $p = 0.002$ , Figure 3a). Moreover, infected monarchs had rounder wings than uninfected monarchs when feeding on *A. curassavica*, *A. syriaca* and *A. incarnata*, but had more elongated wings than uninfected monarchs fed *A. speciosa* (infection\*species:  $F_{3,212} = 4.54$ ,  $p = 0.004$ , Figure 3b). Finally, there were minor differences between male and

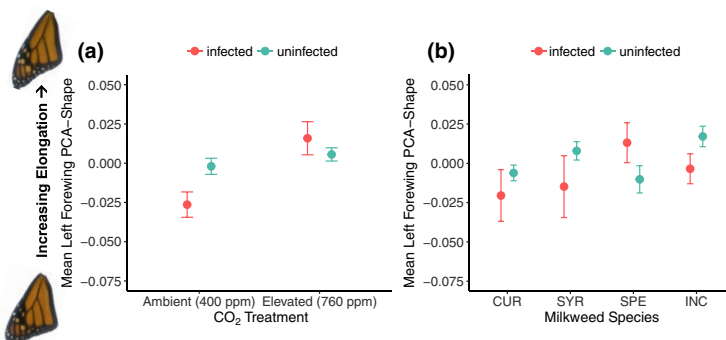
female butterflies in wing shapes dependent on plant species and infection (sex\*infection\*species:  $F_{3,213} = 2.96$ ,  $p = 0.033$ , Figure S3 in the Supporting Information). However, the three-way interaction term explained only a small portion of variance in the model when compared to the main effects reported above. Wing aspect ratio correlated most strongly with PCA-shape ( $r = 0.999$ ,  $N = 237$ ,  $p < 0.0001$ ); thus, we summarize the model results for wing aspect ratios in Table 1 and Supporting Information Table S2.

In contrast to wing shape, none of our treatments affected the size of monarch wings. Male wings were only slightly significantly larger than those of females (sex:  $F_{1,231} = 3.47$ ,  $p = 0.064$ ). Likewise, wing sizes were unaffected by  $\text{CO}_2$  treatment ( $\text{CO}_2$ :  $F_{1,31} = 0.31$ ,  $p = 0.579$ ), milkweed host plant species (species:  $F_{3,202} = 2.09$ ,  $p = 0.102$ ), infection status (infection:  $F_{1,226} = 2.42$ ,  $p = 0.121$ ) or the interaction between these treatments ( $\text{CO}_2$ \*species\*infection:  $F_{3,204} = 0.98$ ,  $p = 0.403$ ).

The wing loading values of male monarchs were 5% higher than those of female monarchs (sex:  $F_{1,15} = 17.13$ ,  $p = 0.0008$ ). Notably, monarchs reared on *A. syriaca* had a 5% higher wing loading than did those reared on other milkweed species (species:  $F_{3,15} = 4.77$ ,  $p = 0.0153$ , Figure 4a). Wing loading was unaffected by  $\text{CO}_2$



**FIGURE 2** The main effects of (a)  $\text{CO}_2$  treatment, (b) sex and (c) milkweed species on a composite measure of monarch forewing shape. Points represent mean PCA-shape values  $\pm 1$  SE. With increasing PCA-shape values, wings become more elongated and angular. Milkweed species codes are the same as above



**FIGURE 3** The interactions between (a)  $\text{CO}_2$  treatment and infection by OE, and (b) milkweed species and infection on a composite measure of monarch forewing shape. Points represent mean PCA-shape values  $\pm 1$  SE. Red points indicate mean shape values of infected monarchs, while blue points represent uninfected monarchs. Milkweed species codes are the same as above

**TABLE 1** The (a) main and (b) interactive effects of CO<sub>2</sub> treatment, butterfly sex and host plant on monarch wing aspect ratios (wing length/wing width), a component of monarch wing shape

(a) Main effects		
CO <sub>2</sub> treatment	$F_{1,214} = 15.82$	$p < 0.0001$
	Aspect ratio	
Ambient	$1.95 \pm 0.002$	
Elevated	$1.96 \pm 0.002$	
Butterfly sex	$F_{1,213} = 15.50$	$p < 0.0001$
	Aspect ratio	
Female	$1.97 \pm 0.002$	
Male	$1.93 \pm 0.002$	
Milkweed species	$F_{3,212} = 3.78$	$p = 0.0113$
	Aspect ratio	
<i>A. curassavica</i>	$1.94 \pm 0.003$	
<i>A. incarnata</i>	$1.97 \pm 0.003$	
<i>A. speciosa</i>	$1.95 \pm 0.004$	
<i>A. syriaca</i>	$1.96 \pm 0.003$	
(b) Two-way interactions		
Infection*CO <sub>2</sub>	$F_{1,212} = 9.46$	$p = 0.0024$
	CO <sub>2</sub> treatment	Aspect ratio
Infected	Ambient	$1.93 \pm 0.004$
	Elevated	$1.97 \pm 0.005$
Uninfected	Ambient	$1.95 \pm 0.002$
	Elevated	$1.96 \pm 0.002$
Infection*Species	$F_{3,212} = 4.54$	$p = 0.004$
	Milkweed species	Aspect ratio
Infected	<i>A. curassavica</i>	$1.93 \pm 0.008$
	<i>A. incarnata</i>	$1.95 \pm 0.005$
	<i>A. speciosa</i>	$1.97 \pm 0.006$
	<i>A. syriaca</i>	$1.94 \pm 0.0102$
Uninfected	<i>A. curassavica</i>	$1.95 \pm 0.003$
	<i>A. incarnata</i>	$1.97 \pm 0.003$
	<i>A. speciosa</i>	$1.94 \pm 0.004$
	<i>A. syriaca</i>	$1.96 \pm 0.003$

