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RESEARCH ARTICLE

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Elevated atmospheric concentrations of CO_2 increase endogenous immune function in a specialist herbivore

Leslie E. Decker[1,2] | Christopher S. Jeffrey[3] | Kaitlin M. Ochsenrider[3] | Abigail S. Potts[2] | Jacobus C. de Roode[4] | Angela M. Smilanich[5] | Mark D. Hunter[2]

[1]Department of Biology, Stanford University, Stanford, CA, USA

[2]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, USA

[3]Chemistry Department, University of Nevada, Reno, NV, USA

[4]Biology Department, Emory University, Atlanta, GA, USA

[5]Department of Biology, University of Nevada, Reno, NV, USA

Correspondence
Leslie E. Decker
Email: leslieedecker@gmail.com

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Abstract

1. Animals rely on a balance of endogenous and exogenous sources of immunity to mitigate parasite attack. Understanding how environmental context affects that balance is increasingly urgent under rapid environmental change. In herbivores, immunity is determined, in part, by phytochemistry which is plastic in response to environmental conditions. Monarch butterflies *Danaus plexippus*, consistently experience infection by a virulent parasite *Ophryocystis elektroscirrha*, and some medicinal milkweed (*Asclepias*) species, with high concentrations of toxic steroids (cardenolides), provide a potent source of exogenous immunity.
2. We investigated plant-mediated influences of elevated CO_2 (eCO_2) on endogenous immune responses of monarch larvae to infection by *O. elektroscirrha*. Recently, transcriptomics have revealed that infection by *O. elektroscirrha* does not alter monarch immune gene regulation in larvae, corroborating that monarchs rely more on exogenous than endogenous immunity. However, monarchs feeding on medicinal milkweed grown under eCO_2 lose tolerance to the parasite, associated with changes in phytochemistry. Whether changes in milkweed phytochemistry induced by eCO_2 alter the balance between exogenous and endogenous sources of immunity remains unknown.
3. We fed monarchs two species of milkweed; *A. curassavica* (medicinal) and *A. incarnata* (non-medicinal) grown under ambient CO_2 (aCO_2) or eCO_2. We then measured endogenous immune responses (phenoloxidase activity, haemocyte concentration and melanization strength), along with foliar chemistry, to assess mechanisms of monarch immunity under future atmospheric conditions.
4. The melanization response of late-instar larvae was reduced on medicinal milkweed in comparison to non-medicinal milkweed. Moreover, the endogenous immune responses of early-instar larvae to infection by *O. elektroscirrha* were generally lower in larvae reared on foliage from aCO_2 plants and higher in larvae reared on foliage from eCO_2 plants. When grown under eCO_2, milkweed plants exhibited lower cardenolide concentrations, lower phytochemical diversity and lower nutritional quality (higher C:N ratios). Together, these results suggest that the loss of exogenous immunity from foliage under eCO_2 results in increased endogenous immune function.

5. Animal populations face multiple threats induced by anthropogenic environmental change. Our results suggest that shifts in the balance between exogenous and endogenous sources of immunity to parasite attack may represent an underappreciated consequence of environmental change.

KEYWORDS

Asclepias, cardenolides, *Danaus plexippus*, ecoimmunology, haemocytes, *Ophryocystis elektroscirrha*, phenoloxidase

1 | INTRODUCTION

Hosts must defend themselves against attack from parasites while embedded within dynamic communities and ecosystems. Despite increasing evidence that environmental change alters host–parasite interactions (Altizer et al., 2013), the primary mechanisms underlying disease responses remain unresolved. Ecoimmunology is a burgeoning field that concentrates on the role of environmental context in determining the strength, activity and variability of the host immune response (Brock et al., 2014; Lazzaro & Little, 2009). Central to this field is the reality that organisms must balance energetic investment in the immune response with investments in other life-history traits (Kraaijeveld et al., 2002; Schmid-Hempel, 2003). In the context of this balance, any factor that generates further energetic deficits may lead to compromised immune function.

Anthropogenic environmental change can alter host immunity in a context-dependent manner, yielding variable patterns (Adamo & Lovett, 2011; Gherlenda et al., 2015; Jolles et al., 2015). The current literature on immunity and environmental change focuses heavily on the direct physiological effects of warming and pollutants on host immune enzymatic activity (Martin et al., 2010; Richard et al., 2015; Wojda, 2017). However, environmental change may indirectly alter aspects of the host environment including population density, stress and diet quality, consequently impacting host immune function (Kraaijeveld et al., 2002; Schmid-Hempel, 2003). Higher temperatures, more variable rainfall and elevated atmospheric concentrations of carbon dioxide (CO_2) have direct effects on plant physiology, which manifest themselves in plant nutritional quality and defensive chemistry (Robinson et al., 2012; Zavala et al., 2013). Thus, organisms that derive energy and nutrition primarily from plants should experience the indirect effects of global change most acutely.

Insect herbivores are especially vulnerable to changes in the chemical quality of their food plants (Hunter, 2016; Mattson, 1980) and regularly face challenge from agents of disease. The endogenous immune response of insects targets parasitoids and parasites and can be broadly divided into humoral and cellular immunity (Beckage, 2011; Strand, 2008). Humoral defences encompass effector molecules such as antimicrobial peptides that act on pathogenic microbes (Kavanagh & Reeves, 2007). In contrast, cellular immunity consists of three phases: phagocytosis, nodule formation and encapsulation enacted by immune cells known as haemocytes (Kacsoh & Schlenke, 2012; Strand, 2008; Vogelweith et al., 2016). As haemocytes undergo apoptosis while surrounding antigens during nodulation and encapsulation, prophenoloxidase is activated into phenoloxidase (PO), an enzyme critical to the production of melanin and other cytotoxic molecules (Nigam et al., 1997). While insects employ phagocytosis and immune effector molecules against smaller parasites and pathogens, the encapsulation response targets multicellular invaders, such as parasitoid eggs and parasites (Strand, 2008).

The strength and variability of the immune response of insect herbivores depend on diet quality (Singer et al., 2014). Because immune defences are energetically costly, the ratio of protein to carbohydrate concentrations (nutritional quality) of host food plants is well known to influence immunity (Cotter et al., 2011; Srygley et al., 2009). The concentration of plant secondary metabolites (PSMs) within the host diet can also alter immune function (Smilanich et al., 2009, 2011; Trowbridge et al., 2016). Certain concentrations and combinations of plant toxins may reduce insect immune function (Lampert & Bowers, 2015), while others may strengthen immune induction (Lampert, 2012; Ojala et al., 2005). Given the large diversity of PSMs, and the many modes of their chemical action on insect performance, it is still unclear how plant secondary metabolism and nutritional quality combine to influence insect immunity.

