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12   Elevated atmospheric concentrations of CO$_2$ increase endogenous immune function in a specialist

13   herbivore

14

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32

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, hemocyte 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

63   the loss of exogenous immunity from foliage under eCO_2 results in increased endogenous

64   immune function.

65

66   5.   Animal populations face multiple threats induced by anthropogenic environmental change. Our

67   results suggest that shifts in the balance between exogenous and endogenous sources of

68   immunity to parasite attack may represent an underappreciated consequence of environmental

69   change.

70

71 **Key-words:** *Asclepias*, Cardenolides, *Danaus plexippus,* Ecoimmunology, Hemocytes, *Ophryocystis*

72 *elektroscirrha,* Phenoloxidase.

73 **INTRODUCTION**

74

75 Hosts must defend themselves against attack from parasites while embedded within dynamic

76 communities and ecosystems. Despite increasing evidence that environmental change alters host-

77 parasite interactions (Altizer, Ostfeld, Johnson, Kutz, & Harvell, 2013), the primary mechanisms

78 underlying disease responses remain unresolved. Ecoimmunology is a burgeoning field that

79 concentrates on the role of environmental context in determining the strength, activity and variability of

80 the host immune response (Brock, Murdock, & Martin, 2014; Lazzaro & Little, 2009). Central to this field

81 is the reality that organisms must balance energetic investment in the immune response with

82 investments in other life history traits (Kraaijeveld, Ferrari, & Godfray, 2002; Schmid-Hempel, 2003). In

83 the context of this balance, any factor that generates further energetic deficits may lead to

84 compromised immune function.

85

86 Anthropogenic environmental change can alter host immunity in a context-dependent manner, yielding

87 variable patterns (Adamo & Lovett, 2011; Gherlenda, Haigh, Moore, Johnson, & Riegler, 2015; Jolles,

88 Beechler, & Dolan, 2015). The current literature on immunity and environmental change focuses heavily

89 on the direct physiological effects of warming and pollutants on host immune enzymatic activity

90 (Martin, Hopkins, Mydlarz, & Rohr, 2010; Richard, Le Bris, Guérard, Lambert, & Paillard, 2015; Wojda,

91 2017). However, environmental change may indirectly alter aspects of the host environment including

92 population density, stress, and diet quality, consequently impacting host immune function (Kraaijeveld

93 et al., 2002; Schmid-Hempel, 2003). Higher temperatures, more variable rainfall, and elevated

94 atmospheric concentrations of carbon dioxide (CO_2) have direct effects on plant physiology, which

95   manifest themselves in plant nutritional quality and defensive chemistry (Robinson, Ryan, & Newman,

96   2012; Zavala, Nabity, & DeLucia, 2013). Thus, organisms that derive energy and nutrition primarily from

97   plants, should experience the indirect effects of global change most acutely.

98

99   Insect herbivores are especially vulnerable to changes in the chemical quality of their food plants

100 (Hunter, 2016; Mattson, 1980), and regularly face challenge from agents of disease. The endogenous

101 immune response of insects targets parasitoids and parasites and can be broadly divided into humoral

102 and cellular immunity (Beckage, 2008; Strand, 2008). Humoral defenses encompass effector molecules

103 such as antimicrobial peptides that act on pathogenic microbes (Kavanagh & Reeves, 2007). In contrast,

104 cellular immunity consists of three phases: phagocytosis, nodule formation, and encapsulation enacted

105 by immune cells known as hemocytes (Kacsoh & Schlenke, 2012; Strand, 2008; Vogelweith, Moret,

106 Monceau, Thiéry, & Moreau, 2016). As hemocytes undergo apoptosis while surrounding antigens during

107 nodulation and encapsulation, prophenoloxidase is activated into phenoloxidase (PO), an enzyme critical

108 to the production of melanin and other cytotoxic molecules (Nigam, Maudlin, Welburn, & Ratcliffe,

109 1997). While insects employ phagocytosis and immune effector molecules against smaller parasites and

110 pathogens, the encapsulation response targets multicellular invaders, such as parasitoid eggs and

111 parasites (Strand, 2008).

112

113 The strength and variability of the immune response of insect herbivores depends on diet quality (Singer

114 *et al.* 2014). Because immune defenses are energetically costly, the ratio of protein to carbohydrate

115 concentrations (nutritional quality) of host food-plants is well-known to influence immunity (Cotter,

116 Simpson, Raubenheimer, & Wilson, 2011; Srygley, Lorch, Simpson, & Sword, 2009). The concentration of

117 plant secondary metabolites (PSMs) within the host diet can also alter immune function (Smilanich,

118 Dyer, Chambers, & Bowers, 2009; Smilanich, Vargas, Dyer, & Bowers, 2011; Trowbridge, Bowers, &

119 Monson, 2016). Certain concentrations and combinations of plant toxins may reduce insect immune

120 function (Lampert & Bowers, 2015), while others may strengthen immune induction (Lampert, 2012;

121 Ojala, Julkunen-Tiitto, Lindstrom, & Mappes, 2005). Given the large diversity of PSMs, and the many

122 modes of their chemical action on insect performance, it is still unclear how plant secondary metabolism

123 and nutritional quality combine to influence insect immunity.

124

125 In addition to influencing the strength of endogenous immunity, PSMs can serve as a potent source of

126 exogenous immunity by serving as medicines (de Roode, Lefèvre, & Hunter, 2013; Huffman & Seifu,

127   1989; Singer et al., 2014). As a consequence, the coevolutionary relationships between herbivores, their

128   host plants, and their natural enemies may ultimately determine the relative effects of PSMs on

129   endogenous and exogenous immunity (Hunter, 2016). The monarch butterfly, *Danaus plexippus*, is a

130   specialist insect herbivore known to utilize the secondary chemistry of its host plants, *Asclepias,* as a

131   defense against infection by a virulent, protozoan parasite, *Ophryocystis elektroscirrha* (Barriga,

132   Sternberg, Lefèvre, de Roode, & Altizer, 2016; Lefèvre et al., 2012, Appendix A). Monarchs become

133   infected with *O. elektroscirrha* after ingesting parasite spores on the surface of egg chorea and

134   milkweed (*Asclepias*) tissues (Altizer & Oberhauser, 1999). Spores lyse within the larval gut, sporozoites

135   penetrate the larval hypoderm and replicate over the course of the monarch's development

136   (Mclaughlin, Myers, Diw, Sem, & College, 1970). Infected adult monarchs emerge covered in dormant

137   parasite spores and experience reduced pre-adult survival, as well as reduced adult lifespan, fecundity,

138   and flight ability (Altizer & Oberhauser, 1999; Bradley & Altizer, 2005; de Roode, Yates, & Altizer, 2008).