Note. Data are the means  $\pm$  1 SE. Aspect ratios range between 1.6 and 2.1 but average around 1.93 in eastern N. American monarch populations. Simplified linear mixed-effects model structure is as follows: Aspect Ratio  $\sim$  CO<sub>2</sub> + butterfly sex + infection + milkweed species + CO<sub>2</sub>\*infection + milkweed species\*infection + sex\*infection\*milkweed species + random effects = chamber, monarch lineage. We present nonsignificant main effects of infection and the three-way interaction in Supporting Information Table S2.

treatment (CO<sub>2</sub>:  $F_{1,111} = 3.01$ ,  $p = 0.112$ ) or parasite infection (infection:  $F_{1,15} = 3.80$ ,  $p = 0.07$ ).

Female monarch wings were 6% denser than male monarch wings (sex:  $F_{1,228} = 15.74$ ,  $p < 0.0001$ ). Monarchs fed *A. curassavica* had the thinnest wings, while those fed *A. syriaca* had the densest wings (species:  $F_{3,199} = 2.66$ ,  $p = 0.0492$ , Figure 4b). Interestingly, the wings of infected

monarchs were 7% less dense than the wings of uninfected monarchs (infection:  $F_{1,221} = 20.65$ ,  $p < 0.0001$ , Figure 4c). Specific wing area was unaffected by CO<sub>2</sub> treatment (CO<sub>2</sub>:  $F_{1,31} = 0.02$ ,  $p = 0.897$ ).

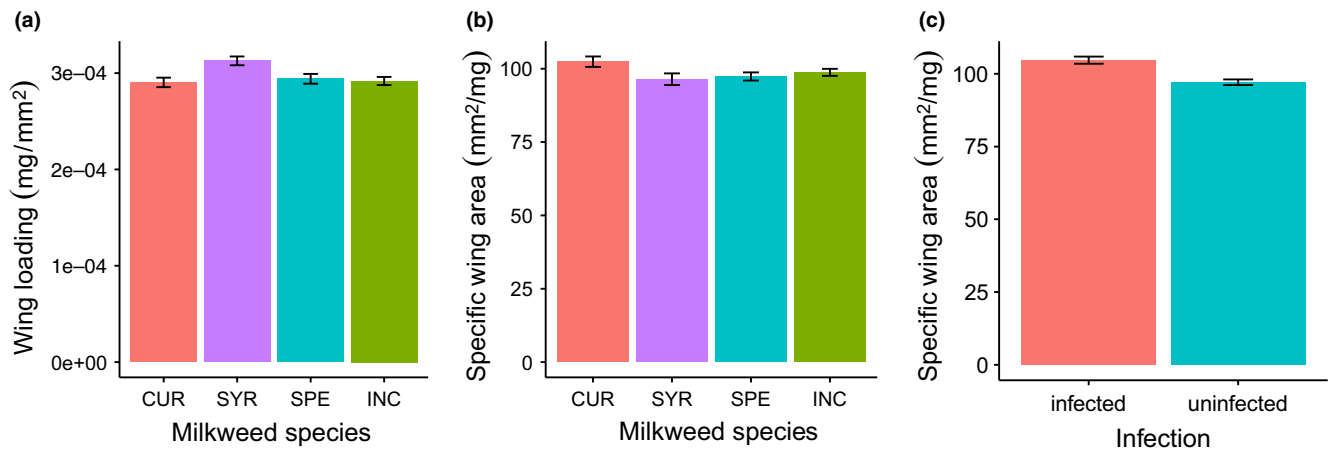
In the simplified model exploring effects of cardenolide sequestration on wing shape, we found no effects of monarch cardenolide sequestration (monarch cardenolides:  $F_{1,230} = 0.05$ ,  $p = 0.816$ ), plant species (species:  $F_{3,228} = 2.19$ ,  $p = 0.090$ ) or their interaction (monarch cardenolides\*species:  $F_{3,230} = 1.62$ ,  $p = 0.184$ , Supporting Information Table S4) on wing shape. Interestingly, monarchs that sequestered higher concentrations of cardenolides had lower wing loading values (monarch cardenolides:  $F_{1,15} = 8.41$ ,  $p = 0.011$ , Figure 5). Also, those monarchs feeding on higher cardenolide milkweed had lower wing loading values (species:  $F_{3,15} = 4.11$ ,  $p = 0.025$ ), but there were no species-dependent effects of monarch cardenolide sequestration on wing loading (monarch cardenolides\*species:  $F_{3,15} = 2.82$ ,  $p = 0.073$ ). Finally, there was a marginally significant trend of less dense wings produced in monarchs sequestering higher concentrations of cardenolides ( $F_{1,229} = 3.59$ ,  $p = 0.059$ ). There were no effects of either milkweed species (species:  $F_{3,227} = 1.95$ ,  $p = 0.122$ ) or the interaction on wing density (monarch cardenolides\*species:  $F_{3,230} = 1.15$ ,  $p = 0.330$ ).