In addition to influencing the strength of endogenous immunity, PSMs can serve as a potent source of exogenous immunity by functioning as medicines (de Roode et al., 2013; Huffman & Seifu, 1989; Singer et al., 2014). As a consequence, the coevolutionary relationships among herbivores, their host plants, and their natural enemies may ultimately determine the relative effects of PSMs on endogenous and exogenous immunity (Hunter, 2016). The monarch butterfly *Danaus plexippus*, is a specialist insect herbivore known to utilize the secondary chemistry of its host plants, *Asclepias*, as a defence against infection by a virulent, protozoan parasite *Ophryocystis elektroscirrha* (Barriga et al., 2016; Lefèvre et al., 2012, Appendix A). Monarchs become infected with *O. elektroscirrha* after ingesting parasite spores on the surface of egg chorea and milkweed *Asclepias* tissues (Altizer & Oberhauser, 1999). Spores lyse within the larval gut; sporozoites penetrate the larval hypoderm and replicate over the course of the monarch's development (Mclaughlin et al., 1970). Infected adult monarchs emerge covered in dormant parasite spores and experience reduced pre-adult survival, as well as reduced adult life span, fecundity and flight ability (Altizer & Oberhauser, 1999; Bradley & Altizer, 2005; de Roode, Yates, et al., 2008). Critically,

certain milkweed species with high concentrations of toxic steroids known as cardenolides reduce *O. elektroscirrha* infection probability, growth rate and virulence (de Roode, Pedersen, et al., 2008; Gowler et al., 2015; Tao et al., 2016). Feeding on high-cardenolide (hereafter medicinal) milkweed also ameliorates the fitness costs of harbouring each additional parasite, a form of defence known as host tolerance (Sternberg et al., 2012). In short, milkweed cardenolides are a potent source of exogenous immunity for monarchs.

In addition to its role in exogenous immunity, one recent study has investigated how milkweed phytochemistry influences monarch endogenous immunity. In this study, monarch larvae were inoculated with parasites, and whole bodies and guts were dissected 24 hr later. Surprisingly, within this timeframe, infection by *O. elektroscirrha* did not upregulate typical immune genes, whereas feeding on medicinal milkweed actually downregulated a handful of canonical immune genes and significantly altered the expression of several detoxification genes (Tan et al., 2019). Among the detoxification genes upregulated by the medicinal milkweed were a glutathione S-transferase (DPOGS210488) and a carboxyl esterase (DPOGS204275). The downregulation of immune genes in response to feeding on a medicinal milkweed species regardless of infection status suggests that coevolution among monarchs, milkweed, and *O. elektroscirrha* has reduced monarch reliance on endogenous immunity, and perhaps, favoured cardenolides as an exogenous functional replacement for endogenous immune activity (Smilanich & Nuss, 2019).

Milkweed phytochemical diversity derives both from the variety of milkweed secondary metabolites in tissues and their plasticity under environmental variation. For example, beyond cardenolides, multiple pregnane glycosides and phenolics have been detected in the foliar and root tissues of *Asclepias incarnata*, a low cardenolide milkweed species naturally occurring in North America (Sikorska, 2003; Warashina & Noro, 2000). Additionally, both *A. incarnata* and *A. curassavica* produce complex mixtures of flavonoid compounds, including flavanol glycosides, with strong antioxidant properties (Haribal & Renwick, 1996). Phytochemical diversity is profoundly sensitive to environmental variation (Hunter, 2016), including that introduced by ongoing environmental change (Robinson et al., 2012; Zavala et al., 2013). As a result, the value of secondary metabolites as sources of exogenous immunity may be compromised in a changing world. Monarch caterpillars feeding on the same medicinal milkweed species described above lose tolerance to their parasite when those milkweeds are grown under eCO_2. Reductions in monarch tolerance under eCO_2 are associated with decreases in the production of certain lipophilic, medicinally active cardenolides in their milkweed host plants (Decker et al., 2018). However, whether the holistic phytochemical changes induced under eCO_2 influence monarch endogenous immunity remains unknown.

In this study, we examine how eCO_2 alters monarch immune function through changes in the medicinal (secondary chemistry) and nutritional (carbon and nitrogen) quality of milkweed. We fed monarchs foliage from two species of milkweed, *A. curassavica* (medicinal) and *A. incarnata* (non-medicinal), grown under ambient or elevated concentrations of CO_2. Larvae were infected with either *O. elektroscirrha* or left as uninfected controls. We then measured aspects of the monarch humoral (PO activity) and cellular (haemocyte concentrations and types) immune response, along with host-PSMs and nutritional quality, to understand the mechanisms underlying changes in monarch immunity under future environmental conditions. We predicted that (a) medicinal *A. curassavica* would suppress the expression of endogenous immunity more than would non-medicinal *A. incarnata*, (b) eCO_2 would reduce the sources of exogenous immunity (cardenolides) provided by milkweeds and (c) reductions in exogenous immunity under eCO_2 would stimulate compensatory endogenous immunity in those monarchs feeding on *A. curassavica* while infected with the parasite.

2 | MATERIALS AND METHODS

We performed a fully factorial manipulation with milkweed species (*A. incarnata* and *A. curassavica*), CO_2 level (ambient or elevated) and *O. elektroscirrha* (infected or uninfected) as treatments. We then ran (a) immune assays, measuring PO activity and haemocyte counts of early-instar monarch larvae and (b) filament assays, measuring melanization of late-instar larvae. A third group of caterpillars was reared to adulthood as assay controls (Table S1, Appendix B).

2.1 | Milkweed sources and growing conditions

Seeds of both milkweed species were obtained from commercial vendors (*A. curassavica*: Victory Seed, OR and *A. incarnata*: Lupine Gardens, WI). Seeds were surface sterilized using a 5% bleach solution and only *A. incarnata* seeds were cold stratified for 6 weeks prior to planting. Seedlings were germinated on moist, sterilized paper towels and planted on 1 June 2017 in deepots™ containing Metromix 360 (SunGro Horticulture) and Osmocote 16:16:16 controlled release fertilizer (ICL Specialty Fertilizers). We grew and watered seedlings daily in the greenhouse at the University of Michigan Biological Station (UMBS, 45.5587°N, 84.6776°W) for 2 weeks before transferring them outside into the CO_2 array.

On 28 May 2017, we distributed potted plants randomly into 40 open-top controlled atmosphere chambers in the field at UMBS. The CO_2 array was comprised of 20 chambers maintained at aCO_2 (410 ppm) and 20 at eCO_2 (810 ppm) from dawn until dusk (Drake et al., 1989). Within the current century, the concentration of atmospheric CO_2 is anticipated to exceed 700 ppm (IPCC, 2013); therefore, we chose an 810 ppm as our target concentration to simulate a near doubling. We monitored CO_2 concentrations in all 20 eCO_2 chambers and one aCO_2 chamber during daylight hours using an LI-COR 320 IRGA (LI-COR). We recorded air temperatures within the chambers using iButton dataloggers (IbuttonLink). Chamber air temperatures averaged 20.81 (±0.05) °C in eCO_2 and 20.80 (±0.05) °C inside aCO_2 chambers, well within temperatures experienced by monarchs in eastern North America (Faldyn et al., 2018).

We grew three plants of each treatment group (2 milkweed species × 2 parasite treatments × 3 assay groups = 12 treatments) within each chamber, for 36 plants/chamber. We planned to rear one caterpillar per treatment group on the three plants from each chamber (20 replicate larvae per treatment); however, final replicate numbers were variable due to larval mortality (Table S1, Appendix C). We began excising foliage for assays after approximately 1 month of growth in the CO_2 array (27 June 2017). Cuttings were placed in 710 ml plastic containers containing one monarch.