139   Critically, certain milkweed species with high concentrations of toxic steroids known as cardenolides

140   reduce *O. elektroscirrha* infection probability, growth rate, and virulence (de Roode, Pedersen, Hunter,

141   & Altizer, 2008; Gowler, Leon, Hunter, & de Roode, 2015; Tao, Hoang, Hunter, & de Roode, 2016).

142   Feeding on high-cardenolide (hereafter medicinal) milkweed also ameliorates the fitness costs of

143   harboring each additional parasite, a form of defense known as host tolerance (Sternberg et al., 2012).

144   In short, milkweed cardenolides are a potent source of exogenous immunity for monarchs.

145

146   In addition to its role in exogenous immunity, one recent study has investigated how milkweed

147   phytochemistry influences monarch endogenous immunity. In this study, monarch larvae were

148   inoculated with parasites, and whole bodies and guts were dissected 24 hours later. Surprisingly, within

149   this timeframe infection by *O. elektroscirrha* did not upregulate typical immune genes, whereas feeding

150   on medicinal milkweed actually down-regulated a handful of canonical immune genes and significantly

151   altered the expression of several detoxification genes (Tan et al., 2019). Among the detoxification genes

152   upregulated by the medicinal milkweed were a glutathione S-transferase (DPOGS210488) and a carboxyl

153   esterase (DPOGS204275). The downregulation of immune genes in response to feeding on a medicinal

154   milkweed species regardless of infection status, suggests that coevolution between monarchs, milkweed

155   and *O. elektroscirrha* has reduced monarch reliance on endogenous immunity, and perhaps, favored

156   cardenolides as an exogenous functional replacement for endogenous immune activity (Smilanich &

157   Nuss, 2019).

158

159 Milkweed phytochemical diversity derives both from the variety of milkweed secondary metabolites in

160 tissues and their plasticity under environmental variation. For example, beyond cardenolides, multiple

161 pregnane glycosides and phenolics have been detected in the foliar and root tissues of *Asclepias*

162 *incarnata*, a low cardenolide milkweed species naturally occurring in North America (Sikorska, 2003;

163 Warashina & Noro, 2000). Additionally, both *A. incarnata* and *A. curassavica* produce complex mixtures

164 of flavonoid compounds, including flavanol glycosides, with strong antioxidant properties (Haribal &

165 Renwick, 1996). Phytochemical diversity is profoundly sensitive to environmental variation (Hunter,

166 2016), including that introduced by ongoing environmental change (Robinson et al., 2012; Zavala et al.,

167 2013). As a result, the value of secondary metabolites as sources of exogenous immunity may be

168 compromised in a changing world. Monarch caterpillars feeding on the same medicinal milkweed

169 species described above lose tolerance to their parasite when those milkweeds are grown under

170 elevated (eCO$_2$). Reductions in monarch tolerance under eCO$_2$ are associated with decreases in the

171 production of certain lipophilic, medicinally active cardenolides in their milkweed hostplants (Decker, de

172 Roode, & Hunter, 2018). However, whether the holistic phytochemical changes induced under eCO$_2$

173 influence monarch endogenous immunity remains unknown.

174

175 In this study, we examine how eCO$_2$ alters monarch immune function through changes in the medicinal

176 (secondary chemistry) and nutritional (carbon and nitrogen) quality of milkweed. We fed monarchs

177 foliage from two species of milkweed, *A. curassavica* (medicinal) and *A. incarnata* (non-medicinal),

178 grown under ambient or elevated concentrations of CO$_2$. Larvae were infected with either *O.*

179 *elektroscirrha* or left as uninfected controls. We then measured aspects of the monarch humoral (PO

180 activity) and cellular (hemocyte concentrations and types) immune response, along with host-plant

181 secondary metabolites and nutritional quality, to understand the mechanisms underlying changes in

182 monarch immunity under future environmental conditions. We predicted that (1) medicinal *A.*

183 *curassavica* would suppress the expression of endogenous immunity more than would non-medicinal *A.*

184 *incarnata*, (2) eCO$_2$ would reduce the sources of exogenous immunity (cardenolides) provided by

185 milkweeds*,* and (3) reductions in exogenous immunity under eCO$_2$ would stimulate compensatory

186 endogenous immunity in those monarchs feeding on *A. curassavica* while infected with the parasite.

187

188 **MATERIALS AND METHODS**

189

190   We performed a fully factorial manipulation with milkweed species (*A. incarnata* and *A. curassavica*),

191   CO_2 level (ambient or elevated), and *O. elektroscirrha* (infected or uninfected) as treatments. We then

192   ran (i) immune assays, measuring PO activity and hemocyte counts of early-instar monarch larvae, and

193   (ii) filament assays, measuring melanization of late-instar larvae. A third group of caterpillars were

194   reared to adulthood as assay controls (Table S1, Appendix B).

195

196   ***Milkweed Sources and Growing Conditions***

197   Seeds of both milkweed species were obtained from commercial vendors (*A. curassavica*: Victory Seed,

198   OR and *A. incarnata*: Lupine Gardens, WI). Seeds were surface sterilized using a 5% bleach solution and

199   only *A. incarnata* seeds were cold stratified for six weeks prior to planting. Seedlings were germinated

200   on moist, sterilized paper towels and planted on 1-June-2017 in deepots ™ containing Metromix 360

201   (SunGro Horticulture, Vancouver, BC) and Osmocote 16:16:16 controlled release fertilizer (ICL Specialty

202   Fertilizers, Dublin, OH). We grew and watered seedlings daily in the greenhouse at the University of

203   Michigan Biological Station (UMBS, 45.5587° N, 84.6776° W) for two weeks before transferring them

204   outside into the CO_2 array.

205

206   On May 28th, 2017 we distributed potted plants randomly into 40 open-top controlled atmosphere

207   chambers in the field at UMBS. The CO_2 array was comprised of 20 chambers maintained at aCO_2 (410

208   ppm) and 20 at eCO_2 (810 ppm) from dawn until dusk (Drake, Leadley, Arp, Nassiry, & Curtis, 1989).

