The price of defence: toxins, visual signals and oxidative state in an aposematic butterfly

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Running headline: Oxidative stress and warning signal honesty

SUMMARY

In a variety of aposematic species, the conspicuousness of an individual's warning signal and the quantity of its chemical defence are positively correlated. This apparent honest signalling in aposematism is predicted by resource competition models which assume that the production and maintenance of aposematic defences compete for access to antioxidant molecules that have dual functions as pigments directly responsible for colouration and in protecting against oxidative lipid damage. Here we study a model aposematic system—the monarch butterfly (*Danaus plexippus*) and make use of the variable phytochemistry of its larval host-plant, milkweeds (Asclepiadaceae), to manipulate the concentration of sequestered cardenolides. We test two fundamental assumptions of resource competition models: that (1) the possession of secondary defences is associated with costs in the form of oxidative lipid damage and reduced antioxidant defences; and (2) that oxidative damage or decreases in antioxidant defences can reduce the capacity of individuals to produce aposematic displays. Monarch caterpillars that sequestered the highest concentrations of cardenolides exhibited higher levels of oxidative lipid damage as adults. The relationship between warning signals, cardenolide concentrations and oxidative damage differed between the sexes. In male monarchs conspicuousness was explained by an interaction between oxidative damage and sequestration: as males sequester more cardenolides, those with high levels of oxidative damage become less conspicuous, while those that sequester lower levels of cardenolides equally invest in conspicuous with increasing oxidative damage. There was no significant effect of oxidative damage or concentration of sequestered cardenolides on female conspicuousness. Our results demonstrate physiological linkage between the production of coloration and protection from autotoxicity, that warning signals can be honest indicators of defensive capability, and that the relationships are different between the sexes.

Key-words: aposematism, cardenolides, cardiac glycosides, resource competition, oxidative lipid damage, monarch butterfly, honest signalling.

Introduction

Many species of animals, plants and micro-organisms possess chemical defences that reduce the likelihood of their being eaten [1]. Aposematic species, such as the monarch butterfly (*Danaus plexippus*), signal their chemical defences to predators with conspicuous warning colours [2]. In some species, the conspicuousness of the warning signal and the quantity of chemical defence are positively correlated, for example in dendrobatid frogs [3-5], marine opisthobranchs [6], ladybird beetles [7, 8], and paper wasps *Polistes dominula* [9]. The identification of these relationships has led to renewed interest in the idea that warning signals may inform predators about levels of prey defence and that such signals are honest indicators of defensive capability [10]. Several theoretical studies have, however, predicted the opposite: that well defended prey should reduce investment in signals, because signalling carries an additional conspicuousness cost, and such prey stand a good chance of surviving any attack unharmed [11-13]. Negative signal-defence correlations have been reported across Dendrobatid species of poison dart frogs [14] including the granular poison dart frog *Dendrobates granuliferus* [15]. Using a novel theoretical model Blount et al [16] could explain both the positive and negative correlations between warning colours and chemical defences if these two traits are linked through the competitive use of a shared resource. One possible shared resource is energy, which can be limiting for the sequestration or biosynthesis of toxins [17]. Alternatively, sequestration of toxins could impose metabolic costs in terms of oxidative lipid damage and reduced antioxidant defences – an idea that has has received limited empirical attention in regard to warning signals.

Many plant allelochemicals are powerful pro-oxidants, which, when ingested, can cause oxidative lipid damage [18]. In their resource competition framework, Blount et al [16] envisaged that pigments used in prey warning signals (e.g., carotenoids, flavonoids, melanins, and pteridines) might play a dual role imparting colour as well as acting as antioxidants that

prevent self-damage when storing toxins. In their model, if antioxidants are required to enable high levels of toxicity, signal reliability can be explained if the brightest and most toxic species gain access to more of the limiting resource than those that are less bright and less toxic. The prediction of signal honesty breaks down in their model when prey have very abundant resources, in which case it pays to divert these resources increasingly into toxins and not into warning signals, because less conspicuous but highly toxic prey encounter predators less often and have high chances of surviving attacks [16]. Because directly controlling antioxidant availability is challenging experimentally, whether colour-toxin relationships are influenced by the resource state of prey is poorly understood [14, 15], and has not yet been examined experimentally [11, 12, 16, 17, 19].

Here we manipulate diet-derived toxins available to an individual to test whether toxin sequestration and the production and maintenance of warning signals are linked via oxidative state. We achieve this by using a model aposematic system, the monarch butterfly, that selectively metabolises cardenolides present in its host-plants and stores them in its body [20, 21], and by using the known inter- and intraspecific variability in cardenolide concentration and cardenolide polarity of the monarch's larval milkweed host-plants (Asclepiadaceae; [21, 22]) to manipulate the toxins available to an individual to sequester. We test whether (1) the quantity of sequestered secondary defence by caterpillars is associated with oxidative lipid damage and reduced antioxidant defences in adults; and (2) whether oxidative lipid damage incurred during sequestration affects the capacity of adult butterflies to produce aposematic displays.