2.2 | Monarch sources and rearing methods

Monarchs were the grand-offspring of lab-reared butterflies collected from St. Marks, FL and Lawrence, KS. We distributed monarchs from five full-sib family lines evenly across treatments. A darkened monarch egg (indicates close proximity to hatching) was assigned randomly and attached to the surface of each leaf cutting. Three days after neonates hatched on their plant cuttings, we began the inoculation process. Each larva was transferred to a petri dish containing a 95 cm^2 piece of moist filter paper and a 70.6 mm^2 leaf disk taken from the larva's assigned host plant, cleaned with a 5% bleach solution and rinsed thoroughly with water. For those larvae designated as inoculated, we placed 10 parasite spores on the surface of the leaf disk, while uninoculated larvae received spore-free leaf disks. Immediately after the leaf disk was taken from the plant for inoculation, we collected foliage for chemical analyses (Appendix D). The petri dishes containing larvae and leaf disks were kept in an incubator maintained at 26°C with 16-hr daylight. Upon consuming the entire leaf disk (all spores), larvae were returned to their cleaned containers with new plant cuttings. We continued to feed monarchs in the control (Appendix B) and filament treatments ad libitum until pupation or until filament insertion (see below), replacing foliage and cleaning each container every 2–3 days. Monarchs designated for the immune assays (see below) were sacrificed 48 hr following inoculation to determine the initial early-instar immune response to *O. elektroscirrha* infection. We chose this period to ensure that all larvae had completed inoculation and had adequate time to mount an immune response (Beckage, 2011).

2.3 | Foliar chemical analyses

At the time of parasite inoculations (above), six leaf disks from each monarch's assigned plant were punched into 1 ml of methanol and stored at −10°C for cardenolide extraction (*see detailed description in* Appendix D; Tao & Hunter, 2012). Another six disks were taken, dried and weighed to estimate sample dry mass of the disks collected for chemical analysis.

We calculated two metrics of cardenolide chemistry from our milkweed foliar samples; total cardenolide concentration and cardenolide polarity. Total cardenolide concentrations were calculated as the sums of the individual cardenolide peak areas separated by UPLC corrected by the concentration of an internal digitoxin standard and normalized by the dry sample mass. The biological activity of cardenolides is determined, in part, by the polarity of the different sugar moieties attached to the steroid skeleton of the compound (Agrawal et al., 2012). Because animal cell membranes are outwardly hydrophobic, the most lipophilic (nonpolar) cardenolides are thought to be the most toxic (Sternberg et al., 2012; Tao et al., 2016). We calculated an index of cardenolide polarity for each sample as: Polarity = sum(P_i RT_i), where P_i is the area and RT_i is the retention time of the *i*th cardenolide peak, following Rasmann and Agrawal (2011).

At the same time that we removed leaf disks for cardenolide analysis, we harvested three leaves for NMR sampling. We processed the NMR spectral data using MestReNova software (Mestrelab Research) and aligned sample spectra using the solvent peak. Sample spectra were then baseline-corrected, phase-corrected and normalized to the total area of 100, and binned every 0.04 ppm from 0.5 to 14 ppm. As an estimate of whole-plant chemical diversity, we calculated the Simpson diversity index ($D = 1 - \sum(n/N)^2$) of chemical shifts (approximations of secondary metabolites) where n is the integral of a specific binned frequency range, and *N* is the total number of binned frequency ranges measured in the sample (Richards et al., 2015).

Remaining dried foliar tissue was ground to a fine powder and then analysed using a TruMac CN analyser (Leco Corporation) to provide estimates of foliar carbon (C) and nitrogen (N) concentrations. Examining the foliar C:N ratio is a simple approximation of the nutritional quality of many plants (Mattson, 1980), including milkweed (Tao et al., 2015).

2.4 | Immune assays

In early-instar larvae, we performed assays to measure standing and activated phenoloxidase (PO) activity and haemocyte concentration and identity. Using a colorimetric assay, we determined the activity of free, naturally active PO (standing PO activity) and the total PO activity (standing PO + activated prophenoloxidase, activated PO) in monarch haemolymph (Adamo, 2004; Dhinaut et al., 2018; Smilanich et al., 2017). To extract haemolymph, we made incisions in the larval cuticle above the final proleg in the A6 abdominal segment using a hand-pulled Pasteur pipette needle (Smilanich et al., 2009, 2017). With a micropipette, we took 2 μl of haemolyph from each larva and deposited it into 50 μl of chilled phosphate-buffered saline (PBS) solution in a 1.0-ml Eppendorf tube and vortexed the mixture. We then prepared two separate reactions of the haemolymph extract in order to detect standing PO activity and total PO activity after activating the inactive prophenoloxidase dimer using Cetylpyridinium chloride monohydrate (CPC). To each well of a 96-well plate, one 50-μl aliquot of the haemolymph-PBS mixture was added along with 300 μl of L-DOPA (g L-DOPA in mL deionized water). We incubated the plate

for 20 min at room temperature. To the second designated well of each sample, we also added 17 μl of 10% CPC to activate prophenoloxidase present in the haemolymph during the incubation step. Using an ELx800 Absorbance Microplate Reader (BioTek), we measured absorbance of the samples at 490 nm every 30 s for 180 min. In our analyses, we used the slope of the linear portion of the absorbance curve (30–106 min) as our measure of standing PO and total PO activity. We calculated the activity of activated PO by subtracting standing PO from total PO activity.

Circulating haemocytes aid in the recognition and phagocytosis of microbial parasites and encapsulation of parasitoids. Monarchs typically produce four differentiated haemocyte types: phagocytic granulocytes, capsule-forming plasmocytes, oenocytoids that contain components of the PO cascade, and spherule cells that potentially contain cuticular components (Strand, 2008). The density and frequency of different haemocytes can indicate insect melanization and encapsulation ability (Kacsoh & Schlenke, 2012). To identify and count haemocytes, we took an additional 4 μl of haemolymph and added it to 8 μl of chilled anticoagulant solution (0.684 g EDTA, 0.346 g citric acid dissolved in 180 ml PBS, pH 7.4). Within 24 hr, we performed counts using a Neubauer Bright-Line haemocytometer (Cambridge Instruments, Inc.) and 10 μl of the sample. We counted the total number of haemocytes present in the entire central gridded area and recorded the different haemocyte types present in the haemolymph following the descriptions of Strand (2008) and Vogelweith et al. (2016). Haemocyte categorizations and counts were all performed randomly and blindly by the same individual.

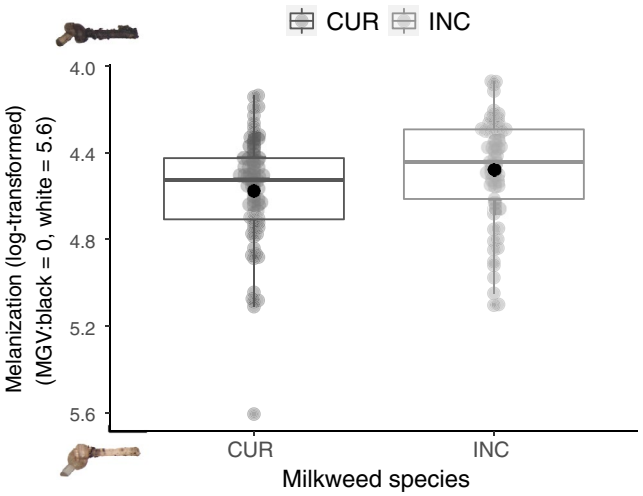

FIGURE 1 The melanization response of late-instar monarch larvae was weaker when monarchs fed on medicinal milkweed. Note the flipped y-axis: Higher mean gray values (MGV) represent lighter pigmented filaments or lower melanization responses. Images on axes are of actual filaments inserted into monarchs representing dark (highly melanated) and light (un-melanated) examples. Milkweed species codes are as follows: CUR = *A. curassavica* (dark grey) and INC = *A. incarnata* (light grey). Box plot hinges represent first and third quartiles, error bars are 95% confidence intervals and bars are median values. Black points represent mean values ± 1 *SE*.