209   Within the current century, the concentration of atmospheric CO_2 is anticipated to exceed 700 ppm

210   (IPCC 2013), therefore, we chose a 810 ppm as our target concentration to simulate a near doubling. We

211   monitored CO_2 concentrations in all 20 eCO_2 chambers and one aCO_2 chamber during daylight hours

212   using a LI-COR 320 IRGA (LI-COR, Lincoln, NE, USA). We recorded air temperatures within the chambers

213   using iButton dataloggers (IbuttonLink, Whitewater, WI, USA). Chamber air temperatures averaged

214   20.81 (± 0.05) ºC in eCO_2 and 20.80 (± 0.05) ºC inside aCO_2 chambers, well within temperatures

215   experienced by monarchs in eastern North America (Faldyn, Hunter, & Elderd, 2018).

216

217   We grew three plants of each treatment group (2 milkweed species x 2 parasite treatments x 3 assay

218   groups = 12 treatments) within each chamber, for 36 plants/chamber. We planned to rear one

219   caterpillar per treatment group on the three plants from each chamber (20 replicate larvae per

220   treatment), however final replicate numbers were variable due to larval mortality (Table S1, Appendix

221   C). We began excising foliage for assays after approximately one month of growth in the CO_2 array (27-

222   June-2017). Cuttings were placed in 710 mL plastic containers containing one monarch.

223

224   ***Monarch Sources and Rearing Methods***

225   Monarchs were the grand-offspring of lab-reared butterflies collected from St. Marks, FL and Lawrence,

226   KS. We distributed monarchs from five full-sib family lines evenly across treatments. A darkened

227   monarch egg (indicates close proximity to hatching) was assigned randomly and attached to the surface

228   of each leaf cutting. Three days after neonates hatched on their plant cuttings, we began the inoculation

229   process. Each larva was transferred to a petri dish containing a 95 cm^2 piece of moist filter paper and a

230   70.6 mm^2 leaf disk taken from the larva's assigned host plant, cleaned with a 5% bleach solution and

231   rinsed thoroughly with water. For those larvae designated as inoculated, we placed 10 parasite spores

232   on the surface of the leaf disk, while uninoculated larvae received spore-free leaf disks. Immediately

233   after the leaf disk was taken from the plant for inoculation, we collected foliage for chemical analyses

234   (Appendix D). The petri dishes containing larvae and leaf disks were kept in an incubator maintained at

235   26°C with 16-hour daylight. Upon consuming the entire leaf disk (all spores), larvae were returned to

236   their cleaned containers with new plant cuttings. We continued to feed monarchs in the control

237   (Appendix B) and filament treatments *ad libitum* until pupation or until filament insertion (see below),

238   replacing foliage and cleaning each container every 2-3 days. Monarchs designated for the immune

239   assays (see below) were sacrificed 48 hours following inoculation to determine the initial early-instar

240   immune response to *O. elektroscirrha* infection. We chose this period to ensure that all larvae had

241   completed inoculation and had adequate time to mount an immune response (Beckage, 2008).

242

243   ***Foliar Chemical Analyses***

244   At the time of parasite inoculations (above), 6 leaf disks from each monarch's assigned plant were

245   punched into 1 mL of methanol and stored at -10°C for cardenolide extraction (*see detailed description*

246   *in* Appendix D; Tao & Hunter 2012). Another 6 disks were taken, dried and weighed to estimate sample

247   dry mass of the disks collected for chemical analysis.

248

249   We calculated two metrics of cardenolide chemistry from our milkweed foliar samples; total cardenolide

250   concentration and cardenolide polarity. Total cardenolide concentrations were calculated as the sums of

251   the individual cardenolide peak areas separated by UPLC corrected by the concentration of an internal

252   digitoxin standard and normalized by the dry sample mass. The biological activity of cardenolides is

253   determined, in part, by the polarity of the different sugar moieties attached to the steroid skeleton of

254   the compound (Agrawal, Petschenka, Bingham, Weber, & Rasmann, 2012). Because animal cell

255   membranes are outwardly hydrophobic, the most lipophilic (nonpolar) cardenolides are thought to be

256   the most toxic (Sternberg et al., 2012; Tao et al., 2016). We calculated an index of cardenolide polarity

257   for each sample as: Polarity $= \mathrm{sum}(P_i\, RT_i)$, where P_i is the area and RT_i is the retention time of the ith

258   cardenolide peak, following Rasmann & Agrawal (2011).

259

260   At the same time that we removed leaf disks for cardenolide analysis, we harvested three leaves for

261   NMR sampling. We processed the NMR spectral data using MestReNova software (Mestrelab Research)

262   and aligned sample spectra using the solvent peak. Sample spectra were then baseline-corrected, phase-

263   corrected, and normalized to the total area of 100, and binned every 0.04 ppm from 0.5 to 14 ppm. As

264   an estimate of whole-plant chemical diversity, we calculated the Simpson diversity index ($D = 1 - \sum$

265     $(n/N)^2$) of chemical shifts (approximations of secondary metabolites) where n is the integral of a specific

266   binned frequency range, and N is the total number of binned frequency ranges measured in the sample

267   (Richards et al., 2015).

268

269   Remaining dried foliar tissue was ground to a fine powder and then analyzed using a TruMac CN

270   Analyzer (Leco Corporation, St. Joseph, MI) to provide estimates of foliar carbon (C) and nitrogen (N)

271   concentrations. Examining the foliar C:N ratio is a simple approximation of the nutritional quality of

272   many plants (Mattson, 1980), including milkweed (Tao, Ahmad, de Roode, & Hunter, 2015).

273

274   ***Immune Assays***

275   In early-instar larvae, we performed assays to measure standing and activated phenoloxidase (PO)

276   activity and hemocyte concentration and identity. Using a colorimetric assay we determined the activity

277   of free, naturally active PO (standing PO activity) and the total PO activity (standing PO + activated

278   prophenoloxidase, activated PO) in monarch hemolymph (Adamo, 2004; Dhinaut, Chogne, & Moret,

279   2018; Smilanich et al., 2017). To extract hemolymph, we made incisions in the larval cuticle above the

280   final proleg in the A6 abdominal segment using a hand-pulled Pasteur pipette needle (Smilanich et al.,

281   2017, 2009). With a micropipette, we took 2 µL of hemolyph from each larva and deposited it into 50 µL

282   of chilled phosphate-buffered saline (PBS) solution in a 1.0 mL Eppendorf tube and vortexed the

283   mixture. We then prepared two separate reactions of the hemolymph extract in order to detect

284   standing PO activity and total PO activity after activating the inactive prophenoloxidase dimer using

285   Cetylpyridinium chloride monohydrate (CPC). To each well of a 96-well plate, one 50 µL aliquot of the

286   hemolymph-PBS mixture was added along with 300 µL of L-DOPA (g L-DOPA in mL deionized water). We

287   incubated the plate for 20 minutes at room temperature. To the second designated well of each sample,

288   we also added 17 µL of 10% CPC to activate prophenoloxidase present in the hemolymph during the

289   incubation step. Using an ELx800 Absorbance Microplate Reader (BioTek, VT) we measured absorbance

290   of the samples at 490 nm every 30 seconds for 180 minutes. In our analyses, we used the slope of the

291   linear portion of the absorbance curve (30-106 minutes) as our measure of standing PO and total PO

292   activity. We calculated the activity of activated PO by subtracting standing PO from total PO activity.

