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Phytochemical changes in milkweed induced by elevated CO_2 alter wing morphology but not toxin sequestration in monarch butterflies

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42 **Abstract:**

43 1. Environmental change has the potential to influence trophic interactions by altering the

44   defensive phenotype of prey.

45 2. Here, we examine the effects of a pervasive environmental change driver, elevated

46   atmospheric concentrations of CO_2 (eCO_2), on toxin sequestration and flight morphology

47   of a specialist herbivore.

48 3. We fed monarch butterfly larvae, *Danaus plexippus*, foliage from four milkweed,

49   *Asclepias*, species of varying chemical defense profiles grown under either ambient or

50   eCO_2. We also infected a subset of these herbivores with a protozoan parasite,

51   *Ophryocystis elektroscirrha*, to understand how infection and environmental change

52   combine to alter herbivore defenses. We measured changes in phytochemistry induced by

53   eCO_2 and assessed cardenolide, toxic steroid, sequestration and wing morphology of

54   butterflies.

55 4. Monarchs compensated for lower plant cardenolide concentrations under eCO_2 by

56   increasing cardenolide sequestration rate, maintaining similar cardenolide composition

57   and concentrations in their wings under both CO_2 treatments. We suggest that these

58   increases in sequestration rate are a byproduct of compensatory feeding aimed at

59   maintaining a nutritional target in response to declining dietary quality.

60   5. Monarch wings were more suitable for sustained flight (more elongated) when reared on

61   plants grown under eCO_2 or when reared on *A. syriaca* or *A. incarnata* rather than on *A.*

62   *curassavica* or *A. speciosa*. Parasite infection engendered wings less suitable for

63   sustained flight (wings became rounder) on three of four milkweed species. Wing loading

64   (associated with powered flight) was higher on *A. syriaca* than on other milkweeds,

65   whereas wing density was lower on *A. curassavica*. Monarchs that fed on high

66   cardenolide milkweed developed rounder, thinner wings, which are less efficient at

67   gliding flight.

68   6. Ingesting foliage from milkweed high in cardenolides may provide protection from

69   enemies through sequestration yet come at a cost to monarchs manifested as lower

70   quality flight phenotypes: rounder, thinner wings with lower wing loading values.

71   7. Small changes in morphology may have important consequences for enemy evasion and

72   migration success in many animals. Energetic costs associated with alterations in defense

73   and morphology may, therefore, have important consequences for trophic interactions in

74   a changing world.

75

76   **Key-words:** *Asclepias*, cardenolides, environmental change, elevated CO_2, *Danaus plexippus*,

77   monarch butterfly, predator-prey**,** plant secondary metabolites.

78   **Introduction**

79   Environmental change influences trophic interactions through multiple mechanisms (Gilman,

80   Urban, Tewksbury, Gilchrist, & Holt, 2010; Tylianakis, Didham, Bascompte, & Wardle, 2008).

81   For example, elevated atmospheric carbon dioxide (eCO_2) has both direct and indirect effects on

82   organismal physiology, and behavior. Plants grown under eCO_2 produce tissues with lower

83   nitrogen concentrations (Drake, Gonzalez-Meler, & Long, 1997; Robinson, Ryan, & Newman,

84   2012), causing herbivores to increase the amount of foliage they consume (Docherty et al., 1996;

85   Johnson, Lopaticki, & Hartley, 2014). Moreover, eCO_2 also changes the composition and

86   concentration of plant secondary metabolites (PSMs) (Klaiber, Dorn, & Najar-Rodriguez, 2013;

87   Ryan, Rasmussen, & Newman, 2010). Because catabolizing PSMs is energetically costly,

88   changes in these compounds affect the ecology of herbivores (Hunter, 2016). In general,

89 herbivore growth, fecundity, and survival decline under eCO_2 (Jamieson et al., 2017; Robinson

90 et al., 2012). Higher trophic levels are also affected by CO_2-induced shifts in plant quality

91 (Facey, Ellsworth, Staley, Wright, & Johnson, 2014; Ode & Crompton, 2013; Ode, Johnson, &

92 Moore, 2014), often mediated by shifts in prey nutrition and growth (Chen, Ge, & Parajulee,

93 2005; Klaiber, Najar-Rodriguez, Dialer, & Dorn, 2013). For example, eCO_2 reduces alfalfa

94 nutritional quality, which increases armyworm larval development times, resulting in asynchrony

95 between larvae and parasitoid wasps, ultimately reducing parasitoid fitness (Dyer *et al.* 2013).

96 Elevated CO_2 may also benefit higher trophic levels by inhibiting herbivore defense and escape

97 (Hentley, Vanbergen, Hails, Jones, & Johnson, 2014).

98

99 Many specialist herbivores have evolved mechanisms to co-opt PSMs for their own defense

100 (Dyer & Deane Bowers, 1996; Opitz & Müller, 2009; Petschenka & Agrawal, 2016).