2.5 | Filament assay to determine monarch melanization response

To measure the immune defence of 5th instar larvae designated for the filament treatment, we inserted an artificial antigen into monarchs following Klemola et al. (2008). Our simulated antigens were 2 mm long pieces of nylon, which we rubbed with sandpaper, knotted at one end (for ease of handling), sterilized with 100% ethanol and dried before inserting into larvae. Similar to the haemolymph extraction protocol, we made a small incision into the larval cuticle just above the final proleg. We then inserted the filament into the larval haemocoel parallel to the abdomen, taking care not to perforate the midgut or hindgut. Larvae were returned to their cleaned containers and allowed 24 hr to mount an immune response. We removed the implanted filaments using forceps, deposited the filaments into a 70% ethanol solution and stored samples at −20°C for 3 months.

To quantify filament melanization, we photographed filaments under a dissecting microscope using an iPhone 6s (Apple Inc.) with an iDu LabCam Microscope Adapter (iDu) in a dark room. We calibrated Adobe Photoshop to calculate distance measures based on a pixel-to-millimetre ratio. We then quantified the mean gray value (MGV, 0 = black to 255 = white) of a roughly 0.500 mm^2 rectangle selected from the tip of the filament that was directly inserted into the insect (*see pictures on y-axis of* Figure 1).

2.6 | Statistical analyses

For all analyses, we used linear mixed models (LMMs; LME4 package) always starting with models that included chamber identity, date and monarch genotype (when appropriate) as random effects. Random effects were removed when model fits were singular due to complex random effects structures. We performed model selection using the ANOVA function, fitting linear mixed models with maximum likelihood. We implemented all statistical tests in R version 3.3.2 (R Core Team, 2020), all variables were transformed to best achieve normality of error using the Shapiro–Wilk test and examined visually. Model fits were visually inspected using qqplots and homogeneity of variance was evaluated by plotting residuals against fitted values (Zuur et al., 2010). In instances of multiple comparison, we performed post hoc tests among least-squared means using the EMMEANS package (Lenth, 2020).

We assessed the melanization response of late-instar monarchs as a measure of mean gray value (MGV) over a standardized area of filament by running an LMM with CO_2 treatment, infection by *O. elektroscirrha*, milkweed species and their interactions as fixed effects. Melanization values were natural log-transformed.

We assessed the effects of milkweed species and CO_2 treatment on (a) total foliar cardenolide concentration (log-transformed),

(b) cardenolide polarity, (c) whole-PSM diversity detected with H^1-NMR and (d) foliar C:N ratio (log-transformed) using LMMs.

To investigate the effects of CO_2 treatment, infection by *O. elektroscirrha* and milkweed species on the PO activity of larvae, we ran LMMs with the three treatments and their interactions as fixed effects and the activity of (a) total PO, (b) activated PO, and (c) standing PO as response variables. Standing PO did not respond to any of our treatments (Table S2) and is, therefore, not reported here. We used LMMs to assess the effects of our treatments on (a) total haemocyte concentration (log-transformed), (b) granulocyte (log-transformed), (c) oenocytoid (log-transformed), (d) spherule cell (square-root transformed) and (e) plasmocyte (log-transformed) concentrations. In each of these LMMs, CO_2 treatment, infection by *O. elektroscirrha*, milkweed species and their interactions were fixed effects.

To explore the effects of foliar chemical traits on monarch immunity, we used LMMs with all measured immunological traits as response variables and our four foliar traits (above) as fixed effects. To avoid detecting spurious correlations that result from differences between plant species, we originally included milkweed species as a fixed effect in these models. However, the species term did not improve model fit and was removed. We report only those models that showed significant effects of foliar quality on immune function.

3 | RESULTS

3.1 | 'Medicinal' milkweed inhibited the melanization response of late-instar monarch larvae

In partial support of our first prediction that medicinal *A. curassavica* would reduce the expression of endogenous immunity, the melanization response around a sterile filament (simulated antigen) was 13% lower (−0.39 ± 0.19 effect size) in monarch larvae feeding on *A. curassavica* than on *A. incarnata* (species: $F_{1,133} = 5.84$, $p = 0.017$, Figure 1; Table S2). Monarch genotype accounted for 0.002 ± 0.04 of the variance in the melanization response. In other words, feeding on a milkweed species that provided high exogenous immunity against parasites reduced modestly the strength of one aspect of endogenous immune defence against parasitoids.

3.2 | Foliar chemical defences and nutritional quality declined under elevated CO_2

In support of our second prediction that eCO_2 would reduce the sources of exogenous immunity, eCO_2 induced a 23% reduction in foliar cardenolide concentrations (quantified with UPLC-UV detection) in *A. curassavica* and a 30% reduction in *A. incarnata* (CO_2: $F_{1,98} = 7.88$, $p = 0.006$, Figure 2a; Table S4). Because eCO_2 induced reductions of similar magnitude in both species, there was no interaction between milkweed species and CO_2 treatment on cardenolide production (species*CO_2: $F_{1,265} = 1.26$, $p = 0.2632$). The mean polarity index of cardenolides produced by *A. curassavica* was twice that of *A. incarnata* (species: $F_{1,264} = 104.40$, $p < 0.0001$, Figure 2b); a high polarity index indicates an abundance of lipophilic cardenolides. However, CO_2 treatment had no effect on the mean polarity value of cardenolides (CO_2: $F_{1,94} = 2.79$, $p = 0.0982$, Figure 2b) and caused no significant interaction (species*CO_2: $F_{1,263} = 2.48$, $p = 0.1166$).

