Supporting Information

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

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Appendix A. Additional background

Monarchs face attack from other enemies vulnerable to the insect immune response. Several microbial parasites and pathogens including a nuclear polyhedrosis virus (NPV), gram-positive bacteria such as *Bacillus*, gram-negative bacteria such as *Pseudomonas* and a microsporidian *Nosema* species infect monarchs (Karen Suzanne Oberhauser, Nail, & Altizer, 2015). Additionally, there are 12 species of tachinid flies, one brachonid wasp and one chalcid wasp known to parasitize monarchs (Arnaud, 1978; Stenoien, McCoshum, Caldwell, De Anda, & Oberhauser, 2015). The tachinid fly, *Lespesia archippivora*, has received the most attention (Borkin, 1982; K. Oberhauser, Gebhard, Cameron, & Oberhauser, 2009; K. S. Oberhauser, 2012; Prysby, 2004; Smithers, 1973). This parasitoid attacks middle to late instar larvae and pupae, and may parasitize anywhere from 10-90% of monarchs in a given population (K. S. Oberhauser, 2012).

Due to the intimate nature of parasitoid development within the monarch larvae, the nutritional and medicinal quality of the monarch host diet likely influences parasitoid success (K. S. Oberhauser et al., 2015). However, we know very little about general rates of parasitoid survival within monarchs (Hunter, Malcolm, & Hartley, 1996; Sternberg, Lefèvre, Rawstern, & De Roode, 2011), and monarch immune defense against parasitoid infection. Interestingly, *O. elektroscirrha* infection reduces the mortality caused by the tachinid parasitoid, *L. archippivora* to late-instar monarch larvae (Sternberg et al., 2011). We need additional studies to understand the complex interactions among co-infection, monarch immunity, and diet chemistry.

Appendix B. ***Monarch Performance Measures (only Assay Control Caterpillars)***

Approximately 24 hours following the formation of the chrysalis, we removed all frass and remaining foliage from each container. We sexed monarchs 24 hours after eclosion and transferred them to 5.75 x 9.5 cm glassine envelopes. Monarchs were then stored in an incubator kept at 15°C with 16-hour daylight for the remainder of their adult lives. We checked each monarch daily and recorded date of death. The lifespan of monarchs under these starvation conditions correlates strongly with lifetime reproductive fitness (de Roode *et al.* 2008b). Therefore, we estimated parasite virulence as the reduction in the lifespan of infected monarchs as compared to uninfected monarchs (de Roode, Pedersen, Hunter, & Altizer, 2008). Two months after death, we quantified the spore loads of each monarch following well-established methods (de Roode et al., 2008). The tolerance of monarchs to the parasite was measured as the slope of the regression between spore load and longevity, with separate regressions for monarch treatment groups feeding on each milkweed species and CO_2 treatment (Sternberg et al., 2012; Sternberg, Li, Wang, Gowler, & de Roode, 2013; Appendix D).

Within the Assay Control group (Table S1), we investigated the effects of our treatments on monarch performance. To determine if monarch tolerance to infection varied by CO_2 treatment and milkweed species, we used an LMM with the lifespan of infected monarchs as the response variable and spore load (log_{10}-transformed), CO_2 treatment, and milkweed species as fixed effects. Any significant interaction between either CO_2 treatment or milkweed species and spore load indicates effects on monarch tolerance to infection. To test for effects of CO_2 treatment, and milkweed species on parasite virulence, we ran an LMM with monarch lifespan as the response variable and parasite treatment, CO_2 treatment, and milkweed species as fixed effects. Similar to tolerance, an interaction of either of these treatments with infection indicates a difference in the magnitude reduction in lifespan (fitness) induced by infection (our definition of a change in virulence). To estimate any treatment effects on monarch resistance, we used an LMM with spore load (log_{10}-transformed) as the response variable and CO_2 treatment, and milkweed species as fixed effects. Lastly, we used GLMMs with binomial error distributions and logit link functions to assess the effects of CO_2 treatment, and milkweed species on the binary response variables of monarch survival and infection probability.