101 Sequestration by insect herbivores involves the modification, transfer, and storage of toxic

102 compounds at high metabolic costs, potentially reducing immune responses to other enemies

103 (Greeney, Dyer, & Smilanich, 2012; Smilanich, Dyer, Chambers, & Bowers, 2009). Monarch

104 butterflies, *Danaus plexippus,* store toxic steroids (cardenolides) derived from the foliage of their

105 milkweed, *Asclepias,* host plants (Reichstein, Euw, Parsons, & Rothschild, 1968). Cardenolides

106 disrupt the function of Na^+/K^+-channels in animal cells (Agrawal, Petschenka, Bingham, Weber,

107 & Rasmann, 2012) and render monarchs well-defended prey (Malcolm & Brower, 1989). The

108 concentration and composition of cardenolides sequestered by monarchs depends on milkweed

109 species, the amount of tissue consumed, and sequestration efficiency (Agrawal, Ali, Rasmann, &

110 Fishbein, 2015; Bowers & Collinge, 1992; Camara, 1997; Malcolm, 1990, 1994). Environmental

111 factors that alter phytochemistry and consumption rates could also influence sequestration and

112 affect vulnerability to enemies. Despite a growing body of work illustrating the effects of

113 environmental change on milkweed chemistry (Andrews, 2015; Tao, Berns, & Hunter, 2014;

114 Vannette & Hunter, 2011), little is known about how monarch sequestration will respond to

115 future environmental conditions (*but see* Tao & Hunter, 2015).

116

117 In addition to sequestration, the seasonal migration of monarchs may also reduce mortality

118 imposed by natural enemies. In eastern North America, monarchs migrate up to 4,500 km from

119 their summer breeding grounds to overwintering sites in Mexico every fall (Urquhart & Urquhart

120   1978; Brower & Malcolm 1991; Flockhart *et al.* 2017). For many flying animals, foraging,

121   escape, and migration are strongly influenced by wing size, shape, and wing loading; the ratio

122   between body mass and wing area (Berwaerts, Van Dyck, & Aerts, 2002; Robert Dudley, 2002).

123   Subtle changes in wing size and shape can affect drag, lift, and flight behavior (Srygley &

124   Thomas, 2002). To maximize energy use efficiency, flying animals both glide and actively

125   propel (Kovac, Vogt, Ithier, Smith, & Wood, 2012; Park, Bae, Lee, Jeon, & Choi, 2010). Larger,

126   more elongated wing shapes, with high wing length to width ratio (aspect ratio), allow for

127   optimal gliding flight (Kerlinger, 1989). Migratory eastern N. American monarchs have larger

128   and more elongated wings (higher aspect ratios) than non-migratory conspecifics (Altizer &

129   Davis, 2010; Li, Pierce, & de Roode, 2016). Migratory monarchs also have higher wing loading

130   values, correlated with larger energy reserves for stronger powered flight (Dudley & Srygley,

131   2008). However, despite an extensive body of literature detailing the importance of dietary

132   chemistry for insect fitness (Awmack & Leather, 2002), few studies have explored the effects of

133   diet on wing morphology and flight ability (Boggs & Freeman, 2005; Johnson, Solensky,

134   Satterfield, & Davis, 2014; Pellegroms, Van Dongen, Van Dyck, & Lens, 2009). Food restriction

135   reduces monarch wing size (Johnson et al., 2014), but no study to date has examined the effects

136   of changing phytochemistry on monarch wing morphology.

137

138   Natural enemies may also influence toxin sequestration and morphology of herbivores. While

139   sequestration can reduce insect immunity to parasites (*reviewed in* Greeney *et al.* 2012), it is

140   unclear how parasite infection alters sequestration. Throughout their range, monarchs suffer

141   infection by a debilitating, protozoan parasite, *Ophryocystis elektroscirrha,* that reduces monarch

142   lifespan, fecundity, and flight ability (Bradley & Altizer, 2005; de Roode, Chi, Rarick, & Altizer,

143   2009). Heavily infected butterflies are more likely to die during migration, termed "migratory

144   culling" (Altizer, Hobson, Davis, De Roode, & Wassenaar, 2015). Thus, monarchs that do arrive

145   at overwintering grounds support lower pathogen loads (Altizer, Bartel, & Han, 2011). For

146   moderately infected monarchs, there is no clear effect of infection on wing morphology (Bradley

147   & Altizer, 2005). *Ophryocystis elektroscirrha* likely depletes the energy reserves necessary for

148   flight (Altizer et al., 2015). However, additional stressors, such as reductions in diet quality

149   induced by eCO_2, may influence the impact of infection on flight ability.

150

151   Here, we investigate the effects of eCO_2 on the defensive phenotype of monarch butterflies. We

152   fed larvae foliage from four milkweed species with varying phytochemistry grown under either

153   ambient CO_2 (aCO_2) or eCO_2. We also infected a subset of monarchs with *O. elektroscirrha*, to

154   understand how infection and environmental change combine to alter monarch defense,

155   including wing traits associated with migration. We measured changes in phytochemistry

156   induced by eCO_2 and assessed sequestration and morphology of butterflies. We expected

157   sequestration profiles to mirror changes in plant chemistry induced by eCO_2 because factors that

158   alter phytochemistry and consumption rates should also influence the types and amounts of

159   PSMs monarchs sequester. We also predicted that changes in cardenolides and reductions in the

160   nutritional quality of larval host-plants grown under eCO_2 (Decker, de Roode, & Hunter, 2018;

161   Robinson et al., 2012) would cause declines in the quality of the insect flight phenotype: smaller,

162   thinner and rounder wings with lower wing loading values. Feeding on lower quality food with

163   different types and amounts of cardenolides may engender a metabolic cost inflicting stress upon

164   the insect and inducing morphological changes. Finally, we hypothesized that the metabolic costs

165   of infection would exacerbate any deleterious effects of eCO_2 on cardenolide sequestration or

166   wing morphology.