293

294   Circulating hemocytes aid in the recognition and phagocytosis of microbial parasites, and encapsulation

295   of parasitoids. Monarchs typically produce four differentiated hemocyte types: phagocytic granulocytes,

296   capsule-forming plasmocytes, oenocytoids that contain components of the PO cascade, and spherule

297   cells that potentially contain cuticular components (Strand, 2008). The density and frequency of

298   different hemocytes can indicate insect melanization and encapsulation ability (Kacsoh & Schlenke,

299   2012). To identify and count hemocytes, we took an additional 4 µL of hemolymph and added it to 8 µL

300   of chilled anticoagulant solution (0.684 g EDTA, 0.346 g citric acid dissolved in 180 mL PBS, pH 7.4).

301   Within 24 hours, we performed counts using a Neubauer Bright-Line hemocytometer (Cambridge

302   Instruments, Inc.) and 10 µL of the sample. We counted the total number of hemocytes present in the

303   entire central gridded area and recorded the different hemocyte types present in the hemolymph

304   following the descriptions of Strand (2008) and Vogelweith *et al.* (2016). Hemocyte categorizations and

305   counts were all performed randomly and blindly by the same individual.

306

307   ***Filament Assay to Determine Monarch Melanization Response***

308   To measure the immune defense of 5th instar larvae designated for the Filament treatment, we inserted

309   an artificial antigen into monarchs following Klemola *et al.* (2008). Our simulated antigens were 2 mm

310   long pieces of nylon, which we rubbed with sandpaper, knotted at one end (for ease of handling),

311   sterilized with 100% ethanol, and dried before inserting into larvae. Similar to the hemolymph extraction

312   protocol, we made a small incision into the larval cuticle just above the final proleg. We then inserted

313   the filament into the larval haemocoel parallel to the abdomen, taking care not to perforate the midgut

314   or hindgut. Larvae were returned to their cleaned containers and allowed 24 hours to mount an immune

315   response. We removed the implanted filaments using forceps, deposited the filaments into a 70%

316   ethanol solution and stored samples at – 20ºC for three months.

317

318 To quantify filament melanization we photographed filaments under a dissecting microscope using an

319 iPhone 6s (Apple Inc., Cupertino, CA, USA) with an iDu LabCam Microscope Adapter (iDu, Detroit, MI,

320 USA) in a dark room. We calibrated Adobe Photoshop to calculate distance measures based on a pixel-

321 to-millimeter ratio. We then quantified the mean gray value (MGV, 0 = black to 255 = white) of a roughly

322 0.500 mm^2 rectangle selected from the tip of the filament that was directly inserted into the insect (*see*

323 *pictures on y-axis of* Fig. 1).

324

325 ***Statistical Analyses***

326 For all analyses, we used linear mixed models (LMMs; lme4 package) always starting with models that

327 included chamber identity, date, and monarch genotype (when appropriate) as random effects. Random

328 effects were removed when model fits were singular due to complex random effects structures. We

329 performed model selection using the anova function, fitting linear mixed models with maximum

330 likelihood. We implemented all statistical tests in R version 3.3.2 (R Core Team, 2019), all variables were

331 transformed to best achieve normality of error using the Shapiro-Wilk test and examined visually. Model

332 fits were visually inspected using qqplots and homogeneity of variance was evaluated by plotting

333 residuals against fitted values (Zuur, Ieno, & Elphick, 2010). In instances of multiple comparison, we

334 performed post-hoc tests among least-squared means using the emmeans package (Lenth 2020).

335

336 We assessed the melanization response of late-instar monarchs as a measure of Mean Gray Value

337 (MGV) over a standardized area of filament by running an LMM with CO_2 treatment, infection by *O.*

338 *elektroscirrha*, milkweed species, and their interactions as fixed effects. Melanization values were

339 natural log- transformed.

340

341 We assessed the effects of milkweed species and CO_2 treatment on a) total foliar cardenolide

342 concentration (log-transformed), b) cardenolide polarity, c) whole-plant secondary metabolite diversity

343 detected with H^1-NMR, and d) foliar C:N ratio (log-transformed) using LMMs.

344

345 To investigate the effects of CO_2 treatment, infection by *O. elektroscirrha*, and milkweed species on the

346 PO activity of larvae, we ran LMMs with the three treatments and their interactions as fixed effects and

347 the activity of a) total PO, b) activated PO , and c) standing PO as response variables. Standing PO did

348 not respond to any of our treatments (Table S2) and is, therefore, not reported here. We used LMMs to

349   assess the effects of our treatments on a) total hemocyte concentration (log-transformed), b)

350   granulocyte (log-transformed), c) oenocytoid (log-transformed), d) spherule cell (square-root

351   transformed), and e) plasmocyte (log-transformed) concentrations. In each of these LMMs, CO_2

352   treatment, infection by *O. elektroscirrha*, milkweed species, and their interactions were fixed effects.

353

354   To explore the effects of foliar chemical traits on monarch immunity we used LMMs with all measured

355   immunological traits as response variables and our four foliar traits (above) as fixed effects. To avoid

356   detecting spurious correlations that result from differences between plant species, we originally

357   included milkweed species as a fixed effect in these models. However, the species term did not improve

358   model fit, and was removed. We report only those models that showed significant effects of foliar

359   quality on immune function.