Our data show only a visual pattern supporting the previously reported loss of tolerance to *O. elektroscirrha* infection in monarchs fed *A. curassavica* grown under elevated CO_2 (Fig. S2; Decker, de

Roode, & Hunter, 2018). However, there was no statistical support for this trend detected (spore load*milkweed species*CO_2: $F_{1,99} = 0.55$, $p = 0.460$). There was no effect of milkweed species (milkweed species*infection: $F_{1,115} = 0.83$, $p = 0.6340$), CO_2 treatment (CO_2*infection: $F_{1,115} = 0.2$, $p = 0.6232$), or their interaction (milkweed species* CO_2*infection: $F_{1,116} = 0.48$, $p = 0.4920$) on parasite virulence likely because of a small sample size. The spore loads of infected monarchs did not respond to CO_2 treatment ($F_{1,23} = 0.06$, $p = 0.8118$), milkweed species ($F_{1,23} = 2.39$, $p = 0.1357$) or their interaction ($F_{1,23} = 0.74$, $p = 0.3975$). Monarchs fed *A. incarnata* foliage had a 3% lower survival rate than those monarchs fed *A. curassavica* as larvae (milkweed species: $\chi^2 = 4.24$, $p = 0.0395$). There was no effect of CO_2 treatment ($\chi^2 = 1.36$, $p = 0.24321$) or infection ($\chi^2 = 1.41$, $p = 0.2347$) on monarch survival.

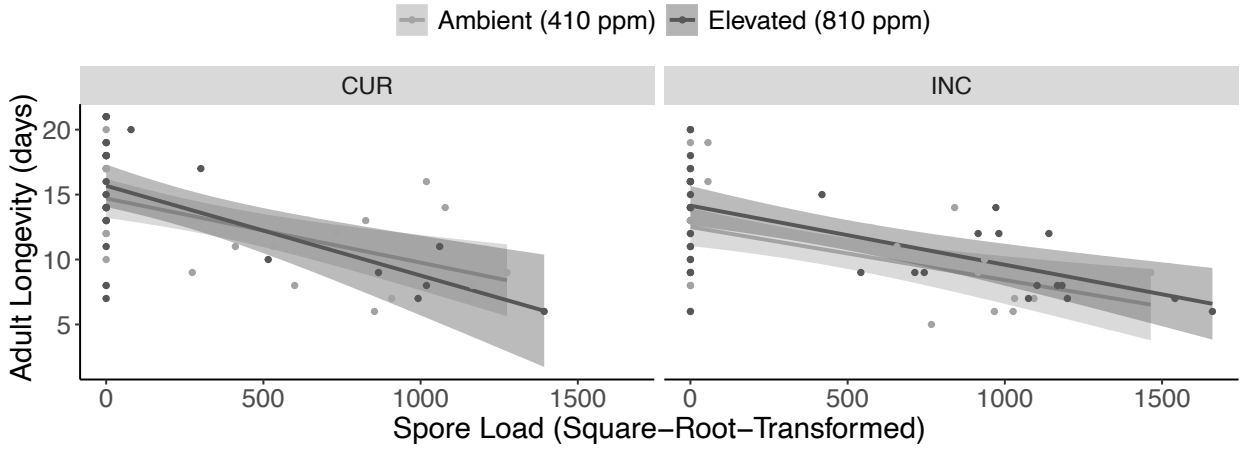

Figure S1. We depict the visual trend congruent with the previously reported loss of tolerance to *O. elektroscirrha* infection in monarchs fed *A. curassavica* grown under elevated CO_2 (Decker, de Roode, & Hunter, 2018; N=310). In our study the resulting sample size of monarchs that experienced no immune assay manipulation was nearly half the size of the population used to previously detect this pattern (N = 154). Light gray lines and points correspond to tolerance slopes of monarchs reared on plants grown under ambient CO_2 (410 ppm) and dark gray lines and points correspond to tolerance slopes of monarchs reared on plants grown under elevated CO_2 (810 ppm).

Appendix C. Patterns of Larval Mortality

We used GLMs with binomial error distributions and logit link functions to assess the effects of CO_2 treatment and milkweed species on larval mortality recorded in the control and filament assayed groups separately. We first tried to incorporate the random effects of chamber and genotype using generalized linear mixed effects models, but model fits were singular, so we proceeded with generalized linear models.

Within control monarchs, mortality of larvae feeding on *A. incarnata* (7.1%) differed from those feeding on *A. curassavica* (3.7) by 3.4% (species: $\chi^2 = 4.24$, $p = 0.04$). Mortality was randomly distributed among the remaining treatment combinations (Table S9). Within monarchs that received the filament assay, there were large differences between infected and uninfected larvae contingent upon the species of milkweed upon which monarchs were reared (parasite treatment*species: $\chi^2 = 4.86$, $p = 0.027$). Infected monarchs fed *A. incarnata* had the highest mortality rate (35%), compared to uninfected larvae feeding on the same species (15%). While the largest difference in mortality rates was between uninfected monarchs fed *A. curassavica* (5%) and uninfected monarchs on the same milkweed species (26%). Overall, infected monarchs within the filament assay had a 1% lower mortality than uninfected monarchs (parasite treatment*species: $\chi^2 = 4.72$, $p = 0.028$).