167   **Materials and Methods**

168   *Milkweed and Monarch Source Materials*

169   We analyzed the wings of monarchs reared on milkweeds grown under ambient (400 ppm) or

170   elevated (760 ppm) CO_2 at the University of Michigan Biological Station (UMBS). We provide

171   details of the UMBS CO_2 array in Decker *et al.* (2018). In 2015, we grew four milkweed species

172   in a 40 chamber array (Drake *et al.* 1989), with 20 chambers maintained at aCO_2 and 20 at eCO_2.

173   Within those chambers, we grew milkweed that varied in cardenolide concentrations, ranging

174   from high to low: *A. curassavica, A. syriaca, A. speciosa,* and *A. incarnata*. Seeds were planted

175   in the UMBS greenhouse and, 18 days later, seedlings were transferred outside into the chamber

176   array for the remainder of the experiment. Each chamber contained 24 milkweeds (4 species x 6

177   plants per species).

178

179   The monarchs used in this study were the F_1 offspring of seven full-sib crosses between monarch

180   lineages from eastern N. America (St Marks, FL). Monarch larvae were assigned to one of 16

181   treatments (2 parasite treatments x 4 host plant species x 2 levels of CO_2 x 20 replicate

182   chambers) making for 320 monarchs reared in total. Not all monarchs survived to adulthood and

183   some inoculated monarchs resisted infection, inflating the sample size of uninfected monarchs.

184   Final sample sizes varied for each species by CO_2 treatment between N=19-27 for uninfected

185   and N=5-11 for infected monarchs (Table S1 in the Supporting Information). Each individual

186   monarch larva was assigned three plants of the same treatment grown in the same chamber on

187   which to complete development.

188

189   Monarch larvae were inoculated with *O. elektroscirrha* on cuttings from one of their three

190   assigned plants. After 42 days of growth in the array, we placed plant cuttings in individual

191   containers kept under aCO_2. A darkened monarch egg (darkening indicates eggs ready to hatch)

192   was attached to a leaf on each cutting to ensure that neonates consumed their assigned plant

193   before parasite inoculation. Three days after hatching, larvae were inoculated with *O.*

194   *elektroscirrha* following the methods of de Roode *et al.* (2008). Parasites originated from one

195   lineage collected from an eastern N. American, wild-caught butterfly. Control larvae were fed

196   leaf disks of the same size with no spores. Foliar chemistry samples were taken from each plant

197   at the same time as inoculations (see below) and assumed to adequately reflect the chemistry of

198   the other two assigned plants the larvae consumed in later instars.

199

200   Larvae were fed cuttings from their assigned host-plants *ad libitum* until pupation. Adult

201   butterflies were sexed, weighed, and kept in 5.75 x 9.5 cm glassine envelopes at 15°C for the

202   remainder of their adult lives (de Roode, Gold, & Altizer, 2007). Three weeks after death, we

203   removed and stored the monarch wings at -20°C, and estimated parasite spore load from bodies

204   using established methods following de Roode et al. (2008). We scanned the left forewing of

205   each monarch on an HP scanJet 6300C (Hewlett-Packard, Palo Alto, USA), weighed the wing,

206   and stored it in 1mL centrifuge tubes for cardenolide analyses.

207

208   *Cardenolide Chemical Analysis*

209   We quantified foliar cardenolide concentrations (Vannette & Hunter, 2011; Zehnder & Hunter,

210   2009) from the first milkweed that monarchs consumed, and in left forewings, because wing

211   cardenolides correlate tightly with body cardenolide concentrations and wings are typically the

212   first tissues tasted by bird predators upon attack (Fink & Brower 1981). We detail our chemical

213   analysis in Appendix S1 in the Supporting Information.

214

215   *Wing Morphometrics*

216   We measured forewing morphometrics because monarchs position forewings to cover their

217   hindwings during soaring flight (Altizer & Davis, 2010), allowing forewing size and shape to

218   have the largest influence on flight ability. Additionally, preliminary work established that

219   milkweed chemistry only affects forewing morphology (Berns, Zelditch, & Hunter, 2014).

220

221   We scanned the left forewing of each specimen with a ruler for scale and calibrated Adobe

222   Photoshop (Adobe, San Jose, USA) to calculate distance measures based on a pixel-to-millimeter

223   ratio. We took four basic measures of forewing morphology: first, length of the butterfly wing

224   from wing apex to thorax insertion (mm); second, width of the forewing at the longest axis

225   perpendicular to the length measurement (mm); third, total forewing area (mm^2); and fourth,

226   wing perimeter (mm, Fig. S1 in the Supporting Information). For slightly damaged wings, we

227   estimated wing edges to create a complete outline. Butterflies with substantial wing damage were

228   discarded from all analyses.

229

230   We calculated two metrics of forewing shape: wing aspect ratio (length divided by width), and

231   roundness (area to perimeter ratio: 4π area/perimeter^2) (Altizer & Davis 2010). We also

232   calculated wing loading (body mass/wing area), a common aeronautical measure indicative of

233   maneuverability and flight performance. Finally, we examined butterfly wing density which we

234   termed specific wing area (wing area/wing mass).