Using H^1-NMR, we estimated the holistic diversity of secondary metabolite structural features within the milkweed plants using Simpson's diversity index of binned chemical shift values. The diversity of metabolites declined in both species of milkweed under eCO_2 (CO_2: $F_{1,38} = 6.93$, $p = 0.0122$, Figure 2c; Table S4) but was higher in *A. incarnata* (species: $F_{1,435} = 13.21$, $p = 0.0003$, Figure 2c). Qualitative structural analysis of crude ^{1}H-NMR spectra revealed that *A. incarnata* contained a much higher proportion of flavonoids (diagnostic ^{1}H-NMR resonances 6.5–7.8 ppm) to steroidal glycosides, steroidal compounds (0.5–3.0 ppm) and

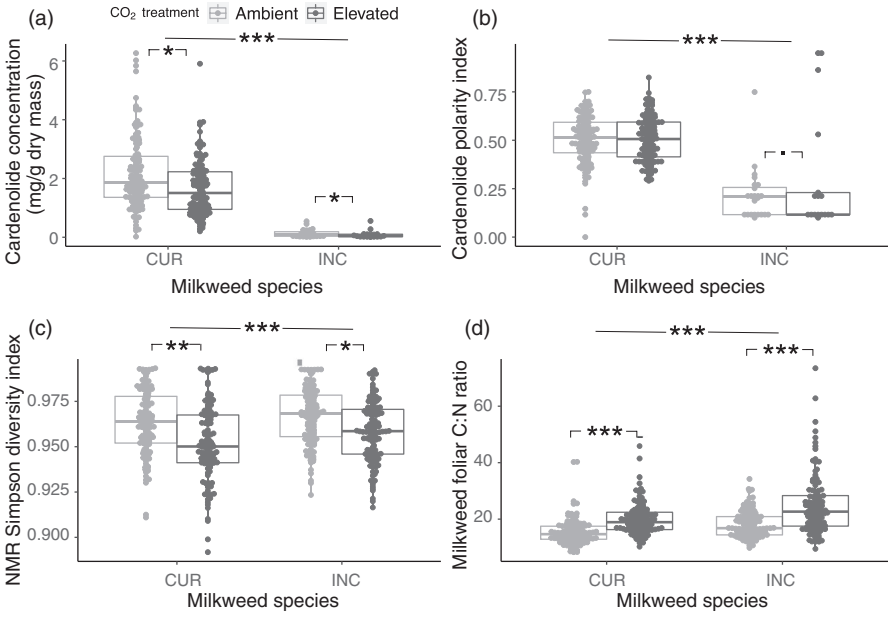

FIGURE 2 The effects of CO_2 treatment and milkweed species on (a) initial foliar cardenolide concentrations (mg/g dry mass), (b) cardenolide polarity index, (c) the Simpson diversity of all foliar secondary metabolites detected with H^1-NMR and (d) the ratio of foliar carbon to nitrogen (C:N). Dark grey points and lines represent plants grown under eCO_2 and light grey are those grown under aCO_2. Asterisks indicate significant differences detected with post hoc tests

glycosides (3.5–4.2 ppm, Figure 3), resulting in greater interclass phytochemical diversity when compared to *A. curassavica*. As with cardenolide concentrations, eCO_2 caused similar declines in the chemical diversity of both milkweed species (species*CO_2: $F_{1,435} = 1.36$, $p = 0.2437$, Figure 2c). Losses of cardenolide structures, revealed by ^{1}H-NMR spectra (diagnostic methyl doublet resonances at 1.1–1.2 ppm) and UPLC-UV detection, likely contributed to reductions of interclass phytochemical diversity captured by the Simpson index (Figure 3).

Consistent with two decades of research on CO_2, the nutritional quality of milkweed foliage declined under eCO_2 (CO_2: $F_{1,38} = 75.11$, $p < 0.0001$, Figure 2d; Table S4). Specifically, the foliar C:N ratio increased by 29% in *A. curassavica* and by 38% in *A. incarnata*. Across both CO_2 treatments, *A. incarnata* had 22% higher foliar C:N ratios than *A. curassavica* (species: $F_{1,437} = 41.34$, $p < 0.0001$, Figure 4d), but there was no species-specific response of the foliar C:N ratio to CO_2 treatment (species*CO_2: $F_{1,437} = 0.10$, $p = 0.7571$, Figure 2d).

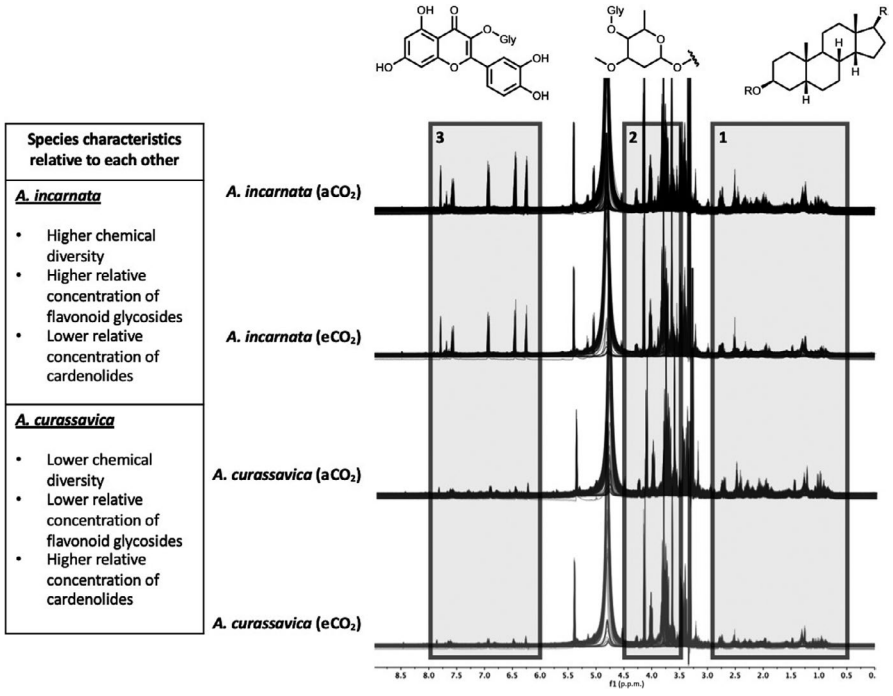

FIGURE 3 Superimposed ^{1}H-NMR spectra of plant crude extracts for each treatment group (top to bottom: *A. incarnata*, ambient CO_2 (aCO_2); *A. incarnata*, elevated CO_2 (eCO_2); *A. curassavica*, aCO_2; *A. curassavica*, (eCO_2). Qualitative structural analysis revealed chemical shift data (ppm) consistent with literature reported values for steroidal (1), glycosylated (2) and flavonoid (3) regions of the ^{1}H-NMR spectra. Both *A. incarnata* and *A. curassavica* show reduced concentrations of cardenolides and chemical diversity under eCO_2. *A. incarnata* had higher proportions of flavonoid glycosides in both CO_2 treatments resulting in greater interclass diversity compared to *A. curassavica*, which had higher proportions of cardenolides compared to *A. incarnata*