It is important to note that all assays were performed by a single individual to maintain consistency. This was especially critical for filament insertion in the melanization assay. The filament assay incurred the highest mortality within those monarchs reared to adulthood contingent on milkweed species and infection. This could be due to increased stress experienced by the insects from the initial incision, insertion, and removal of filaments compounded with infection and cardenolide toxicity. Further work with larger sample sizes is needed to determine if milkweed species and infection treatment significantly alter larval survival enough to influence the effects of our treatments on melanization.

Appendix D. Elaborated Chemical Methods

To extract cardenolides, we ground foliage for 3 minutes, sonicated the sample for 1 hour at 60°C, and centrifuged the sample for 6 minutes. We then transferred the supernatant to a new 1 mL ependorff tube and evaporated the sample under vacuum at 45°C until dry. Samples were resuspended in 150 μL of methanol spiked with 0.15 mg/mL digitoxin internal standard. We separated cardenolide compounds of interest using ultra performance liquid chromatography (UPLC, Waters Inc., Milford, MA, USA) with an Acquity BEH C18 column (1.7 μm, 2.1 x 50 mm, Waters Inc., Milford, MA, MA, USA). Each 2 μL sample injection was eluted for 9 minutes with a constant flow rate of 0.7 mL per minute under a mobile phase of 20% acetonitrile (ACN): 80% water for 3 minutes followed by a gradient that increased to 45% ACN: 55% water over the remainder of the run. Cardenolides were quantified using a diode array detector scanning between 200 and 300 nm; peaks that absorbed symmetrically with maxima between 216-222 nm were considered cardenolides.

At the same time that we removed leaf disks for cardenolide analysis, we harvested three additional leaves for ^{1}H-NMR analysis. We dried these leaves in a 30°C drying oven and ground the tissue to a fine powder. We then transferred 200 mg of each sample to a centrifuge tube and added 3 mL of deuterated extraction buffer (25% KH_2PO_4 90 mM, pH 6 in D_2O, 75% CD_3OD with Tetramethylsilane; Santos Pimenta, Schilthuizen, Verpoorte, & Choi, 2014). We vortexed each sample for 30 seconds, sonicated and then centrifuged each sample for 15 minutes each. We filtered the supernatant into an NMR tube and prepared the sample for NMR analysis on a 400 MHz Varian Instrument (Aligent Technologies). We processed the NMR spectral data using MestReNova software (Mestrelab Research) and aligned sample spectra using the methanol solvent peak (CHD_2OD) at 3.31 ppm. 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).

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Appendix E: Additional Tables and Figures

Table S1. The total number of larvae used in an experiment investigating the effects of infection by *Ophryocystis elektroscirrha*, elevated CO_2 and milkweed species on monarch immunity. The table shows experimental treatments, initial sample sizes, and the number of individuals that survived to be assayed (melanization or PO/Hemocytes) or that survived to adulthood (Assay Controls).

Assay	CO_2 Treatment	Milkweed Species	Parasite Treatment	Surviving N	Initial N
Melanization	ambient	*A. curassavica*	uninfected	14	20
			infected	19	20
		A. incarnata	uninfected	15	20
			infected	12	20
	elevated	*A. curassavica*	uninfected	15	20
			infected	19	20
		A. incarnata	uninfected	18	20
			infected	12	19
PO & Hemocytes	ambient	*A. curassavica*	uninfected	18	20
			infected	20	20
		A. incarnata	uninfected	16	20
			infected	20	20
	elevated	*A. curassavica*	uninfected	20	20
			infected	19	20
		A. incarnata	uninfected	19	20
			infected	19	20
Assay Control	ambient	*A. curassavica*	uninfected	20	20
			infected	19	20
		A. incarnata	uninfected	18	20
			infected	20	20
	elevated	*A. curassavica*	uninfected	20	20
			infected	19	20
		A. incarnata	uninfected	18	20
			infected	20	20

Table S2. ANOVA tables of linear mixed effects models used to investigate the indirect effects of elevated CO_2 on the immune response of monarchs to infection by *Ophryocystis elektroscirrha* contingent on the species of milkweed monarchs were fed. Melanization was measured as a mean gray value of a roughly 0.500 mm^2 rectangle selected from the tip of the filament that was directly inserted into the insect. We also measured standing phenoloxidase (PO), an enzyme critical to the production of melanin and cytotoxic molecules; its activated precursor, activated prophenoloxidase (proPO); and total PO to quantify immune response potential. Model selection was performed using the anova package in R with maximum likelihood fitting. Tables also include random effects estimates ±1 standard deviation.