235

236   *Analyses*

237   We used linear mixed models (LMMs; R version 3.3.2.; package: lme4) to assess effects of our

238   treatments on phytochemistry, toxin sequestration, and wing morphology. Chamber identity was

239   included as a random effect in all models (Littell, Stroup, & Freund, 2002; Vannette & Hunter,

240   2011). For models with monarch traits, we also included monarch lineage as a random effect

241   because it was not specifically manipulated and had uninformative factor levels. We transformed

242   all (dependent and independent) variables when necessary to achieve homogeneity of variance,

243 simplified models when possible, and visually inspected model residuals to confirm best fit

244 (Crawley, 2012). We present model structures, results, and random effects in Table S3 in the

245 Supporting Information.

246

247 To determine the effects of CO_2 and milkweed species on foliar cardenolide concentration, we

248 ran LMMs with foliar cardenolide concentration (square root transformed) as a response variable

249 and CO_2 treatment and milkweed species as fixed effects. Because there was a significant

250 interaction between milkweed species and CO_2, we analyzed each plant species separately to

251 determine which species drove the interaction. We used similar LMMs to test for effects of CO_2,

252 host-plant species and infection on monarch wing cardenolides (square-root-transformed).

253 Additionally, we tested whether CO_2 treatment or infection altered the relationship between

254 foliar and wing cardenolides by including these factors (CO_2 treatment and infection) as fixed

255 effects in an LMM with foliar cardenolide concentration (square root transformed) as an

256 independent variable and wing cardenolide concentration (square root transformed) as the

257 dependent variable. A significant interaction between foliar cardenolide concentration and either

258 of the factors (CO_2 treatment or infection) indicates a change in the slope of the relationship

259 between plant and butterfly cardenolides dependent on that factor.

260

261 Some monarchs exposed to *O. elektroscirrha* resist infection and are spore-free as adults. We

262 compared the wing cardenolides of control monarchs (never exposed to the parasite) with those

263 of monarchs that were exposed to the parasite but had no spores. We ran an LMM with monarch

264 cardenolide concentration (square root transformed) as the dependent variable and parasite

265 exposure (control and exposed but uninfected) as a fixed effect. There were no significant

266 differences between resistant monarchs and control monarchs in the sequestration of total

267 cardenolides ($F_{1,190} = 0.90$, $p = 0.345$). Therefore, in all analyses of sequestration, we grouped

268 these two monarch treatments (control and exposed but uninfected) into one "uninfected" status.

269 We followed a similar procedure to determine whether or not monarch sex influenced

270 sequestration chemistry and found no effect of monarch sex on sequestered cardenolide

271 concentrations ($F_{1,250} = 0.24$, $p = 0.624$). Therefore, monarch sex was not included in models

272 that explored treatment effects on toxin sequestration.

273

274   We used permutational multivariate analysis of variance (PerMANOVA; Anderson, 2001) to

275   compare the effects of CO_2 treatment, milkweed species and, for butterfly cardenolides,

276   infection status on the assemblage of cardenolide compounds produced in milkweed and

277   sequestered by monarchs. For plant cardenolide composition, we performed a PerMANOVA

278   with CO_2 treatment, milkweed species and their interaction as independent variables, and Bray-

279   Curtis distance of percentage weight of each foliar cardenolide peak as dependent variables. We

280   chose Bray-Curtis as our ordination because it includes relative abundance information and

281   accounts for peak identity. To determine which factors altered wing cardenolide composition, we

282   performed a PerMANOVA with CO_2 treatment, milkweed species, infection status and their

283   interactions as independent variables, and the Bray-Curtis distance of percentage weight of each

284   sequestered wing cardenolide peak as dependent variables.

285

286   We followed Altizer & Davis (2010) and used Principal Component Analysis (PCA) to reduce

287   butterfly morphology measures into one PCA axis explaining forewing size (PCA-size) and

288   another PCA axis explaining forewing shape (PCA-shape). Forewing length, width, area and

289   perimeter were used to create the PCA-size axis that explained 99.6% of the total variance, while

290   forewing area and roundness were used to create the PCA-shape axis that explained 95.2% of the

291   total variance. Higher values of PCA-size represent larger wings, and higher values of PCA-

292   shape represent more elongated wings. We first ran LMMs similar to those used above for

293   sequestration results, to test whether there was a difference between control monarchs and those

294   that did not become infected in the size and shape of their wings. We ran two LMMs with either

295   PCA-shape or PCA-size as the dependent variables and parasite exposure (control or cleared) as

296   a fixed effect to examine the possibility that resisting infection could result in morphological

297   differences. There were no significant differences between resistant monarchs and control

298   monarchs in the size ($F_{1,166} = 0.35$, $p = 0.552$) and shape of wings ($F_{1,180} = 1.90$, $p = 0.170$). We

299   then ran models with these PCA axes as response variables, and CO_2 treatment, milkweed

300   species, infection and monarch sex as fixed effects. Due to sample size limitations (see Table

301   S1), we could not include the four-way interaction in any of our full models, but we did initially

302   include all other interaction terms between CO_2 treatment, milkweed species, infection, and

303   monarch sex.

304

305 To examine effects of the treatments on monarch wing loading and specific wing area, we ran

306 LMMs with wing loading (log-transformed) and specific wing area as response variables, with

307 identical fixed effects as above. We ran two LMMs with either wing loading or specific wing

308 area as response variables and CO_2 treatment, milkweed species, infection, and monarch sex as

309 fixed effects.

310

311 Finally, we tested whether cardenolide sequestration affected wing shape, loading, and density.

312 We ran three LMMs with either PCA-shape, wing loading or wing density (log-transformed) as

313 dependent variables, and monarch cardenolide concentration (square root transformed),

314 milkweed species and their interaction as fixed effects.