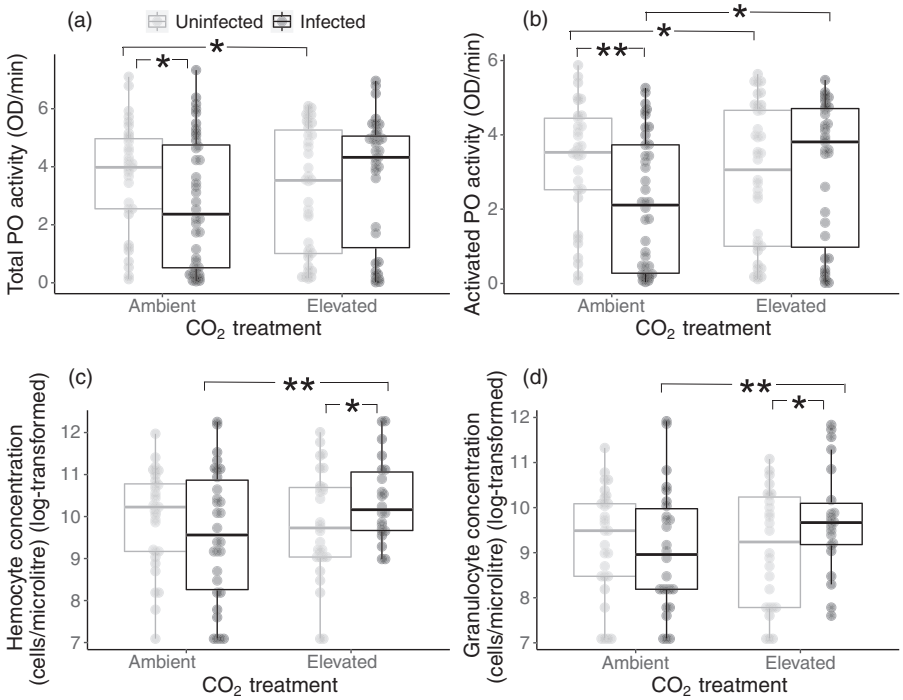

FIGURE 4 The interactive effects of infection by *Ophryocystis elektroscirrha* and CO_2 treatment on monarch (a) total phenoloxidase (PO) activity, (b) activated prophenoloxidase (activated PO) activity, (c) total haemocyte concentration (log-transformed) and (d) granulocyte concentration (log-transformed). Ambient CO_2 concentrations averaged 410 ppm and elevated CO_2 concentrations averaged 810 ppm. Black points and lines represent infected monarchs and light grey are controls. Asterisks indicate significant differences detected with post hoc tests

3.3 | Foliage from plants grown under eCO_2 increased the relative strength of monarch endogenous immunity

In partial support of our third prediction that reductions in exogenous immunity (medicinal phytochemical protection) under eCO_2 would stimulate compensatory endogenous immunity, certain monarch immune responses to infection were higher in larvae reared on foliage from eCO_2 plants and lower in larvae reared on aCO_2 plants (Figure 4). Specifically, larvae challenged with the parasite and reared on eCO_2 plants exhibited 43% higher activated PO activity (infection*CO_2: $F_{1,137} = 9.46$, $p = 0.0003$, Figure 4b; Table S2), and 30% higher total haemocyte concentrations (infection*CO_2: $F_{1,108} = 4.97$, $p = 0.028$, Figure 4c; Table S3) than infected larvae reared on aCO_2 plants. The concentration of one phagocytic haemocyte type, granulocytes (phagocytic cells), was 7.5% higher in larvae challenged with the parasite and fed eCO_2 plants (infection*CO_2: $F_{1,95} = 4.12$, $p = 0.0452$, Figure 4d; Table S3). Furthermore, feeding on eCO_2 plants released total PO activity from a 25% immune suppression experienced by infected larvae under ambient conditions (infection*CO_2: $F_{1,137} = 5.93$, $p = 0.016$, Figure 4a; Table S2).

In contrast to this general pattern and our predictions, the concentration of oenocytoids circulating in monarch haemolymph was highest in infected larvae feeding on *A. curassavica* grown under aCO_2 (infection*milkweed species*CO_2: $F_{1,54} = 4.60$, $p = 0.036$, Figure 5a; Table S3). Across parasite treatments, mean oenocytoid concentrations in monarchs fed *A. curassavica* were 33% lower under eCO_2 (species*CO_2: $F_{1,54} = 3.99$, $p = 0.051$, Figure 5b; Table S3). Oenocytoids are much rarer in lepidopteran haemolymph but are thought to be directly involved in PO production (Altizer & de Roode, 2015; Strand, 2008). Monarchs fed *A. curassavica* produced 48% more oenocytoids than those fed *A. incarnata* (species: $F_{1,54} = 8.15$, $p = 0.006$, Table S3).

Based on Prediction 1, and the melanization responses reported for late-instar larvae (above), we expected that our indices of endogenous immunity would be higher on larvae fed the non-medicinal *A. incarnata* than the medicinal *A. curassavica*; this