log(**Melanization**) ~ infection + species + CO_2 + infection *species + random = 1|genotype

	infection	***species***	CO_2	infection * species	Random Effect ±SD
F	$F_{1,131} = 2.19$	$\boldsymbol{F_{1,133} = 5.84}$	$F_{1,133} = 0.45$	$F_{1,\ 132} = 2.72$	genotype
p	0.142	***0.017***	0.506	0.102	0.001713±0.04139

Standing Phenoloxidase ~ infection + species + CO_2 + species*CO_2 + infection*CO_2 + random = 1|chamber

	infection	species	CO_2	species*CO_2	infection*CO_2	Random Effect ±SD
F	$F_{1,108} = 2.02$	$F_{1,110} = 0.21$	$F_{1,39} = 0.16$	$F_{1,\ 110} = 2.18$	$F_{1,\ 108} = 0.00$	chamber
p	0.158	0.6466	0.6943	0.1430	0.9769	0.051 ± 0.226

Activated Phenoloxidase ~ infection + species + CO_2 + infection*CO_2 + random = 1|date

	infection	species	CO_2	***infection* CO_2***	Random Effect ±SD
F	$F_{1,136} = 2.39$	$F_{1,137} = 0.17$	$F_{1,138} = 0.01$	$\boldsymbol{F_{1,\ 137} = 9.46}$	date
p	0.124	0.677	0.938	***0.0003***	1.224 ± 1.106

Total Phenoloxidase ~ infection + species + CO_2 + infection*CO_2 + random = 1|date

	infection	species	CO_2	***infection* CO_2***	Random Effect ±SD
F	$F_{1,136} = 0.25$	$F_{1,138} = 0.01$	$F_{1,139} = 0.15$	$\boldsymbol{F_{1,\ 137} = 5.93}$	date
p	0.621	0.940	0.702	***0.016***	1.435 ± 1.198

Table S3. ANOVA tables of linear mixed effects models used to investigate the indirect effects of elevated CO_2 on the cellular immune response of monarchs to infection by *Ophryocystis elektroscirrha* contingent on the species of milkweed monarchs were fed. Model selection was performed using the anova package in R with maximum likelihood fitting, and ANOVA tables were produced with the R package LmerTest, using Type III sums of squares with Satterthwaite approximation for degrees of freedom. Tables also include random effects estimates ±1 standard deviation.

*log(**Total Hemocytes**) ~ infection + species + CO_2 + infection*CO_2 + random* = 1|*chamber*

	infection	*species*	CO_2	***infection*CO₂***	Random Effect ±SD
F	$F_{1,108}$= 0.24	*$F_{1,108}$= 3.01*	$F_{1,108}$= 2.18	***$F_{1,108}$= 4.97***	chamber
p	0.623	*0.085*	0.142	***0.028****	0.00±0.00

*log(**Granulocytes**) ~ infection + species + CO_2 + infection*species + species*CO_2 + infection*CO_2 + random* =1|*genotype*

	infection	species	CO_2	infection*species	*species*CO_2*	***infection*CO₂***	Random Effect ±SD
F	$F_{1,95}$= 1.51	$F_{1,95}$= 1.35	$F_{1,96}$= 1.83	$F_{1,96}$ = 0.76	$F_{1,94}$= 3.40	***$F_{1,95}$= 4.12***	genotype
p	0.223	0.248	0.179	0.386	*0.068*	***0.045****	0.001±0.031

*log(**Oenocytoids**) ~ infection + species + CO_2 + infection*species + species*CO_2 + infection*CO_2 + random* = 1|*genotype*

	infection	***species***	CO_2	infection*species	***species*CO₂***	*infection*CO_2*	***species*infection*CO₂***	Random Effect ±SD
F	***$F_{1,54}$= 5.96***	***$F_{1,54}$= 8.15***	$F_{1,54}$= 2.21	$F_{1,54}$= 0.48	***$F_{1,54}$= 3.99***	*$F_{1,54}$= 3.36*	***$F_{1,54}$= 4.60***	genotype
p	***0.018***	***0.006******	0.143	0.490	***0.051****	*0.072*	***0.036****	0.001±0.031

*Square-root(**Spherule Cells**) ~ infection + species + CO_2 + infection*species + infection*CO_2 + random* = 1|*genotype*

	infection	species	***CO_2***	*infection*species*	infection*CO_2	Random Effect ±SD
F	$F_{1,78}$= 1.47	$F_{1,78}$= 0.34	***$F_{1,78}$= 5.15***	*$F_{1,78}$= 3.57*	$F_{1,78}$= 1.67	genotype
p	0.229	0.560	***0.026****	*0.063*	0.200	0.00±0.00