360

361

362   **RESULTS**

363

364   ***"Medicinal" milkweed inhibited the melanization response of late-instar monarch larvae***

365   In partial support of our first prediction that medicinal *A. curassavica* would reduce the expression of

366   endogenous immunity, the melanization response around a sterile filament (simulated antigen) was 13%

367   lower (-0.39±0.19 effect size) in monarch larvae feeding on *A. curassavica* than on *A. incarnata* (species:

368    $F_{1, 133}$ = 5.84, p = 0.017, Fig. 1, Table S2). Monarch genotype accounted for 0.002 ±0.04 of the variance in

369   the melanization response. In other words, feeding on a milkweed species that provided high exogenous

370   immunity against parasites reduced modestly the strength of one aspect of endogenous immune

371   defense against parasitoids.

372

373   ***Foliar chemical defenses and nutritional quality declined under elevated CO_2***

374   In support of our second prediction that eCO_2 would reduce the sources of exogenous immunity, eCO_2

375   induced a 23% reduction in foliar cardenolide concentrations (quantified with UPLC-UV detection) in *A.*

376   *curassavica* and a 30% reduction in *A. incarnata* (CO_2: $F_{1,98}$ = 7.88, p = 0.006, Fig. 2a, Table S4)*.* Because

377   eCO_2 induced reductions of similar magnitude in both species, there was no interaction between

378   milkweed species and CO_2 treatment on cardenolide production (species* CO_2: $F_{1,265}$ = 1.26, p = 0.2632).

379   The mean polarity index of cardenolides produced by *A. curassavica* was twice that of *A. incarnata*

380   (species: $F_{1,264}$ = 104.40, p < 0.0001, Fig. 2b); a high polarity index indicates an abundance of lipophilic

381   cardenolides. However, CO_2 treatment had no effect on the mean polarity value of cardenolides (CO_2:

382    $F_{1,94}$ = 2.79, p = 0.0982, Fig. 2b) and caused no significant interaction (species* CO_2: $F_{1,263}$ = 2.48, p =

383   0.1166).

384

385   Using H^1-NMR, we estimated the holistic diversity of secondary metabolite structural features within the

386   milkweed plants using Simpson's diversity index of binned chemical shift values. The diversity of

387   metabolites declined in both species of milkweed under eCO_2 (CO_2: $F_{1,38}$ = 6.93, p = 0.0122, Fig. 2c, Table

388   S4) but was higher in *A. incarnata* (species: $F_{1,435}$ = 13.21, p = 0.0003, Fig. 2c). Qualitative structural

389   analysis of crude ^{1}H-NMR spectra revealed that *A. incarnata* contained a much higher proportion of

390   flavonoids (diagnostic ^{1}H-NMR resonances 6.5-7.8 ppm) to steroidal glycosides, steroidal compounds

391   (0.5-3.0 ppm), and glycosides (3.5-4.2 ppm, Fig. 3), resulting in greater interclass phytochemical diversity

392   when compared to *A. curassavica*. As with cardenolide concentrations, eCO_2 caused similar declines in

393   the chemical diversity of both milkweed species (species* CO_2: $F_{1,435}$ = 1.36, p = 0.2437, Fig. 2c). Losses of

394   cardenolide structures, revealed by ^{1}H-NMR spectra (diagnostic methyl doublet resonances at 1.1-1.2

395   ppm) and UPLC-UV detection, likely contributed to reductions of interclass phytochemical diversity

396   captured by the Simpson index (Fig. 3).

397

398   Consistent with two decades of research on CO_2, the nutritional quality of milkweed foliage declined

399   under eCO_2 (CO_2: $F_{1,38}$ = 75.11, p < 0.0001, Fig. 2d, Table S4). Specifically, the foliar C:N ratio increased by

400   29% in *A. curassavica* and by 38% in *A. incarnata*. Across both CO_2 treatments, *A. incarnata* had 22%

401   higher foliar C:N ratios than *A. curassavica* (species: $F_{1,437}$ = 41.34, p < 0.0001, Fig. 4d), but there was no

402   species-specific response of the foliar C:N ratio to CO_2 treatment (species* CO_2: $F_{1,437}$ = 0.10, p = 0.7571,

403   Fig. 2d).

404

405   ***Foliage from plants grown under eCO₂ increased the relative strength of monarch endogenous***

406   ***immunity***

407   In partial support of our third prediction that reductions in exogenous immunity (medicinal

408   phytochemical protection) under eCO_2 would stimulate compensatory endogenous immunity, certain

409   monarch immune responses to infection were higher in larvae reared on foliage from eCO_2 plants and

410   lower in larvae reared on aCO_2 plants (Fig. 4). Specifically, larvae challenged with the parasite and reared

411   on eCO_2 plants exhibited 43% higher activated PO activity (infection* CO_2: $F_{1,\ 137}$ = 9.46, p = 0.0003, Fig.

412   4b, Table S2), and 30% higher total hemocyte concentrations (infection* CO_2: $F_{1,108}$ = 4.97, p = 0.028, Fig.

413   4c, Table S3) than did infected larvae reared on aCO_2 plants. The concentration of one phagocytic

414   hemocyte type, granulocytes (phagocytic cells), was 7.5% higher in larvae challenged with the parasite

415   and fed eCO_2 plants (infection*CO_2: $F_{1,95}$ = 4.12, p = 0.0452, Fig. 4d, Table S3). Further, feeding on eCO_2

416   plants released Total PO activity from a 25% immune suppression experienced by infected larvae under

417   ambient conditions (infection*CO_2: $F_{1, 137}$ = 5.93, p = 0.016, Fig. 4a, Table S2).

418

419   In contrast to this general pattern and our predictions, the concentration of oenocytoids circulating in

420   monarch hemolymph was highest in infected larvae feeding on *A. curassavica* grown under aCO_2

421   (infection*milkweed species*CO_2: $F_{1,54}$ = 4.60, p = 0.036, Fig. 5a, Table S3). Across parasite treatments,

422   mean oenocytoid concentrations in monarchs fed *A. curassavica* were 33% lower under eCO_2

423   (species*CO_2: $F_{1,54}$ = 3.99, p = 0.051, Fig. 5b, Table S3). Oenocytoids are much rarer in lepidopteran

424   hemolymph but are thought to be directly involved in PO production (Altizer & de Roode, 2015; Strand,

425   2008). Monarchs fed *A. curassavica* produced 48% more oenocytoids than those fed *A. incarnata*

426   (species: $F_{1,54}$ = 8.15, p = 0.006, Table S3).