Because there is substantial genetic variation in sequestration ability, and because evolutionary history and contemporary species interactions may influence patterns of cardenolide sequestration [23] we used monarchs from the same population. We reared them

on four milkweed species that show within- and between-species, and among-population variation in cardenolide concentration, diversity, and polarity [24-27]. The polarity of cardenolides can influence their toxicity [21] and transport [28], so our four plant species were used to represent a gradient of distinct cardenolide defence, with the aim to create different costs of sequestration.

We reared monarch caterpillars on either *Ascelpias curassavica*, which is a critical hostplant worldwide and contains a diversity of cardenolides, including apolar voruscharin which is detrimental to monarch performance [21, 29-32]; *A. syriaca* (common milkweed) which is the principal native food plant of the monarch east of the Rocky Mountains and is characterized by more polar cardenolides though also contains the apolar labriformin [33, 34]; *A. incarnata* (swamp milkweed) which is a preferred hostplant in the Midwest USA and has low concentrations of cardenolides [35]; or *A. tuberosa* (butterfly milkweed) which is an occasional native foodplant in the wild with low concentrations of cardenolides [21, 36]. Based on the published records of biological activity and chromatic quantification (e.g., HPLC and thin layer chromatography) of cardenolides of milkweeds in different populations/latitudes [37, 38], we predicted that concentrations of cardenolides in the body and wings of newly eclosed adults would differ according to the cardenolides of the host-plant on which they were reared (see methods): *A. curassavica* (*Ac*) ≥ *A. syriaca* (*As*) > *A. incarnata* (*Ai*) ≥ *A. tuberosa* (*At*). Because sequestration, modification, and storage of allelochemicals are believed to be oxidatively stressful for many chemically defended organisms [18], we predicted that body levels of oxidative lipid peroxidation would differ amongst individuals according to host-plant, as follows: *Ac*≥*As*>*Ai*≥*At*. In turn, we predicted that survival to eclosion would differ according to host-plant as follows: *At*≥*Ai*>*As*≥*Ac*, and that the redness, luminance, and conspicuousness of the warning signals would differ: *Ac*≥*As*>*Ai*≥*At*. In addition to testing for treatment effects, we examined associations between

101 individual levels of cardiac glycosides (CGs), oxidative state, and warning signal redness,
102 luminance, and conspicuousness. We predicted a positive association between levels of CGs
103 and and lipid peroxidation, a negative association of CGs with antioxidant defences, and that
104 highly toxic prey will reduce investment in signals if they have high levels of oxidative
105 damage because they cannot bear the oxidative cost of investing in both traits [16].

106 **Materials and methods**

107 ***(a) Milkweed plants***

108 Milkweeds (*A. incarnata, A. tuberosa, A. curassavica, and A. syriaca*) were locally sourced
109 in Amherst, Virginia, USA. We did not measure cardenolide content in the leaves of the
110 plants used in the experiment, but used published records about the spatial repeatability of
111 cardenolide defences that take into account latitudinal clines [37-39]. From these records we
112 predicted that for milkweeds grown in Mid-Atlantic and Southeastern regions of the United
113 States, total cardenolide content would vary in the order *A. curassavica* (*Ac*) ≥ *A. syriaca* (*As*)
114 > *A. incarnata* (*Ai*) ≥ *A. tuberosa* (*At*). The plants were grown without the use of pesticides as
115 described previously [40]. All plants were healthy with undamaged leaves at the start of the
116 experiment.

117 **(b) *Milkweed Feeding Assay and preparation of samples***

118 Eggs were obtained from wild caught Monarch females in Virginia (USA) and larvae were
119 reared by LPB on single plants. All larvae had abundant food during the experiment. Larvae
120 were monitored daily for pupation starting at Day 12.

121 Upon eclosion, the date and the individual's sex were recorded. Adults were placed in
122 glassine envelopes and frozen on dry ice until dead, then stored in a freezer at -80°C before
123 being shipped to the UK on dry ice. Adults were stored at -80°C in JDB's laboratory until
124 they were analysed.

125 For analysis, the gut was removed, and the head, thorax and abdomen (hereafter, 'body')
126 were bisected longitudinally. One half of the body was returned to storage at -80ºC for later
127 analysis of cardenolides. The other half of the body was immediately homogenised in ice-
128 cold phosphate-buffered saline (PBS; 5 % w/v), for analyses of oxidative stress. The wings
129 were removed using dissecting scissors and shipped on dry ice to HMR's laboratory for
130 analyses of colouration (see below). Wings were then returned (on dry ice) to JDB's
131 laboratory for cardenolide analysis.