*log(**Plasmocytes**) ~ infection + species + CO_2 + infection*species + species*CO_2 + random* = 1|*genotype* + 1|*chamber*

	infection	species	CO_2	infection*species	species*CO_2	Random Effect ±SD	
F	$F_{1,75}$= 0.34	$F_{1,75}$= 0.09	$F_{1,34}$= 0.09	$F_{1,78}$= 1.42	$F_{1,75}$= 0.72	genotype	chamber
p	0.560	0.762	0.769	0.238	0.400	0.036±0.189	0.114±0.337

*Table S4***.** The effects of CO_2 treatment, milkweed species and their interaction on log-transformed foliar cardenolide concentration, foliar cardenolide polarity, log-transformed foliar cardenolide diversity, entire plant secondary metabolite diversity detected with H^1-NMR, and carbon (C) to nitrogen (N) ratio.

	CO_2		**Species**		**CO_2*Species**	
	F	*P*	F	*P*	F	*P*
log(Cardenolide Concentration)	**$F_{1,98}$ = 7.88**	**p = 0.006****	**$F_{1,265}$ = 622.82**	**p < 0.0001*****	$F_{1,265}$ = 1.26	p = 0.2632
Cardenolide Polarity	$F_{1,94}$ = 2.79	p = 0.0982	**$F_{1,264}$ = 104.40**	**p < 0.0001*****	$F_{1,263}$ = 2.48	p = 0.1166
NMR Simspon Diversity Index	**$F_{1,38}$ = 6.93**	**p = 0.0122***	**$F_{1,435}$ = 13.21**	**p = 0.0003****	$F_{1,435}$ = 1.36	p = 0.2437
log(C:N Ratio)	**$F_{1,38}$= 75.11**	**p < 0.0001*****	**$F_{1,437}$= 41.34**	**p < 0.0001*****	$F_{1,437}$ = 0.10	p = 0.7571

Table S5. ANOVA tables of linear mixed effects models used to investigate relationships between phytochemistry and the melanization response of monarchs to infection by *Ophryocystis elektroscirrha*. Melanization was measured as a mean gray value of a roughly 0.500 mm^2 rectangle selected from the tip of the filament that was directly inserted into the insect. Model selection was performed using the anova package in R with maximum likelihood fitting, and ANOVA tables were produced with the R package LmerTest, using Type III sums of squares with Satterthwaite approximation for degrees of freedom. Tables also include random effects estimates ±1 standard deviation.

log(**Melanization**) ~ log(cardenolides) + infection + random = 1|genotype

	log(cardenolides)	***infection***	Random Effect ±SD
F	$F_{1,77} = 0.25$	$\boldsymbol{F_{1,77} = 7.82}$	genotype
p	0.620	***0.007***	0.005± 0.074

log(**Melanization**) ~ cardenolide polarity + infection + cardenolide polarity * infection + random = 1|genotype

	cardenolide polarity	infection	***cardenolide polarity* infection***	Random Effect ±SD
F	$\boldsymbol{F_{1,132} = 5.98}$	$F_{1,131} = 0.34$	$\boldsymbol{F_{1,132} = 5.90}$	genotype
p	***0.016***	0.563	***0.017***	0.0004± 0.019

log(**Melanization**) ~ NMR Simpson Index + infection + NMR Simpson Index* infection + random = 1|genotype

	NMR Simpson Index	infection	NMR Simpson Index * infection	Random Effect ±SD
F	$F_{1,132} = 1.52$	$F_{1,132} = 0.10$	$F_{1,132} = 0.12$	genotype
p	0.220	0.753	0.731	0.001± 0.039

log(**Melanization**) ~ CN + infection + CN * infection + random = 1|genotype

	CN	infection	CN* infection	Random Effect ±SD
F	$F_{1,131} = 0.39$	$F_{1,132} = 0.13$	$F_{1,131} = 0.05$	genotype
p	0.532	0.718	0.818	0.002± 0.049

Table S6. ANOVA tables of linear mixed effects models used to investigate relationships between phytochemistry and the total PO activity (sum of activated bound proPO and free PO) of monarchs in response to infection by *Ophryocystis elektroscirrha*. Model selection was performed using the anova package in R with maximum likelihood fitting, and ANOVA tables were produced with the R package LmerTest, using Type III sums of squares with Satterthwaite approximation for degrees of freedom. Tables also include random effects estimates ±1 standard deviation.