427

428   Based on Prediction 1, and the melanization responses reported for late-instar larvae (above), we

429   expected that our indices of endogenous immunity would be higher on larvae fed the non-medicinal *A.*

430   *incarnata* than the medicinal *A. curassavica*; this was generally not the case. There were no main or

431   interactive effects of milkweed species on total PO activity, activated PO activity, hemocyte

432   concentration, or granulocyte concentration (Table S2 & S3). However, in partial support of Prediction 1

433   that *A. curassavica* would suppress the expression of endogenous immunity more than would non-

434   medicinal, *A. incarnata*, the concentration of spherule cells (which contain cuticular components for

435   clotting) responded slightly more to infection in larvae fed *A. incarnata* than in larvae fed *A. curassavica*

436   (infection*milkweed species: $F_{1,78}$ = 3.57, p = 0.063, Fig. 3c, Table S3). Additionally, across infection and

437   milkweed species, eCO_2 induced a 60% increase in the concentration of spherule cells in monarchs (CO_2:

438    $F_{1,78}$ = 5.15, p = 0.026, Fig. 3d), consistent with a relative increase in endogenous immunity under eCO_2.

439

440   ***Larval immune responses to parasite infection correlated with measures of phytochemistry***

441   In late-instar larvae, the melanization response of uninfected monarchs correlated negatively with foliar

442   cardenolide polarity whereas the melanization response of infected larvae did not correlate with

443   cardenolide polarity (infection*cardenolide polarity: $F_{1,132}$ = 5.90, p = 0.017, Fig. 6a, Table S5). When

444   cardenolides were included in the model predicting the melanization response of monarchs, we

445   detected a 10% stronger melanization response induced by *Ophryocystis elektroscirrha* infection (Table

446   S6, Fig. S2).

447

448   Despite our predictions that monarch immunity would respond to changes in secondary chemistry,

449   measures of in early-instar endogenous immunity correlated most strongly with foliar nutritional quality

450   (C:N ratios). Infected early-instar larvae increased endogenous immunity as foliar nutritional quality

451   declined (Fig. 6b & c, Table S6 & S8). Specifically, the total PO activity and hemocyte concentrations of

452   infected larvae increased as foliar C:N ratios increased (parasite treatment*CN: $F_{1,140}$ = 3.95, p = 0.049;

453    $F_{1,106}$ = 6.29, p = 0.014, respectively, Fig. 6b & c, Table S6 & S8**)**.

454

455

456   **DISCUSSION**

457

458   Here, we demonstrate that specific endogenous immune responses of monarchs to infection with *O.*

459   *elektroscirrha* are generally reduced when larvae feed on foliage grown under ambient CO_2 and

460   enhanced when larvae feed on foliage grown under elevated CO_2. These results are consistent with a

461   change in the relative strengths of endogenous and exogenous immunity under elevated concentrations

462   of atmospheric CO_2. Our results build upon previous work in the monarch-parasite-milkweed system and

463   provide two major avenues of insight. First, a recent transcriptomics study demonstrated little to no

464   response of monarch larval gene expression within 24h to infection by *O. elektroscirrha* (Tan et al.,

465   2019). Here, we confirm that this lack of response in immune gene expression translates to immune

466   function suppression when infected larvae are reared on foliage from plants grown under aCO_2. Second,

467   the study by Tan et al. (2019) also reported that a subset of monarch immune genes were

468   downregulated when larvae were reared on the medicinal *A. curassavica*. The authors suggested that

469   this reflects reliance on the well-characterized exogenous immunity to parasites that is provided by *A.*

470   *curassavica* (de Roode, Pedersen, et al., 2008; Sternberg et al., 2012). Here, we report that a loss of

471   medicinal phytochemistry in milkweed plants grown under eCO_2 is reflected in a loss of endogenous

472   immune suppression and the upregulation of immune function in infected larvae. Our results therefore

473   combine with previous work to suggest that coevolution between monarchs, milkweed, and *O.*

474   *elektroscirrha* has reduced monarch reliance on endogenous immunity and that future environmental

475   conditions may change the balance of endogenous and exogenous immune function.

476

477 In our study, humoral and cellular immune responses including PO activity and circulating hemocyte

478 density were suppressed in monarch larvae feeding on their typical diet but increased when larvae

479 consumed foliage from plants grown under eCO_2. Foliage from eCO_2 exhibited lower nutritional quality,

480 lower concentrations of cardenolides (UPLC-UV detected), which are known to provide exogenous

481 immunity (Gowler et al., 2015), and lower holistic phytochemical diversity (^{1}H-NMR detected). Here,

482 total PO activity and hemocyte counts correlated directly with losses in foliar nutritional quality (higher

483 C:N ratios). The costs of mounting endogenous immune responses as nutritional quality declines may

484 explain in part the loss of tolerance by monarchs to OE under eCO_2 reported previously (Decker et al.,

485 2018). However, it is impossible to disentangle the singular roles of secondary chemistry and nutritional

486 quality on this relationship in the current study which only captures the holistic response of hosts to

487 infection under future environmental conditions.

488

489 We predicted that expression of endogenous immunity would be greater in larvae fed the non-medicinal

490 milkweed (*A. incarnata*) than the medicinal milkweed (*A. curassavica*). While that was supported weakly

491 by the melanization response of late-instar larvae (Fig. 1), it was not supported by our measures of PO

492 activity or hemocyte counts of early-instar larvae, which were related to CO_2 concentration rather than

493 plant species. Notably, standing PO was unaffected by any of our treatments, however, CO_2

494 concentration did alter the magnitude of activated PO activity (potential PO activity) which could

495 indicate differences in immune plasticity in response global change. We also did not find simple

496 correlations between foliar cardenolide concentrations and endogenous immune function of early

497 instars. This is in contrast to the negative effects of other secondary metabolites such as iridoid

498 glycosides on the circulating PO activity of other lepidopterans (Smilanich et al., 2017, 2009). Instead,

499 the immune response of infected monarchs was positively correlated with declining foliar nutrient

500 concentrations induced by eCO_2 (Fig. 6b &c). Typically, insect immunity follows the opposite pattern,

501 whereby immune responses decline on diets low in nutrients (Beckage, 2008; Strand, 2008). In some

502 instances, diets low in protein but high in carbohydrates (a condition commonly induced in foliage

503 grown under eCO_2) may promote insect melanization induced by PO activity (Mason, Smilanich, &

504 Singer, 2014). Presumably, a diet high in soluble carbohydrates is easier to metabolize than one

505 consisting of the less digestible peptide bonds. Within other insect systems, a trade-off between lipid

506 digestion and immunity has been illustrated (Adamo, Bartlett, Le, Spencer, & Sullivan, 2010; Adamo,

507 Roberts, Easy, & Ross, 2008). Therefore, infected monarchs may have more readily available energy to

508   invest in their immune response because of reduced energetic requirements for digestion when feeding

509   on foliage with higher C:N ratios.