132 ***(c) Determination of oxidative stress and cardenolide concentration***

133 Determining oxidative state requires a range of assays including antioxidant defences and
134 oxidative damage. We assayed three such components: total superoxide dismutase (SOD),
135 which are metalloenzymes that catalyse the dismutation of superoxide into oxygen and
136 hydrogen peroxide and form a crucial part of intracellular antioxidant defences; total
137 antioxidant capacity (TAC), which measures the activity of low molecular weight chain-
138 breaking antioxidants including ascorbate, a-tocopherol, carotenoids and flavonoids, and
139 therefore gives an overview of the antioxidant status of individuals (including diet-derived
140 antioxidants); and malondialdehyde (MDA), which is formed by the β-scission of peroxidised
141 polyunsaturated fatty acids, and therefore is a definitive marker of oxidative damage.

142 *(i) Total superoxide dismutase (SOD)*

143 Total SOD was assayed by measuring the dismutation of superoxide radicals generated by
144 xanthine oxidase and hypoxanthine. We followed the instructions of the kit (#706002;
145 Cayman Chemical, Michigan, USA), except that tissue homogenates were further diluted
146 with the supplied sample buffer (1:100 v/v) to ensure that SOD activity fell within the range
147 of the standard curve. Samples were assayed in duplicate and are reported as units of SOD
148 activity (U) per mg tissue.

149 *(ii) Total antioxidant capacity (TAC)*

150 TAC was assayed by measuring the capacity of tissue homogenate to inhibit the oxidation of
151 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (#709001; Cayman Chemical). The
152 homogenate was further diluted with sample buffer (1:5 v/v) to bring the absorbance values
153 within the range of the standard curve. Samples were assayed in duplicate, as per the kit
154 instructions. Data are reported as nmols of TAC activity (Trolox equivalents) per g tissue.

155 *(iii) Malondialdehyde (MDA)*

156 MDA was measured using HPLC with fluorescence detection as described previously for
157 tissue homogenates (e.g. Blount & Pike 2011). All chemicals were HPLC grade, and
158 chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore,
159 Watford, UK). Samples were derived in 2 mL capacity polypropylene screw-top
160 microcentrifuge tubes. For tissue homogenates and for the standard (1,1,3,3-
161 tetraethoxypropane, TEP; see below) a 20 µl aliquot was added to 20 µl butylated
162 hydroxytoluene solution (BHT; 0.05% w/v in 95 % ethanol), 160 µl phosphoric acid solution
163 (H_3PO_4; 0.44 *M*), and 40 µl thiobarbituric acid solution (TBA; 42 m*M*). Samples were
164 capped, vortex mixed for 5 s, then heated at 100 ^{o}C for 1 h in a dry bath incubator to allow
165 formation of MDA-TBA adducts. Samples were then cooled on ice for 5 min, before 80 µl *n*-
166 butanol was added and tubes were vortex mixed for 20s. Tubes were centrifuged at 12 x g
167 and 4 ^{o}C for 3 min, before the upper (*n*-butanol) phase was collected and transferred to an
168 HPLC vial for analysis. Samples (10 µl) were injected into a Dionex HPLC system (Dionex
169 Corporation, California, USA) fitted with a 5µm ODS guard column and a Hewlett-Packard
170 Hypersil 5µ ODS 100 x 4.6 mm column maintained at 37 ^{o}C. The mobile phase was
171 methanol-buffer (40:60, v/v), the buffer being a 50 m*M* anhydrous solution of potassium
172 monobasic phosphate at pH 6.8 (adjusted using 5 *M* potassium hydroxide solution), running
173 isocratically over 3.5 min at a flow rate of 1 ml min^{-1}. Data were collected using a

174 fluorescence detector (RF2000; Dionex) set at 515 nm (excitation) and 553 nm (emission).
175 For calibration a standard curve was prepared using a TEP stock solution (5 m*M* in 40 %
176 ethanol) serially diluted using 40 % ethanol. Data are presented as nmols MDA per g tissue.