## 4 | DISCUSSION

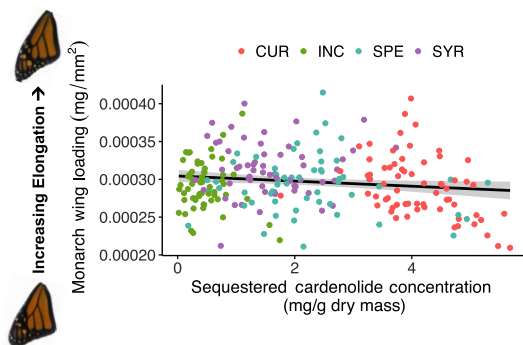
Rapid environmental change may influence trophic interactions by altering the defensive phenotype of prey. Here, we demonstrate that: (a) monarchs maintain the concentration and composition of cardenolides that they sequester despite changes in the phytochemistry of one milkweed species induced by eCO<sub>2</sub>; (b) aspects of monarch morphology important to flight ability such as wing shape, loading and density vary in response to eCO<sub>2</sub>, milkweed host plant species, infection and sex; and 3) feeding on high cardenolide milkweed is associated with the formation of rounder, thinner wings, which are less efficient at gliding flight. We suggest that changes in sequestration rates under eCO<sub>2</sub> are a by-product of compensatory feeding aimed at maintaining a nutritional target in response to declining diet quality. Additionally, monarchs exhibit the cost of sequestering higher concentrations of cardenolides through declining wing loading values. Small changes in wing morphology can have important consequences for migration success (Bradley & Altizer, 2005), including migratory escape from parasites. Therefore, changes in sequestration and morphology may have consequences for monarch defence and migration in a changing world.

### 4.1 | Monarchs increase sequestration rate under eCO<sub>2</sub>

We demonstrate that monarchs can increase their rate of cardenolide sequestration under eCO<sub>2</sub> (Figure 1). Specifically, monarchs sequester a constant concentration and composition of cardenolides from *A. curassavica* despite a 52% reduction in foliar cardenolides and changes in foliar cardenolide composition induced by



**FIGURE 4** The effects of milkweed species on monarch (a) wing loading (body mass/wing area) and (b) specific wing area (wing area/wing mass), a measure of wing density. (c) The effects of OE infection on specific wing area. Bars represent mean values  $\pm$  1 SE. Higher specific wing area values indicate wings that are less dense. Milkweed species codes are the same as above



**FIGURE 5** The relationship between the concentration of cardenolides sequestered in monarch wings and wing loading (body mass/wing area). Points represent individual monarchs, and colours correspond to the species of milkweed host plants fed to each monarch. Milkweed species codes are the same as above

eCO<sub>2</sub>. Monarchs are known to maintain constant concentrations of the cardenolides that they sequester from *A. curassavica* in response to nutrient deposition, another environmental change driver (Tao & Hunter, 2015). Herbivores regulate sequestration by altering both the total amount of foliage consumed and sequestration efficiency (Camara, 1997). Notably, herbivorous arthropods maintain target ratios of carbohydrates to protein in their diet through behavioural shifts in consumption (Simpson et al., 2015). Therefore, it is possible that the monarchs in our study increased the amount of foliage consumed to compensate for reductions in foliar nutrient content under eCO<sub>2</sub> (Hunter, 2001; Johnson, Solensky et al., 2014; Lincoln, Sionit, & Strain, 1984; Zavala, Nabity, & DeLucia, 2013).