315 **Results**

316 *Milkweed Host-Plant Chemistry*

317 Of the 252 milkweeds that supported surviving monarchs (see Table S1), 114 produced

318 measurable cardenolides. To address the large occurrence of zeros in our chemistry dataset, we

319 followed methods detailed in Appendix S1. There was no effect of CO_2 treatment on the

320 proportion of plants that produced detectable foliar cardenolides (CO_2: $\chi^2 = 0.82$, p = 0.366).

321 *Asclepias incarnata* and *A. syriaca* were significantly more likely to express zero cardenolides

322 (species: $\chi^2 = 19.52$, p = 0.0002). The interaction term between CO_2 and species was not

323 significant (CO_2 * species: $\chi^2 = 1.49$, p = 0.684). We therefore chose to exclude the plants that

324 did not produce cardenolides from further analyses of phytochemistry.

325

326 The 114 plants with detectable cardenolides included 65 *A. curassavica*, 19 *A. syriaca*, 18 *A.*

327 *speciosa,* and 12 *A. incarnata*. Elevated CO_2 changed the concentration of foliar cardenolides in

328 a species-specific manner (species*CO_2: $F_{3,106} = 3.05$, p = 0.032, Fig. 1a). Under eCO_2 there was

329 a 52% decline in the foliar cardenolide concentrations of *A. curassavica* ($F_{1,36} = 13.43$, p =

330 0.0008, Fig. 1a). Cardenolide concentrations in *A. syriaca* ($F_{1, 13} = 1.0847$, p = 0.32)*, A. speciosa*

331 ($F_{1, 13} = 0.76$, p = 0.399) and *A. incarnata* ($F_{1, 11} = 0.01$, p = 0.910) remained unaffected by

332 eCO_2. Across CO_2 treatments, *A. curassavica* produced the highest cardenolide concentrations,

333 while *A. incarnata* produced the lowest (species: $F_{3, 106} = 71.72$, p < 0.0001, Fig. 1a). Milkweed

334 species varied in the assemblage of cardenolides that they produced (PERMANOVA, species:

335 $F_{3, 110} = 24.16$, $R^2 = 0.39$, p = 0.001). In addition, the effect of CO_2 treatment on cardenolide

336   composition varied among milkweed species (PERMANOVA, CO_2*species: $F_{3,\,110} = 2.26$, $R^2 =$

337   0.037, $p = 0.004$).

338

339   **_Monarch Wing Chemistry_**

340   Monarchs maintained constant wing cardenolide concentrations between CO_2 treatments

341   (CO_2*species: $F_{3,214}=1.60$, $p=0.191$, Fig. 1b) despite the decline in foliar cardenolide

342   concentration in _A. curassavica_ induced by eCO_2 (Fig. 1a). Critically, monarchs feeding on

343   milkweed foliage grown under eCO_2 sequestered more cardenolides per unit cardenolide

344   available in host-plants (plant cardenolides* CO_2: $F_{1,109}=5.54$, $p=0.020$, Fig. 1c). When feeding

345   on _A. syriaca_, monarchs infected with parasites sequestered 20% less cardenolide in their wings

346   than did uninfected monarchs (infection*species: $F_{3,228}=2.71$, $p=0.0462$, Fig. S2 in the

347   Supporting Information). CO_2 treatment, milkweed species and infection did not interact to

348   influence the concentration of cardenolides sequestered by monarchs (CO_2*species*infection: F

349   $_{3,225} = 0.83$, $p = 0.4803$).

350

351   Despite the interactive effects of CO_2 treatment and milkweed species on the composition of

352   foliar cardenolides, the cardenolide profiles sequestered by monarchs were influenced only by

353   milkweed species (PERMANOVA, species: $F_{3,\,247} = 157.00$, $R^2 = 0.65$, $p = 0.001$). Neither

354   eCO_2 alone (PERMANOVA, CO_2: $F_{1,\,250}= 2.37$, $R^2 = 0.003$, $p = 0.073$), nor its interaction with

355   plant species (PERMANOVA, CO_2*species: $F_{3,\,247}= 1.41$, $R^2 = 0.006$, $p = 0.149$), influenced the

356   cardenolide profiles sequestered by monarchs.

357

358   **_Monarch Wing Morphology_**

359   Monarch wings were more elongated (higher values of PCA-Shape) when larvae fed on

360   milkweed grown under eCO_2 (CO_2: $F_{1,\,214} = 15.82$, $p <0.0001$, Fig. 2a) or when larvae

361   consumed _A. syriaca_ or _A. incarnata_ (species: $F_{3,\,212} = 3.78$, $p = 0.011$, Fig. 2c). Additionally,

362   the wings of female butterflies were more elongated than those of males (sex: $F_{1,\,213} = 15.50$, $p =$

363   0.0001, Fig. 2b).