510

511   Alternatively, the phytochemical changes induced by eCO_2 may have altered the ability of the parasite to

512   evade detection by the monarch host and/or suppress immune activity, two well-documented

513   phenomena of host manipulation by the parasite (*reviewed in* Heil, 2016). Subsequent studies that

514   survey the transcriptomic responses of monarchs to infection under simulated future environmental

515   conditions will improve our understanding of the environmental contingency of these parasitic

516   behaviors. Additionally, these transcriptomic studies may shed light on the complex relationship

517   between detoxification and immunity (Smilanich & Nuss, 2019), that may add further depth to the

518   phytochemical contingency of herbivorous host immunity in a changing world.

519

520   Our results contribute to a growing body of research illustrating costs of secondary metabolite ingestion

521   to the immune response of hosts under parasitoid attack (Hansen, Glassmire, Dyer, Smilanich, &

522   Hansen, 2016; Smilanich et al., 2009). In our study, late-instar monarch larvae reared on high-

523   cardenolide milkweed produced a 13% weaker melanization response against a standardized antigen

524   (Fig. 1). Though statistically significant, this small reduction in melanization could prove less biologically

525   relevant, however we do not know how these reductions in melanization interact with other processes.

526   Although we did not measure sequestration, a large body of literature exists demonstrating that

527   monarchs consuming high-cardenolide milkweed species sequester comparably high concentrations of

528   cardenolides (Agrawal et al., 2012). Thus, our study suggests that the high metabolic demands of

529   consuming and sequestering cardenolides may reduce some endogenous defenses. However, in this

530   case, the "price" of reduced immunity may be worth it if phytochemistry is covering the "costs" of

531   protection from parasite infection.

532

533   Through our qualitative ^{1}H-NMR analysis of milkweed chemotypes, we are able to highlight an additional

534   factor that may contribute to the differences in melanization we observed between monarchs fed the

535   two milkweed species: plant flavonoid diversity. Our structural analyses of crude ^{1}H-NMR spectra

536   confirm that the higher phytochemical diversity reported in *A. incarnata* is due largely to the presence

537   of flavonoid compounds. Critically, the PO cascades that generate encapsulation responses also produce

538   harmful reactive oxygen species that damage surrounding cellular functions but can be neutralized by

539   antioxidant PSMs such as flavonoids (Lampert, 2012). The importance of antioxidant PSMs to herbivore

540 immunity has been demonstrated through artificial diet studies where both specialists and generalist

541 lepidopterans exhibit strengthened immunity in the presence of flavonoids, phenolics, and other

542 antioxidants (Ojala et al., 2005; Smilanich et al., 2009). In the current study, the higher concentrations of

543 flavonoids we detected in *A. incarnata* foliage may, therefore, enhance the melanization response of

544 late-instar monarchs by alleviating the deleterious byproducts of melanization. However, further

545 analysis of metabolomic data as they relate to monarch performance are necessary to support this

546 phytochemical mechanism.

547

548 Monarch butterfly populations currently face multiple threats induced by anthropogenic environmental

549 change (Malcolm, 2017). Here we demonstrate the potential of eCO_2 to compromise their reliance on

550 phytochemistry as a source of exogenous immunity against infection. As a consequence, monarchs

551 induce endogenous sources of immunity that may be energetically costly to produce. Investment in

552 endogenous immunity will likely come at a cost to other important life history traits (Schmid-Hempel,

553 2005) such as growth and reproduction that may ultimately decrease monarch fitness. Our results

554 suggest that shifts in the balance between exogenous and endogenous sources of immunity to parasite

555 attack may represent an underappreciated consequence of environmental change for animals.

556

562

563 **Author Contributions**

564 LED, AMS, JCdR, & MDH designed the experiment; LED, ASP, & KMO collected and processed the data;

565 LED & KMO analyzed the data. LED wrote the manuscript and all authors contributed substantially to

566 drafts and approved the final version.

567

568 **Data Availability Statement**

569 Data are available on Dryad Digital Repository: *https://doi.org/10.5061/dryad.dr7sqv9ww*.

570

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772

Figure Captions

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 examples of dark (highly melanated) and light (un-melanated). Milkweed species codes are as follows: CUR= *A. curassavica* (dark gray) and INC= *A. incarnata* (light gray)*.* 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.

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 gray points and lines represent plants grown under eCO_2 and light gray are those grown under aCO_2. Asterisks indicate significant differences detected with post-hoc tests.

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 hemocyte 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 gray are controls. Asterisks indicate significant differences detected with post-hoc tests.

804   **Figure 5.** The interactive effects of infection by *Ophryocystis elektroscirrha*, milkweed species, and CO_2

805   treatment on a) oenocytoid concentrations. The interaction of b) CO_2 treatment and milkweed species

806   on oenocytoid concentrations, c) infection and milkweed species on spherule cell concentrations, and d)

807   the main effect of CO_2 treatment on spherule cell concentration. Milkweed species codes are as follows:

808   CUR= *A. curassavica* and INC= *A. incarnata*. In a) and b), Black points and lines represent infected

809   monarchs and light gray are control. In d), dark gray points and lines represent plants grown under eCO_2

810   and light gray are those grown under aCO_2. Asterisks indicate significant differences detected with post-

811   hoc tests.

812

813   **Figure 6:** Effects of infection by *Ophryocystis elektroscirrha* on the relationships between a) diet

814   cardenolide polarity index and monarch melanization; b) diet nutritional quality (C:N ratio) and total PO

815   activity; and c) foliar C:N ratio and total hemocyte concentrations. Light gray points and lines represent

816   uninoculated control monarchs and black represents monarchs inoculated with *O. elektroscirrha.* In a),

817   note the flipped y-axis: higher Mean Gray Values (MGV) represent lighter pigmented filaments or lower

818   melanization responses. Shaded bands represent 95% confidence intervals around predicted fit line.

Figure 1.