## 4.2 | Environment influences monarch wing morphology

Our treatments altered indices of monarch wing morphology (wing shape, loading, and density) that are important to both aerial

manoeuvrability and long-distance flight (Berwaerts et al., 2002). Notably, both CO<sub>2</sub> treatment and milkweed species influenced wing shape contingent upon parasite infection status (Figures 2 and 3). Under aCO<sub>2</sub>, infection induced rounder wings lowering the mean aspect ratio from  $1.95 \pm 0.002$  to  $1.93 \pm 0.004$ . However, under eCO<sub>2</sub> both infected and uninfected monarchs developed more angular wings ( $1.97 \pm 0.005$ ,  $1.96 \pm 0.002$ , Figure 3a). No study to date has specifically tested the effects of wing shape on the probability of successful migration from start to finish in monarchs. However, field sampling indicates that earlier arriving migrants tend to have larger, more elongated wing shapes, suggesting that this flight phenotype increases migration success (Satterfield & Davis, 2014). Therefore, future environmental conditions may induce the formation of wing shapes that improve monarch flight efficiency despite infection.

We also report effects of milkweed species on monarch wing shape, loading and density, similar to findings in other flying insects (Benítez, Vargas, & Püschel, 2015; Davis & de Roode, 2018; Soto, Carreira, Soto, & Hasson, 2008). Importantly, monarch wings are rounder and less dense when larvae are reared on *A. curassavica*, a plant exotic to N. America and increasing in prevalence in the southern United States (Satterfield, Maerz, & Altizer, 2015). This species of milkweed does not senesce in autumn and contributes to a loss of monarch migratory behaviour as butterflies encounter viable foliage during their late-season stopovers (Satterfield et al., 2015, 2018). Our data suggest that the offspring of those sedentary monarchs fed *A. curassavica* will develop lower quality flight phenotypes, perhaps furthering the loss of migratory behaviour.

Small differences in wing morphology that affect the efficiency of flight could have large consequences for monarch migration success (Bradley & Altizer, 2005). Eastern N. American monarchs migrate up to 4,500 km through soaring and active flight (Brower & Malcolm, 1991; Gibo, 1986). Monarchs must take shelter during adverse weather conditions, utilize wind patterns and cross large



expanses of unsuitable habitat (Garland & Davis, 2002; Gibo & Pallett, 1979). Therefore, any factor that causes monarchs to remain grounded during beneficial flying conditions or reduces the amount of time monarchs may stay aloft over unsuitable habitat will significantly reduce migration success. In our study, eCO<sub>2</sub> eliminates the shape difference between infected and uninfected individuals, inducing more elongated wings in both groups. If infected individuals become more efficient gliders under environmental change, this might decrease migratory culling, which reduces pathogen prevalence seasonally in the N. American monarch population (Altizer et al., 2011; Bartel, Oberhauser, de Roode, & Altizer, 2011).

Although our treatments altered monarch wing shape, we detected no effect of diet or infection on wing size. All of the butterflies used in this study originated from the migratory eastern N. American population. Therefore, our data substantiate previous studies demonstrating strong selection for larger wings imposed by migration distance within this population (Altizer & Davis, 2010; Li et al., 2016). Forewing size is likely more important to migration success than wing shape because it is conserved among all our treatments and is selected for in the migratory populations of monarchs (Altizer & Davis, 2010; Flockhart et al., 2017; Li et al., 2016). However, aerodynamic theory suggests that wing shape can be important for manoeuvrability and energy conservation (Pennycuik, 2008). Because our monarchs were constrained within these constant wing sizes, perhaps the amount of differentiation in the angular nature of wing shape was limited. Further studies exploring the plasticity of wing shape and size in response to host plant and environmental conditions among different populations of monarchs across the globe are needed to better address this idea.

## ACKNOWLEDGEMENTS

Many thanks to H.B. Streit, A.R. Meier, K.C. Crocker, C.R. Chappell, M.O. Hemken, Y. Yang, J.J. Shi, R. Peterson, J.D. Den Uyl, L. Tao, C.D. Gowler, A.A. Pierce, A.J. Mongue, K. Sanchez, A.E. Stratton. This work was supported by NSF grants DEB-1257160 and DEB-1256115 awarded to J.C.d.R. and M.D.H.

## AUTHORS' CONTRIBUTIONS

L.E.D., M.D.H. & J.C.d.R. designed the experiment; J.C.d.R. provided butterflies and parasites; L.E.D., A.J.S. & M.D.H. collected and analysed the data; L.E.D. wrote the manuscript; all authors contributed to drafts.

## DATA ACCESSIBILITY

Data are available in the Dryad Digital Repository: <https://doi.org/10.5061/dryad.mk3tj78> (Decker, Soule et al., 2018).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Decker LE, Soule AJ, de Roode JC, Hunter MD. Phytochemical changes in milkweed induced by elevated CO<sub>2</sub> alter wing morphology but not toxin sequestration in monarch butterflies. *Funct Ecol*. 2019;33:411–421. <https://doi.org/10.1111/1365-2435.13270>