364

365   While _O. elektroscirrha_ infection had no independent effect on forewing shape (infection: $F_{1,\,212}$

366   $= 0.90$, $p=0.3550$), infected monarchs from aCO_2 plants had rounder wings than butterflies from

367   eCO$_2$ infection treatments (infection*CO$_2$: $F_{1, 212}$ = 9.46, p = 0.002, Fig. 3a). Moreover, infected

368   monarchs had rounder wings than uninfected monarchs when feeding on *A. curassavica, A*

369   *syriaca*, and *A. incarnata*, but had more elongated wings than uninfected monarchs fed *A.*

370   *speciosa* (infection*species: $F_{3, 212}$ = 4.54, p = 0.004, Fig. 3b). Finally, there were minor

371   differences between male and female butterflies in wing shapes dependent on plant species and

372   infection (sex*infection*species: $F_{3, 213}$ = 2.96, p = 0.033, Fig. S3 in the Supporting

373   Information). However, the three-way interaction term explained only a small portion of variance

374   in the model when compared to the main effects reported above. Wing aspect ratio correlated

375   most strongly with PCA-shape (*r* = 0.999, N = 237, p < 0.0001), thus, we summarize the model

376   results for wing aspect ratios in Table 1 and Table S2 in the Supporting Information.

377

378   In contrast to wing shape, none of our treatments affected the size of monarch wings. Male

379   wings were only slightly significantly larger than those of females (sex: $F_{1, 231}$ = 3.47, p =

380   0.064). Likewise, wing sizes were unaffected by CO$_2$ treatment (CO$_2$: $F_{1, 31}$ = 0.31, p = 0.579),

381   milkweed host plant species (species: $F_{3, 202}$ = 2.09, p = 0.102), infection status (infection: $F_{1, 226}$

382   = 2.42, p = 0.121) or the interaction between these treatments (CO$_2$ *species* infection: $F_{3, 204}$ =

383   0.98, p = 0.403).

384

385   The wing loading values of male monarchs were 5% higher than those of female monarchs (sex:

386    $F_{1,15}$ = 17.13, p = 0.0008). Notably, monarchs reared on *A. syriaca* had a 5% higher wing

387   loading than did those reared on other milkweed species (species: $F_{3,15}$ = 4.77, p = 0.0153, Fig.

388   4a). Wing loading was unaffected by CO$_2$ treatment (CO$_2$: $F_{1,11}$ = 3.01, p = 0.112), or parasite

389   infection (infection: $F_{1,15}$ = 3.80, p = 0.07).

390

391   Female monarch wings were 6% denser than male monarch wings (sex: $F_{1, 228}$ = 15.74, p <

392   0.0001). Monarchs fed *A. curassavica* had the thinnest wings while those fed *A. syriaca* had the

393   densest wings (species: $F_{3, 199}$ = 2.66, p = 0.0492, Fig. 4b). Interestingly, the wings of infected

394   monarchs were 7% less dense than the wings of uninfected monarchs (infection: $F_{1, 221}$ = 20.65,

395   p < 0.0001, Fig. 4c). Specific wing area was unaffected by CO$_2$ treatment (CO$_2$: $F_{1, 31}$ = 0.02, p

396   = 0.897).

397

398   In the simplified model exploring effects of cardenolide sequestration on wing shape, we found

399   no effects of monarch cardenolide sequestration (monarch cardenolides: $F_{1,230} = 0.05$, $p = 0.816$),

400   plant species (species: $F_{3,228} = 2.19$, $p = 0.090$) or their interaction (monarch

401   cardenolides*species: $F_{3,230} = 1.62$, $p = 0.184$, Table S4 in the Supporting Information) on wing

402   shape. Interestingly, monarchs that sequestered higher concentrations of cardenolides had lower

403   wing loading values (monarch cardenolides: $F_{1,15} = 8.41$, $p = 0.011$, Fig. 5). Also, those

404   monarchs feeding on higher cardenolide milkweed had lower wing loading values (species: $F_{3,15}$

405    $= 4.11$, $p = 0.025$), but there were no species-dependent effects of monarch cardenolide

406   sequestration on wing loading (monarch cardenolides*species: $F_{3,15} = 2.82$, $p = 0.073$). Finally,

407   there was a marginally significant trend of less dense wings produced in monarchs sequestering

408   higher concentrations of cardenolides ($F_{1,229} = 3.59$, $p = 0.059$). There were no effects of either

409   milkweed species (species: $F_{3,227} = 1.95$, $p = 0.122$) or the interaction on wing density (monarch

410   cardenolides*species: $F_{3,230} = 1.15$, $p = 0.330$).

411

412   **Discussion**

413   Rapid environmental change may influence trophic interactions by altering the defensive

414   phenotype of prey. Here, we demonstrate that: 1) monarchs maintain the concentration and

415   composition of cardenolides that they sequester despite changes in the phytochemistry of one

416   milkweed species induced by eCO_2. 2) Aspects of monarch morphology important to flight

417   ability such as wing shape, loading, and density vary in response to eCO_2, milkweed host plant

418   species, infection, and sex. 3) Feeding on high cardenolide milkweed is associated with the

419   formation of rounder, thinner wings, which are less efficient at gliding flight. We suggest that

420   changes in sequestration rates under eCO_2 are a byproduct of compensatory feeding aimed at

421   maintaining a nutritional target in response to declining diet quality. Additionally, monarchs

422   exhibit the cost of sequestering higher concentrations of cardenolides through declining wing

423   loading values. Small changes in wing morphology can have important consequences for

424   migration success (Bradley & Altizer, 2005), including migratory escape from parasites.

425   Therefore, changes in sequestration and morphology may have consequences for monarch

426   defense and migration in a changing world.