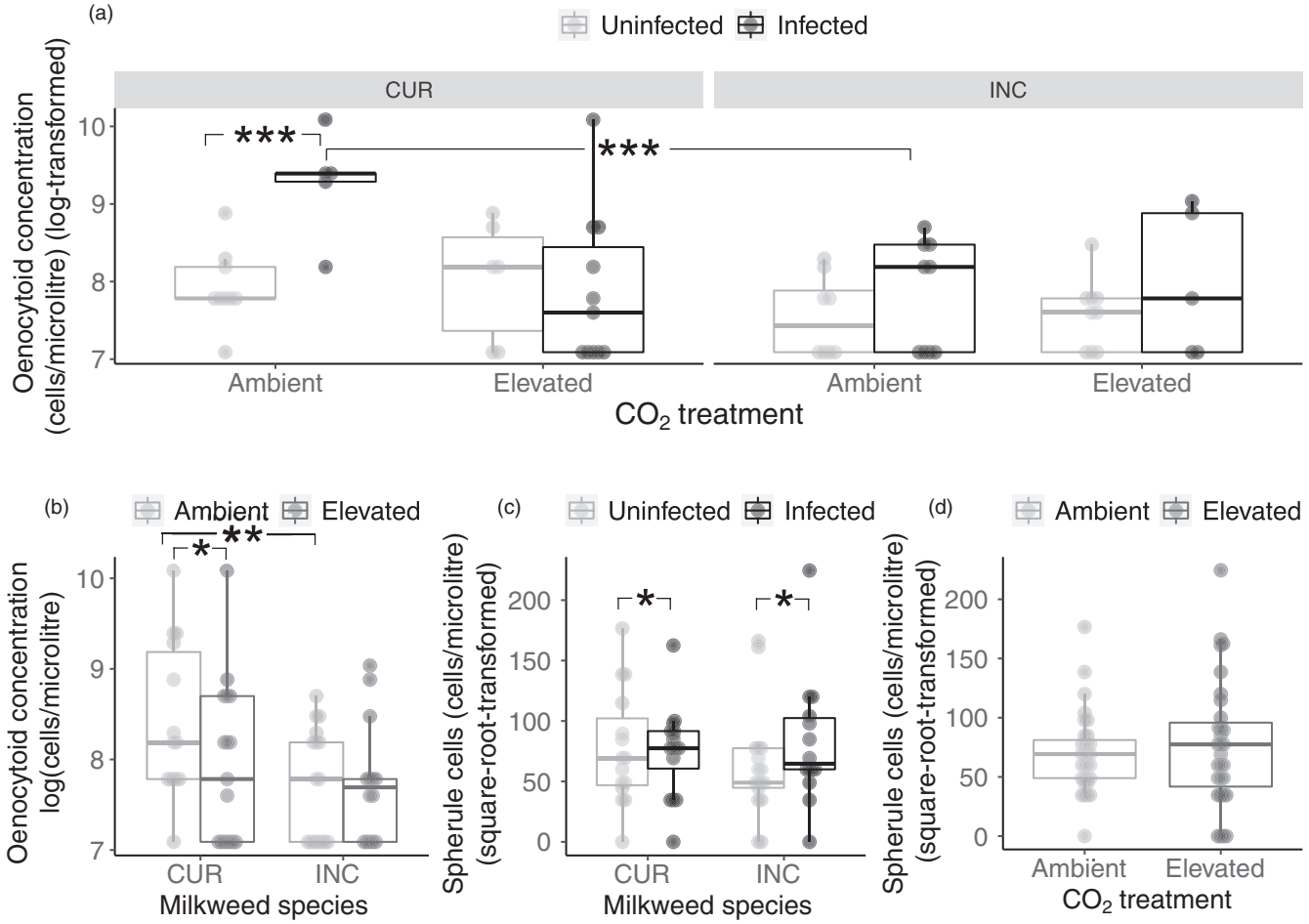

FIGURE 5 The interactive effects of infection by *Ophryocystis elektroscirrha*, milkweed species and CO_2 treatment on (a) oenocytoid concentrations. The interaction of (b) CO_2 treatment and milkweed species on oenocytoid concentrations, (c) infection and milkweed species on spherule cell concentrations and (d) the main effect of CO_2 treatment on spherule cell concentration. Milkweed species codes are as follows: CUR = *A. curassavica* and INC = *A. incarnata*. In (a) and (b), black points and lines represent infected monarchs and light grey are control. In (d), dark grey points and lines represent plants grown under eCO_2 and light grey are those grown under aCO_2. Asterisks indicate significant differences detected with post hoc tests

was generally not the case. There were no main or interactive effects of milkweed species on total PO activity, activated PO activity, haemocyte concentration or granulocyte concentration (Tables S2 and S3). However, in partial support of Prediction 1 that *A. curassavica* would suppress the expression of endogenous immunity more than would non-medicinal, *A. incarnata*, the concentration of spherule cells (which contain cuticular components for clotting) responded slightly more to infection in larvae fed *A. incarnata* than in larvae fed *A. curassavica* (infection*milkweed species: $F_{1,78} = 3.57$, $p = 0.063$, Figure 3c; Table S3). Additionally, across infection and milkweed species, eCO_2 induced a 60% increase in the concentration of spherule cells in monarchs (CO_2: $F_{1,78} = 5.15$, $p = 0.026$, Figure 3d), consistent with a relative increase in endogenous immunity under eCO_2.

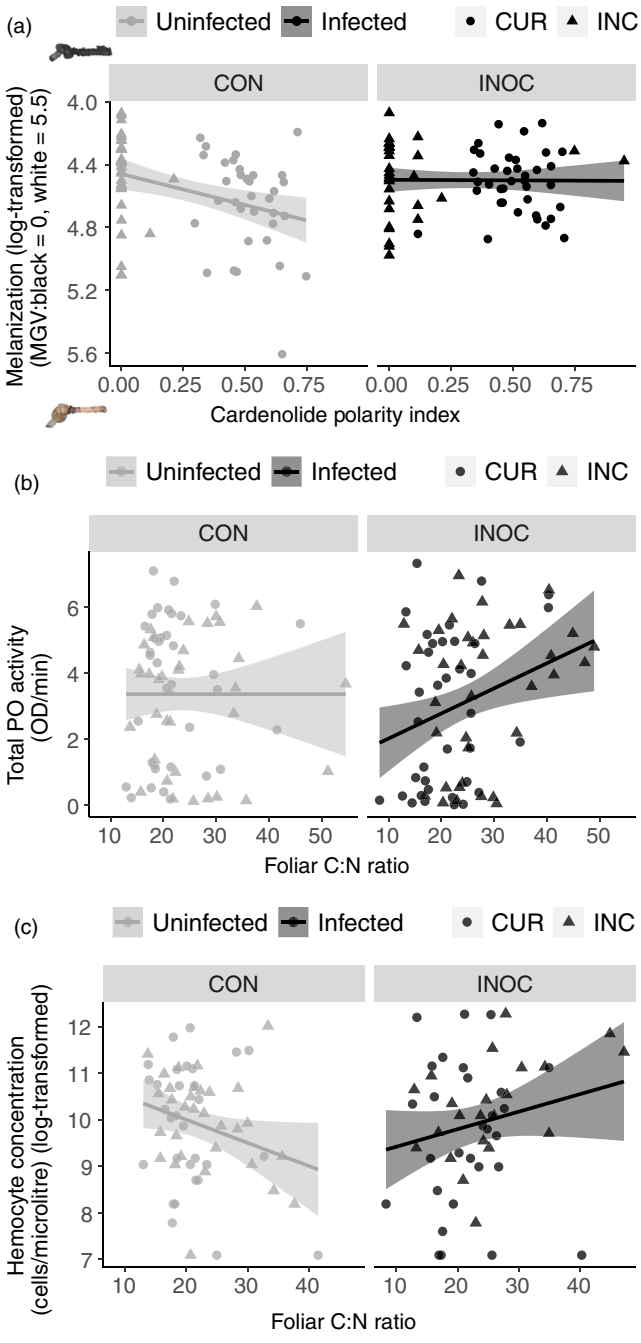

FIGURE 6 Effects of infection by *Ophryocystis elektroscirrha* on the relationships between (a) diet cardenolide polarity index and monarch melanization; (b) diet nutritional quality (C:N ratio) and total PO activity; and (c) foliar C:N ratio and total haemocyte concentrations. Light grey points and lines represent uninoculated control monarchs and black represents monarchs inoculated with *O. elektroscirrha*. In (a), note the flipped y-axis: Higher mean gray values (MGV) represent lighter pigmented filaments or lower melanization responses. Shaded bands represent 95% confidence intervals around predicted fit lines

3.4 | Larval immune responses to parasite infection correlated with measures of phytochemistry

In late-instar larvae, the melanization response of uninfected monarchs correlated negatively with foliar cardenolide polarity whereas the melanization response of infected larvae did not correlate with cardenolide polarity (infection*cardenolide polarity: $F_{1,132} = 5.90$, $p = 0.017$, Figure 6a; Table S5). When cardenolides were included in the model predicting the melanization response of monarchs, we detected a 10% stronger melanization response induced by *O. elektroscirrha* infection (Table S6; Figure S2).

Despite our predictions that monarch immunity would respond to changes in secondary chemistry, measures of early-instar endogenous immunity correlated most strongly with foliar nutritional quality (C:N ratios). Infected early-instar larvae increased endogenous immunity as foliar nutritional quality declined (Figure 6b,c; Tables S6 and S8). Specifically, the total PO activity and haemocyte concentrations of infected larvae increased as foliar C:N ratios increased (parasite treatment*CN: $F_{1,140} = 3.95$, $p = 0.049$; $F_{1,106} = 6.29$, $p = 0.014$, respectively, Figure 6b,c; Tables S6 and S8).

4 | DISCUSSION

Here, we demonstrate that specific endogenous immune responses of monarchs to infection with *O. elektroscirrha* are generally reduced when larvae feed on foliage grown under ambient CO_2 and enhanced when larvae feed on foliage grown under elevated CO_2. These results are consistent with a change in the relative strengths of endogenous and exogenous immunity under elevated concentrations of atmospheric CO_2. Our results build upon previous work in the monarch–parasite–milkweed system and provide two major avenues of insight. First, a recent transcriptomics study demonstrated little to no response of monarch larval gene expression within 24 hr to infection by *O. elektroscirrha* (Tan et al., 2019). Here, we confirm that this lack of response in immune gene expression translates to immune function suppression when infected larvae are reared on foliage from plants grown under aCO_2. Second, the study by Tan et al. (2019) also reported that a subset of monarch immune genes was downregulated when

larvae were reared on the medicinal *A. curassavica*. The authors suggested that this reflects reliance on the well-characterized exogenous immunity to parasites that is provided by *A. curassavica* (de Roode, Pedersen, et al., 2008; Sternberg et al., 2012). Here, we report that a loss of medicinal phytochemistry in milkweed plants grown under eCO_2 is reflected in a loss of endogenous immune suppression and the upregulation of immune function in infected larvae. Our results therefore combine with previous work to suggest that coevolution between monarchs, milkweed and *O. elektroscirrha* has reduced monarch reliance on endogenous immunity and that future environmental conditions may change the balance of endogenous and exogenous immune function.