Total PO ~ log(cardenolides) + infection + random = 1|genotype

	log(cardenolides)	infection	Random Effect ±SD
F	***$F_{1,86}$ = 3.95***	$F_{1,86}$= 2.52	genotype
p	***0.050***	0.116	0.218± 0.467

Total PO ~ cardenolide polarity + infection + cardenolide polarity * infection + random = 1|genotype

	cardenolide polarity	infection	cardenolide polarity* infection	Random Effect ±SD
F	$F_{1,139}$ = 0.23	$F_{1,138}$= 0.39	$F_{1,139}$= 2.19	genotype
p	0.631	0.532	0.141	0.178± 0.422

Total PO ~ NMR Simpson Index + infection + NMR Simpson Index* infection + random = 1|genotype

	NMR Simpson Index	infection	NMR Simpson Index * infection	Random Effect ±SD
F	$F_{1,133}$ = 0.13	$F_{1,140}$= 0.17	$F_{1,140}$ = 0.18	genotype
p	0.719	0.682	0.673	0.177± 0.421

Total PO ~ CN + infection + CN * infection + random = 1|genotype

	CN	***infection***	***CN* infection***	Random Effect ±SD
F	*$F_{1,139}$ = 3.23*	***$F_{1,140}$= 4.72***	***$F_{1,140}$ = 3.95***	genotype
p	*0.074*	***0.031***	***0.049***	0.166± 0.407

Table S7. ANOVA tables of linear mixed effects models used to investigate relationships between phytochemistry and activated PO of monarchs in response to infection by *Ophryocystis elektroscirrha*. Model selection was performed using the anova package in R with maximum likelihood fitting, and ANOVA tables were produced with the R package LmerTest, using Type III sums of squares with Satterthwaite approximation for degrees of freedom. Tables also include random effects estimates ±1 standard deviation.

Activated PO ~ log(cardenolides) + infection + random = 1|genotype

	log(cardenolides)	***infection***	Random Effect ±SD
F	$F_{1,86} = 3.19$	$\boldsymbol{F_{1,86} = 5.70}$	genotype
p	*0.078*	***0.019***	0.070± 0.264

Activated PO ~ cardenolide polarity + infection + cardenolide polarity * infection + random = 1|genotype

	cardenolide polarity	infection	*cardenolide polarity* infection*	Random Effect ±SD
F	$F_{1,139} = 0.00$	$F_{1,138} = 0.03$	$F_{1,139} = 3.00$	genotype
p	0.955	0.859	*0.086*	0.111± 0.333

Activated PO ~ NMR Simpson Index + infection + NMR Simpson Index* infection + random = 1|genotype

	NMR Simpson Index	infection	NMR Simpson Index * infection	Random Effect ±SD
F	$F_{1,130} = 0.40$	$F_{1,140} = 0.03$	$F_{1,140} = 0.04$	genotype
p	0.528	0.864	0.841	0.107± 0.327

Activated PO ~ CN + infection + CN * infection + random = 1|genotype

	CN	infection	CN* infection	Random Effect ±SD
F	$F_{1,139} = 0.62$	$F_{1,141} = 1.49$	$F_{1,141} = 0.50$	genotype
p	0.432	0.224	0.483	0.166± 0.407

Table S8. ANOVA tables of linear mixed effects models used to investigate relationships between phytochemistry and the total hemocyte concentration of monarchs in response to infection by *Ophryocystis elektroscirrha*. Model selection was performed using the anova package in R with maximum likelihood fitting, and ANOVA tables were produced with the R package LmerTest, using Type III sums of squares with Satterthwaite approximation for degrees of freedom. Tables also include random effects estimates ±1 standard deviation.

log(**total hemocytes**) ~ log(cardenolides) + infection + log(cardenolides)* infection + random = 1|genotype

	log(cardenolides)	infection	log(cardenolides)* infection	Random Effect ±SD
F	$F_{1,71} = 0.57$	$F_{1,71} = 1.27$	$F_{1,69} = 0.05$	genotype
p	0.455	0.263	0.820	0.118± 0.344

log(**total hemocytes**) ~ cardenolide polarity + infection + cardenolide polarity * infection + random = 1|genotype

	cardenolide polarity	infection	cardenolide polarity* infection	Random Effect ±SD
F	$F_{1,106} = 3.55$	$F_{1,106} = 1.34$	$F_{1,107} = 2.44$	genotype
p	*0.062*	0.250	0.121	0.001± 0.037

log(**total hemocytes**) ~ NMR Simpson Index + infection + NMR Simpson Index* infection + random = 1|genotype

	NMR Simpson Index	infection	NMR Simpson Index * infection	Random Effect ±SD
F	$F_{1,92} = 0.03$	$F_{1,108} = 1.76$	$F_{1,108} = 1.76$	genotype
p	0.873	0.188	0.188	0.016± 0.126

log(**total hemocytes**) ~ CN + infection + CN * infection + random = 1|genotype

	CN	***infection***	***CN* infection***	Random Effect ±SD
F	$F_{1,106} = 0.11$	$\boldsymbol{F_{1,106} = 5.68}$	$\boldsymbol{F_{1,106} = 6.29}$	genotype
p	0.736	***0.019***	***0.014***	0.018± 0.136

Table S9. Analysis of Deviance tables from Generalized Linear Models used to detect differences in mortality caused by the indirect effects of elevated CO_2, infection by *Ophryocystis elektroscirrha*, and the species of milkweed monarch larvae were fed.