427

428   *Monarchs increase sequestration rate under eCO_2*

429 We demonstrate that monarchs can increase their rate of cardenolide sequestration under eCO_2

430 (Fig. 1). Specifically, monarchs sequester a constant concentration and composition of

431 cardenolides from *A. curassavica* despite a 52% reduction in foliar cardenolides and changes in

432 foliar cardenolide composition induced by eCO_2. Monarchs are known to maintain constant

433 concentrations of the cardenolides that they sequester from *A. curassavica* in response to nutrient

434 deposition, another environmental change driver (Tao & Hunter, 2015). Herbivores regulate

435 sequestration by altering both the total amount of foliage consumed and sequestration efficiency

436 (Camara, 1997). Notably, herbivorous arthropods maintain target ratios of carbohydrates to

437 protein in their diet through behavioral shifts in consumption (Simpson et al., 2015). Therefore, it

438 is possible that the monarchs in our study increased the amount of foliage consumed to

439 compensate for reductions in foliar nutrient content under eCO_2 (Hunter, 2001; Johnson et al.,

440 2014; Lincoln, Sionit, & Strain, 1984; Zavala, Nabity, & DeLucia, 2013).

441

442 ***Environment influences monarch wing morphology***

443 Our treatments altered indices of monarch wing morphology (wing shape, loading, and density)

444 that are important to both aerial maneuverability and long-distance flight (Berwaerts et al.,

445 2002). Notably, both CO_2 treatment and milkweed species influenced wing shape contingent

446 upon parasite infection status (Figs 2 & 3). Under aCO_2, infection induced rounder wings

447 lowering the mean aspect ratio from 1.95 ± 0.002 to 1.93 ± 0.004. However, under eCO_2 both

448 infected and uninfected monarchs developed more angular wings (1.97 ± 0.005, 1.96 ± 0.002,

449 Fig. 3a). No study to date has specifically tested the effects of wing shape on the probability of

450 successful migration from start to finish in monarchs. However, field sampling indicates that

451 earlier arriving migrants tend to have larger, more elongated wing shapes suggesting that this

452 flight phenotype increases migration success (Satterfield & Davis, 2014). Therefore, future

453 environmental conditions may induce the formation of wing shapes that improve monarch flight

454 efficiency despite infection.

455

456 We also report effects of milkweed species on monarch wing shape, loading, and density, similar

457 to findings in other flying insects (Benítez, Vargas, & Püschel, 2015; Davis & de Roode, *in*

458 *Revision*; Soto, Carreira, Soto, & Hasson, 2008). Importantly, monarch wings are rounder and

459 less dense when larvae are reared on *A. curassavica*, a plant exotic to N. America and increasing

460   in prevalence in the southern US (Satterfield, Maerz, & Altizer, 2015). This species of milkweed

461   does not senesce in autumn, and contributes to a loss of monarch migratory behavior as

462   butterflies encounter viable foliage during their late-season stopovers (Satterfield et al., 2018,

463   2015). Our data suggest that the offspring of those sedentary monarchs fed *A. curassavica* will

464   develop lower quality flight phenotypes, perhaps furthering the loss of migratory behavior.

465

466   Small differences in wing morphology that affect the efficiency of flight could have large

467   consequences for monarch migration success (Bradley & Altizer, 2005). Eastern N. American

468   monarchs migrate up to 4,500 km through soaring and active flight (Brower & Malcolm, 1991;

469   Gibo, 1986). Monarchs must take shelter during adverse weather conditions, utilize wind

470   patterns, and cross large expanses of unsuitable habitat (Garland & Davis, 2002; Gibo & Pallett,

471   1979). Therefore, any factor that causes monarchs to remain grounded during beneficial flying

472   conditions or reduces the amount of time monarchs may stay aloft over unsuitable habitat will

473   significantly reduce migration success. In our study, eCO_2 eliminates the shape difference

474   between infected and uninfected individuals, inducing more elongated wings in both groups. If

475   infected individuals become more efficient gliders under environmental change, this might

476   decrease migratory culling which reduces pathogen prevalence seasonally in the N. American

477   monarch population (Altizer et al., 2011; Bartel, Oberhauser, de Roode, & Altizer, 2011).

478

479   Although our treatments altered monarch wing shape, we detected no effect of diet or infection

480   on wing size. All of the butterflies used in this study originated from the migratory eastern N.

481   American population. Therefore our data substantiate previous studies demonstrating strong

482   selection for larger wings imposed by migration distance within this population (Altizer & Davis,

483   2010; Li et al., 2016). Forewing size is likely more important to migration success than wing

484   shape because it is conserved among all our treatments and is selected for in the migratory

485   populations of monarchs (Altizer & Davis, 2010; Flockhart et al., 2017; Li et al., 2016).

486   However, aerodynamic theory suggests that wing shape can be important for maneuverability

487   and energy conservation (Pennycuick, 2008). Because our monarchs were constrained within

488   these constant wing sizes, perhaps the amount of differentiation in the angular nature of wing

489   shape was limited. Further studies exploring the plasticity of wing shape and size in response to

490   host-plant and environmental conditions among different populations of monarchs across the

491   globe are needed to better address this idea.

492

498

499   **Authors' Contributions**:

500   LED, MDH & JCdR designed the experiment. JCdR provided butterflies and parasites. LED,

501   AJS & MDH collected and analyzed the data. LED wrote the manuscript; all authors contributed

502   to drafts.

503

504   **Data Accessibility**: Data are available in the Dryad Digital Repository.