Figure 2.
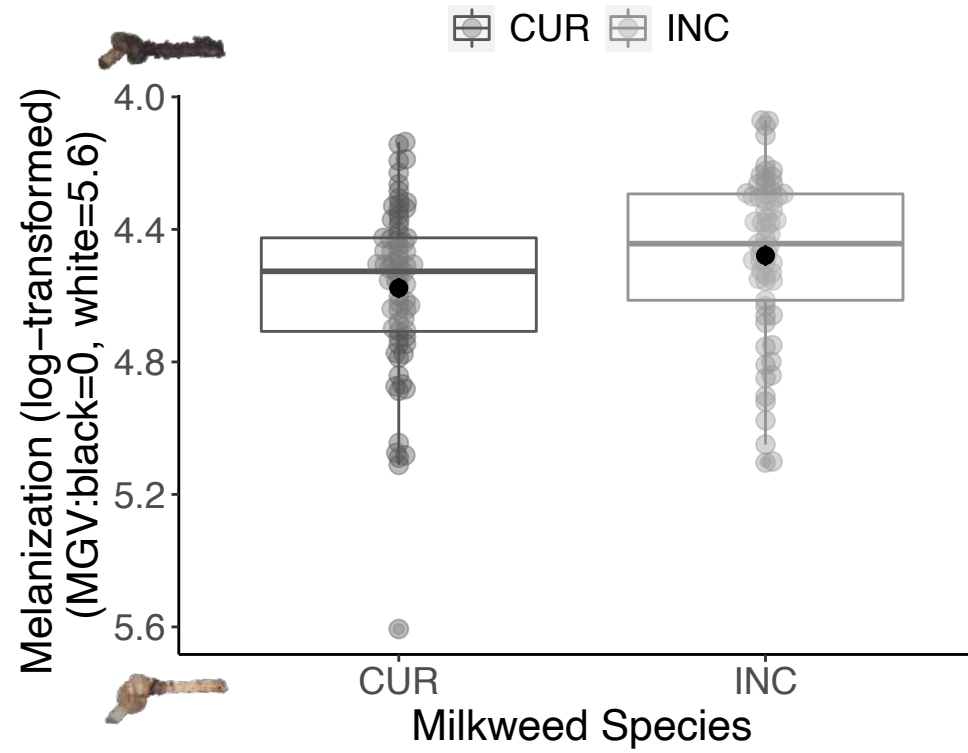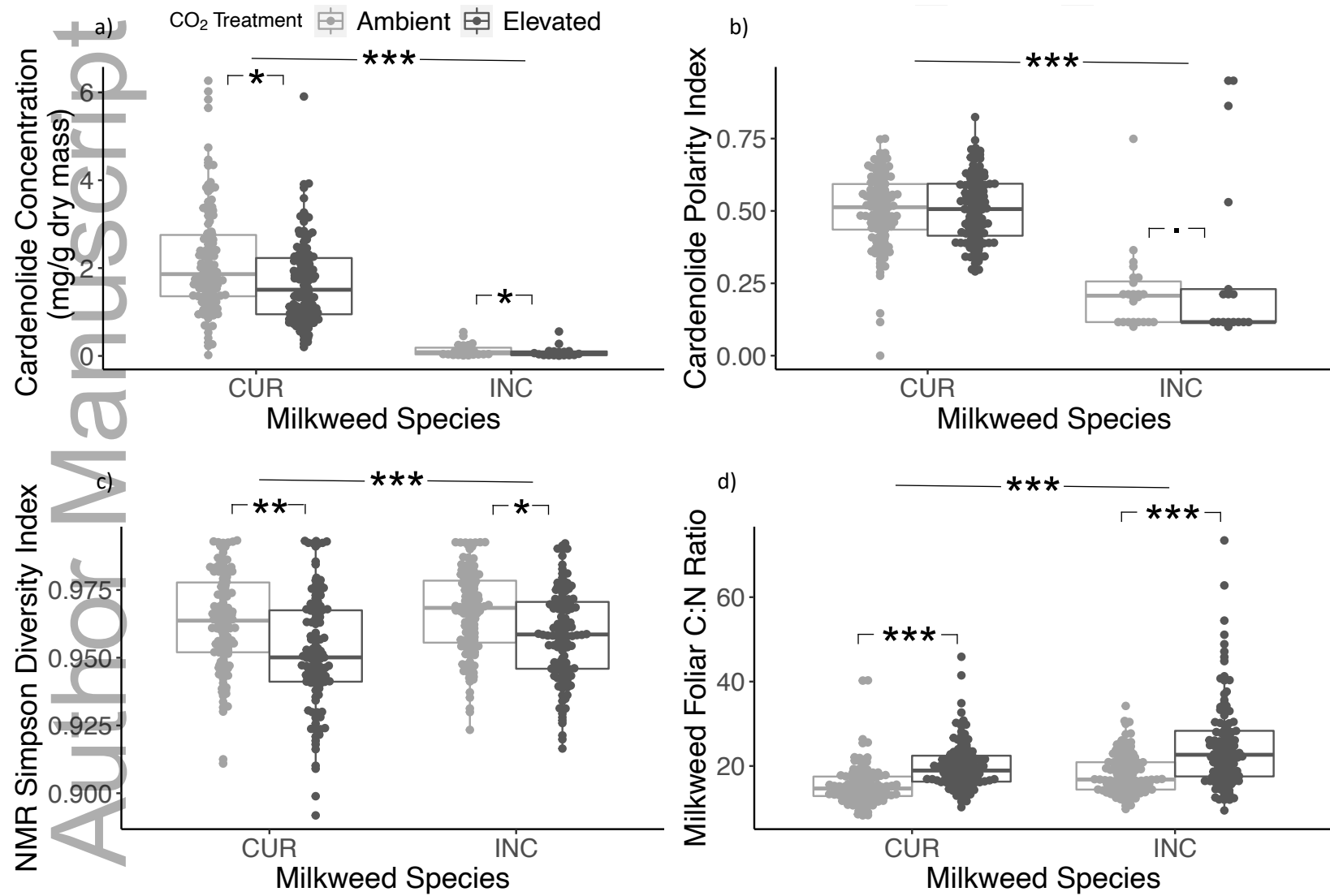
Figure 3.

Figure 4.

a) Total PO Activity (OD/min) vs CO₂ Treatment (Ambient, Elevated) for Uninfected and Infected groups.

b) Activated PO Activity (OD/min) vs CO₂ Treatment (Ambient, Elevated).

c) Hemocyte Concentration (cells/microliter) (log-transform) vs CO₂ Treatment (Ambient, Elevated).

d) Granulocyte Concentration (cells/microliter) (log-transformed) vs CO₂ Treatment (Ambient, Elevated).

Figure 5.