	GLM of Control Larval Mortality			**GLM of Filament Assay Larval Mortality**		
	χ^2	Df	Pr(>χ^2)	χ^2	Df	Pr(>χ^2)
CO_2	1.3619	1	0.24321	0.4116	1	0.52118
species	**4.2398**	**1**	**0.03949***	0.4116	1	0.52118
infection	1.4119	1	0.23473	**4.7229**	**1**	**0.02976***
CO_2*species	1.5371	1	0.21505	0.1137	1	0.73599
CO_2*infection	1.0244	1	0.31149	0.0844	1	0.77148
species*infection	2.4052	1	0.12093	**4.8598**	**1**	**0.02749***
CO_2*species*infection	0	1	0.99997	0.0769	1	0.78155

Table S10. Summary of how results support or refute predictions made in the main text.

	Prediction	***Supported***	***Results***	***Associated Figures***
1	*Medicinal A. curassavica would suppress the expression of endogenous immunity more than would non-medicinal A. incarnata*	Partially	*The melanization response of late-instar larvae was reduced on medicinal milkweed in comparison to non-medicinal milkweed. However, effects were weak and not present in the early-instar measures of immunity.*	Figure 1
2	*eCO_2 would reduce the sources of exogenous immunity (cardenolides and phytochemical diversity) provided by milkweed*	Yes	*When grown under eCO_2, milkweed plants exhibited lower cardenolide concentrations, lower phytochemical diversity, and lower nutritional quality (higher C:N ratios).*	Figure 2 Figure 3
3	*Reductions in exogenous immunity under eCO_2 would stimulate compensatory endogenous immunity in those monarchs feeding on A. curassavica while infected with the parasite.*	Partially	*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. However, the reduction in immunity under aCO_2 did not depend on the species of milkweed on which monarchs were reared. This may suggest the importance of a loss in plant nutritional quality as a driving factor of immune strength under eCO_2.*	Figure 4 Figure 5 Figure 6