505   DOI:10.5061/dryad.mk3tj78

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730 **SUPPORTING INFORMATION**

731 Additional supporting information may be found in the online version of this article.

732

733 Appendix S1. Details of cardenolide extraction and the generalized linear mixed model used to

734 determine the distribution of plants that produced undetectable cardenolides.

735

736 Figure S1. An illustration of the four basic morphometric measures taken in Adobe Photoshop.

737

738 Figure S2. Effects of infection by *Ophryocystis elektroscirrha* on the total concentration of

739 cardenolides sequestered by monarchs fed four species of milkweed.

740

741 Figure S3. The interaction between milkweed host-plant species, infection and sex on a

742 composite measure of monarch forewing shape.

743

744 Table S1. Sample sizes of surviving monarchs that were used to explore the effects of milkweed

745 species, elevated CO_2, and infection, on toxin sequestration and wing morphology

746

747 Table S2. The three-way interaction between treatments on mean monarch wing Aspect Ratios

748

749 Table S3. Table of all models used in this study with accompanying ANOVA tables.

750

751 Table S4. Table of model results testing the relationship between cardenolide sequestration in

752 wings and milkweed species on monarch wing shape, loading and density.

753 **Captions**

754   **Table 1.** The a) main and b) interactive effects of CO_2 treatment, butterfly sex, and host plant on

755   monarch wing aspect ratios (wing length/wing width), a component of monarch wing shape.

756   Data are the means ± 1 SE. Aspect ratios range between 1.6 to 2.1 but average around 1.93 in

757   eastern N. American monarch populations. Simplified linear mixed effects model structure is as

758   follows: Aspect Ratio ~ CO_2 + butterfly sex + infection + milkweed species + CO_2 * infection +

759   milkweed species*infection + sex*infection*milkweed species + random effects = chamber,

760   monarch lineage. We do not present nonsignificant main effects of infection. Table S2 presents

761   the three-way interaction.

762

763   **Fig. 1.** Effects of eCO_2 on (a) foliar cardenolide concentrations (mg/g dry mass), (b) monarch

764   wing cardenolide concentrations (mg/g dry mass), and (c) the relationship between foliar and

765   wing cardenolide concentrations. Bars represent mean values ± 1 SE. Traits were transformed

766   before analyses but are presented as untransformed values for ease of interpretation. Grey bars

767   represent plants grown under aCO_2 and orange bars are those from eCO_2 or the monarchs that

768   fed on those plants. Milkweed species codes: CUR = *A. curassavica*, SYR = *A. syriaca*, SPE =

769   *A. speciosa*, INC= *A. incarnata*.

770

771   **Fig. 2.** The main effects of (a) CO_2 treatment, (b) sex and (c) milkweed species on a composite

772   measure of monarch forewing shape. Points represent mean PCA-shape values ± 1 SE. With

773   increasing PCA-shape values wings become more elongated and angular. Milkweed species

774   codes are the same as above.

775

776   **Fig. 3.** The interactions between (a) CO_2 treatment and infection by OE, and (b) milkweed

777   species and infection on a composite measure of monarch forewing shape. Points represent mean

778   PCA-shape values ± 1 SE. Red points indicate mean shape values of infected monarchs while

779   blue points represent uninfected monarchs. Milkweed species codes are the same as above.

780

781   **Fig. 4.** The effects of milkweed species on monarch (a) wing loading (body mass/wing area) and

782   (b) specific wing area (wing area/wing mass), a measure of wing density. (c) The effects of OE

783   infection on specific wing area. Bars represent mean values ± 1 SE. Higher specific wing area

784   values indicate wings that are less dense. Milkweed species codes are the same as above.

785

786   **Fig. 5.** The relationship between the concentration of cardenolides sequestered in monarch wings

787   and wing loading (body mass/wing area). Points represent individual monarchs and colors

788   correspond to the species of milkweed host-plants fed to each monarch. Milkweed species codes

789   are the same as above.

Tables:

Table 1.

Main Effects				Two-way Interactions		
CO_2 treatment	$F_{1, 214} = 15.82$	$p < 0.0001$		**Infection*CO_2**	$F_{1, 212} = 9.46$	$p = 0.0024$
	Aspect Ratio			**Infection Status**	**CO_2 treatment**	**Aspect Ratio**
Ambient	1.95 ± 0.002			Infected	Ambient	1.93 ± 0.004
Elevated	1.96 ± 0.002				Elevated	1.97 ± 0.005
Butterfly Sex	$F_{1, 213} = 15.50$	$p < 0.0001$		Uninfected	Ambient	1.95 ± 0.002
	Aspect Ratio				Elevated	1.96 ± 0.002
Female	1.97 ± 0.002					
Male	1.93 ± 0.002			**Infection*Species**	$F_{3, 212} = 4.54$	$p = 0.004$
Milkweed Species	$F_{3,212} = 3.78$	$p = 0.0113$		**Infection Status**	**Milkweed Species**	**Aspect Ratio**
	Aspect Ratio			Infected	A. curassavica	1.93 ± 0.008
A. curassavica	1.94 ± 0.003				A. incarnata	1.95 ± 0.005
A. incarnata	1.97 ± 0.003				A. speciosa	1.97 ± 0.006
A. speciosa	1.95 ± 0.004				A. syriaca	1.94 ± 0.0102
A. syriaca	1.96 ± 0.003			Uninfected	A. curassavica	1.95 ± 0.003
					A. incarnata	1.97 ± 0.003
					A. speciosa	1.94 ± 0.004
					A. syriaca	1.96 ± 0.003