In our study, humoral and cellular immune responses including PO activity and circulating haemocyte density were suppressed in monarch larvae feeding on their typical diet but increased when larvae consumed foliage from plants grown under eCO_2. Foliage from eCO_2 exhibited lower nutritional quality, lower concentrations of cardenolides (UPLC-UV detected), which are known to provide exogenous immunity (Gowler et al., 2015), and lower holistic phytochemical diversity (^{1}H-NMR detected). Here, total PO activity and haemocyte counts correlated directly with losses in foliar nutritional quality (higher C:N ratios). The costs of mounting endogenous immune responses as nutritional quality declines may explain in part the loss of tolerance by monarchs to OE under eCO_2 reported previously (Decker et al., 2018). However, it is impossible to disentangle the singular roles of secondary chemistry and nutritional quality on this relationship in the current study which only captures the holistic response of hosts to infection under future environmental conditions.

We predicted that expression of endogenous immunity would be greater in larvae fed the non-medicinal milkweed *A. incarnata* than the medicinal milkweed *A. curassavica*. While that was supported weakly by the melanization response of late-instar larvae (Figure 1), it was not supported by our measures of PO activity or haemocyte counts of early-instar larvae, which were related to CO_2 concentration rather than plant species. Notably, standing PO was unaffected by any of our treatments; however, CO_2 concentration did alter the magnitude of activated PO activity (potential PO activity) which could indicate differences in immune plasticity in response global change. We also did not find simple correlations between foliar cardenolide concentrations and endogenous immune function of early instars. This is in contrast to the negative effects of other secondary metabolites such as iridoid glycosides on the circulating PO activity of other lepidopterans (Smilanich et al., 2009, 2017). Instead, the immune response of infected monarchs was positively correlated with declining foliar nutrient concentrations induced by eCO_2 (Figure 6b,c). Typically, insect immunity follows the opposite pattern, whereby immune responses decline on diets low in nutrients (Beckage, 2011; Strand, 2008). In some instances, diets low in protein but high in carbohydrates (a condition commonly induced in foliage grown under eCO_2) may promote insect melanization induced by PO activity (Mason et al., 2014). Presumably, a diet high in soluble carbohydrates is easier to metabolize than one consisting of the less digestible peptide bonds. Within other insect systems, a trade-off between lipid digestion and immunity has been illustrated (Adamo et al., 2008, 2010). Therefore, infected monarchs may have more readily available energy to invest in their immune response because of reduced energetic requirements for digestion when feeding on foliage with higher C:N ratios.

Alternatively, the phytochemical changes induced by eCO_2 may have altered the ability of the parasite to evade detection by the monarch host and/or suppress immune activity, two well-documented phenomena of host manipulation by the parasite (*reviewed in* Heil, 2016). Subsequent studies that survey the transcriptomic responses of monarchs to infection under simulated future environmental conditions will improve our understanding of the environmental contingency of these parasitic behaviours. Additionally, these transcriptomic studies may shed light on the complex relationship between detoxification and immunity (Smilanich & Nuss, 2019) that may add further depth to the phytochemical contingency of herbivorous host immunity in a changing world.

Our results contribute to a growing body of research illustrating costs of secondary metabolite ingestion to the immune response of hosts under parasitoid attack (Hansen et al., 2016; Smilanich et al., 2009). In our study, late-instar monarch larvae reared on high-cardenolide milkweed produced a 13% weaker melanization response against a standardized antigen (Figure 1). Despite the statistical significance, this small reduction in melanization could prove less biologically relevant; however, we do not know how these reductions in melanization interact with other processes. Although we did not measure sequestration, a large body of literature exists demonstrating that monarchs consuming high-cardenolide milkweed species sequester comparably high concentrations of cardenolides (Agrawal et al., 2012). Thus, our study suggests that the high metabolic demands of consuming and sequestering cardenolides may reduce some endogenous defences. However, in this case, the 'price' of reduced immunity may be worth it if phytochemistry is covering the 'costs' of protection from parasite infection.

Through our qualitative ^{1}H-NMR analysis of milkweed chemotypes, we are able to highlight an additional factor that may contribute to the differences in melanization we observed between monarchs fed the two milkweed species: plant flavonoid diversity. Our structural analyses of crude ^{1}H-NMR spectra confirm that the higher phytochemical diversity reported in *A. incarnata* is due largely to the presence of flavonoid compounds. Critically, the PO cascades that generate encapsulation responses also produce harmful reactive oxygen species that damage surrounding cellular functions but can be neutralized by antioxidant PSMs such as flavonoids (Lampert, 2012). The importance of antioxidant PSMs to herbivore immunity has been demonstrated through artificial diet studies where both specialists and generalist lepidopterans exhibit strengthened immunity in the presence of flavonoids, phenolics and other antioxidants (Ojala et al., 2005; Smilanich et al., 2009). In the current study, the higher concentrations of flavonoids we detected in *A. incarnata* foliage may, therefore, enhance the melanization response of late-instar monarchs by alleviating the deleterious by-products of melanization.

However, further analysis of metabolomic data as they relate to monarch performance is necessary to support this phytochemical mechanism.

Monarch butterfly populations currently face multiple threats induced by anthropogenic environmental change (Malcolm, 2017). Here, we demonstrate the potential of eCO_2 to compromise their reliance on phytochemistry as a source of exogenous immunity against infection. As a consequence, monarchs induce endogenous sources of immunity that may be energetically costly to produce. Investment in endogenous immunity will likely come at a cost to other important life-history traits (Schmid-Hempel, 2005) such as growth and reproduction that may ultimately decrease monarch fitness. Our results suggest that shifts in the balance between exogenous and endogenous sources of immunity to parasite attack may represent an underappreciated consequence of environmental change for animals.

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AUTHORS' CONTRIBUTIONS

L.E.D., A.M.S., J.C.d.R. and M.D.H. designed the experiment; L.E.D., A.S.P. and K.M.O. collected and processed the data; L.E.D. and K.M.O. analysed the data; L.E.D. wrote the manuscript and all authors contributed substantially to drafts and approved the final version.

DATA AVAILABILITY STATEMENT

Data are available on Dryad Digital Repository https://doi.org/10.5061/dryad.dr7sqv9ww (Decker et al., 2020)

ORCID

Leslie E. Decker https://orcid.org/0000-0003-1325-052X
Abigail S. Potts https://orcid.org/0000-0003-0563-1437
Jacobus C. de Roode https://orcid.org/0000-0002-8423-8918
Mark D. Hunter https://orcid.org/0000-0003-3761-8237

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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