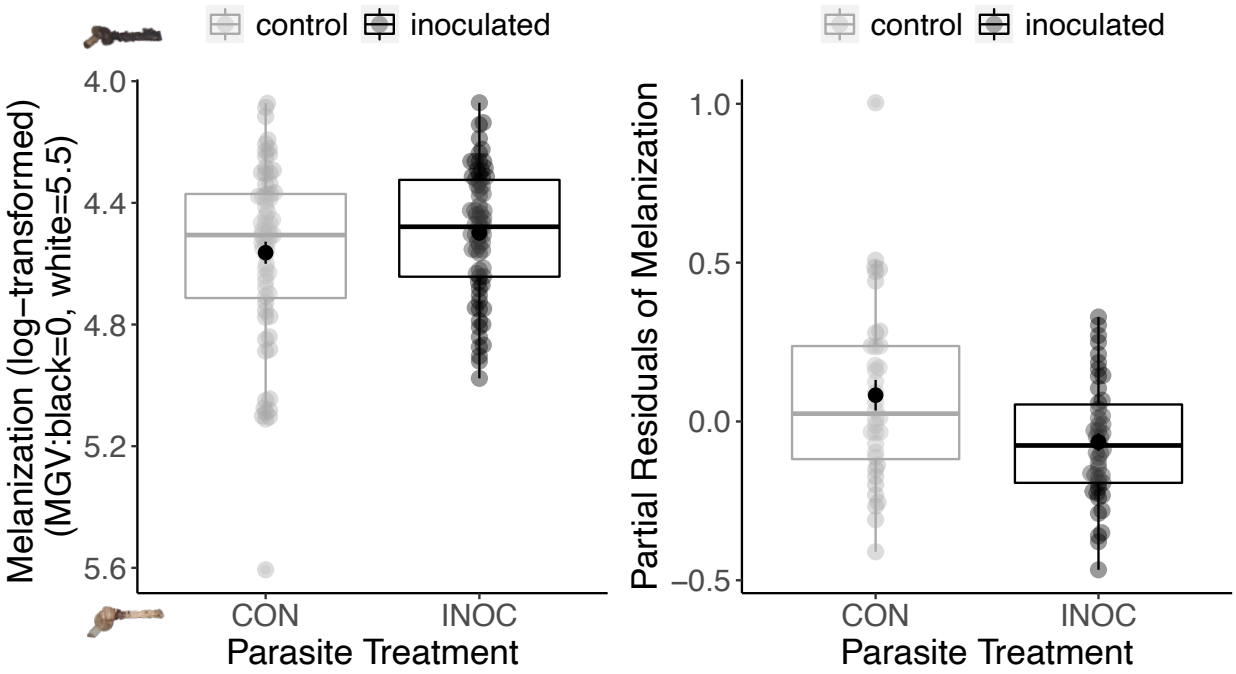

Figure S2. Across all milkweed species, when cardenolides (log-transformed) were included in a model predicting the melanization response of monarchs, infection by *Ophryocystis elektroscirrha* induced a 10% stronger melanization response (Table S5). In a) we present the transformed values of melanization and in b) we present a conditional plot where partial residuals from the model are plotted against the focal predictor while controlling for the other predictors. Note the flipped y-axis in a); higher Mean Gray Values (MGV) represent lighter pigmented filaments or lower melanization responses. Black points and lines represent infected monarchs and light gray are control. Box plot hinges represent first and third quartiles, error bars 95% confidence intervals, and bars median values.

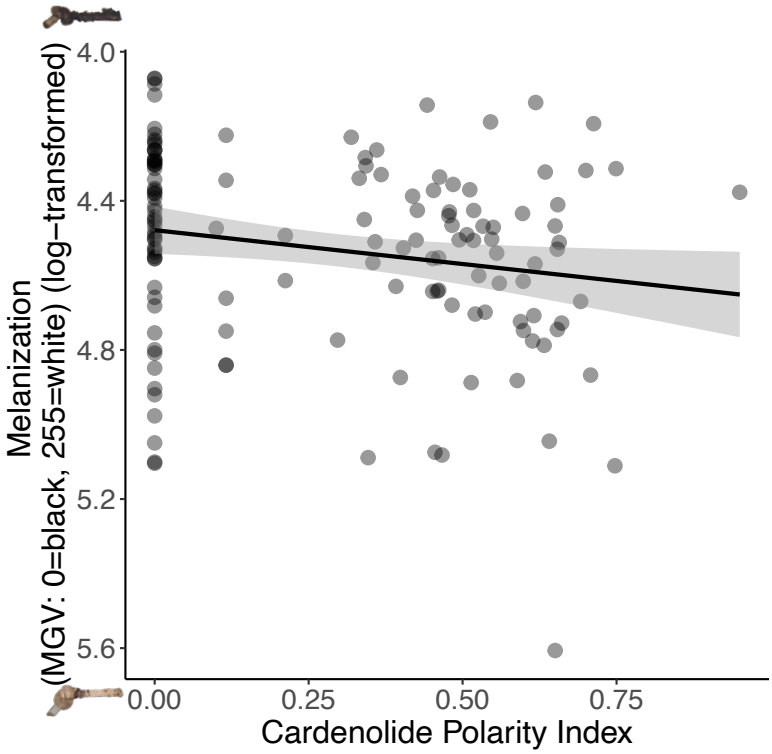

Figure S3. The melanization response of 5th-instar larvae declined with the ingestion of increasingly lipophilic cardenolides (Table S5). Note the flipped y-axis; higher Mean Gray Values (MGV) represent lighter pigmented filaments or lower melanization responses. Shaded band represents 95% confidence intervals around predicted fit line.

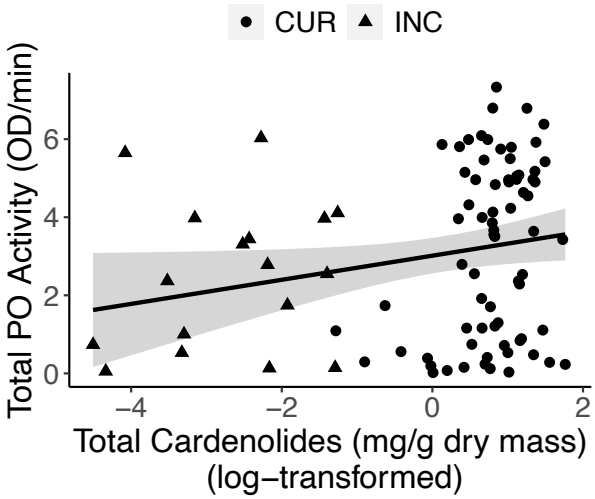

Figure S4. Total PO activity (sum of activated PO and free PO) in monarch hemolymph increased with increasing concentrations of ingested foliar cardenolides (log-transformed) in milkweed hostplants (Table S6). Shaded band represents 95% confidence intervals around predicted fit line.