177 *(iv) Cardenolide analysis*

178 For cardenolide analysis, samples (individual wings and the remaining half of the body) were
179 dried in an oven at 60 °C for 16 h. The dried material was ground to a fine powder using a
180 pestle and mortar, and weighed to the nearest 0.0001 g using a GR-202 electronic balance
181 (A&D Instruments Ltd., Abington, UK). The samples were placed in screw-top
182 polypropylene tubes for de-fatting and cardenolide extraction. To remove fats, 2 ml hexane
183 was added and samples were incubated at 35°C for 30 min, before being centrifuged at 10 x g
184 and 4ºC for 5 min; the supernatant was collected and discarded. Ethanol (1.9 ml) containing
185 20 µg digitoxin (#D5878; Sigma-Aldrich, Dorset, UK) as an internal standard were added to
186 the pellet and it was briefly vortexed to extract cardenolides (Ritland 1994). Samples were
187 placed in an XUB5 ultrasonic bath (Grant Instruments, Shepreth, UK) at 60°C for 60 min,
188 followed by further centrifugation (9.6 x g and 4ºC for 5 min). The supernatant was removed
189 and the remaining pellet dried using a Savant ISS 110 SpeedVac Concentrator at room
190 temperature (Thermo Scientific, Altrincham, UK). Samples were then dissolved in 0.5 ml
191 methanol by vortexing, and 50 µl of the solution was injected into a Dionex HPLC system
192 fitted with a Waters Spherisorb 5µm ODS2 column (150 x 4.6 mm) maintained at 20°C. A
193 multistep gradient of acetonitrile (ACN) and ultra pure water was used as the mobile phase,
194 as follows: initial concentration 20 % ACN held for 5 minutes, ramp to 70 % ACN at 20 mins
195 and held until 25 minutes, ramp to 95 % ACN at 30 mins and held until 35 mins, returning to
196 20 % ACN at 40 mins, with an end time of 50 mins. The flow rate was 0.7 ml min^{-1}. Peaks
197 were collected using a PDA-100 photodiode array detector (Dionex) at an absorbance value
198 of 218 nm. Spectral data between 200-400 nm were collected, and cardenolides were

199 identified as peaks with a symmetrical absorbance band with a maximum absorbance
200 between 217-222 nm [41, 42]. Data are presented as µg total cardenolides per 0.1 g tissue.

201 ***(d) Measurements and visual modelling of colouration***

202 The reflectance of the fore- and hindwings of each butterfly, and of four leaves from one of
203 the representative backgrounds of the leaves of *Asclepias syriaca* (obtained from A.
204 Agrawal), were measured using an Ocean Optics USB2000 spectrophotometer, with
205 specimens illuminated at 45º to normal by a DH1000 balanced halogen deuterium light
206 source. The measuring spot diameter was 3 mm, with spectra recorded at 0.34nm intervals
207 from 300 to 700nm and measured relative to a WS-1 reflectance standard. For each wing, 3-4
208 non-overlapping measurements were taken per orange segment of the wing (figure s1; total
209 number of measurement spots per forewing = 13-14; hind wing = 28-29). For the leaves we
210 took ten non-overlapping measurements. Spectrophotometry data were recorded using Ocean
211 Optics OOBase32.

212 Reflectance spectra were reduced to 1nm intervals ranging from wavelengths 300-700 nm
213 using customised MATLAB code (written by I. C. Cuthill). To determine differences in
214 chroma between treatments, we modelled the predicted photon catches of a generalized
215 passerine bird (passerines being the main avian predators of monarchs [43]) for each
216 spectrum following the Vorobyev-Osorio model [44], using a customized MATLAB code
217 (provided by I. C. Cuthill, 2006). We used the cone types of a blue tit, *Cyanistes caeruleus*
218 [45, longwave, LWS, λmax 563nm; mediumwave, MWS, λmax 503 nm; shortwave, SWS,
219 λmax 448 nm ; ultraviolet, UVS, λmax 371 nm; and double dorsal, DD λmax 563 nm;
220 46].We calculated redness as the ratio of long wave to medium wave cone responses. We also
221 calculated a measure of conspicuousness as ΔS between the butterflies and *A. syriaca* leaves
222 using the Vorobyev-Osorio colour discrimination model, which is based on evidence that
223 colour discrimination is determined by noise arising in the photoreceptors and is independent

224 of light intensity. We used a Weber fraction value of 0.05 for the most abundant cone type
225 [47]. We calculated luminance as the response of the dorsal double cone [48].

226 ***(e) Data analyses***

227 Left- and right-wing cardenolide concentration (from here on CG) were significantly
228 positively correlated (r = 0.96, t = 20.75, df = 38, p = 2.2e-16). There was no significant
229 correlation between asymmetry in wing CG concentrations and the mean CG of the wings (r
230 = 0.10, t = 0.64, df = 38, P = 0.52), and so for each individual we calculated a mean wing CG
231 concentration. Left- and right-wing redness were significantly positive correlated (r = 0.65, t
232 = 5.30, df = 38, P = 5.2e-06), as were left- and right-wing conspicuousness (r = 0.59, t = 4.41,
233 df = 37, P = 8.6e-05), and left- and right-wing luminance (r = 0.396, t = 2.66, df = 38, P =
234 0.0114). For each individual we calculated a mean forewing redness, conspicuousness, and
235 luminance.

236 We log transformed malondialdehyde (MDA) and wing and body CG and to normalise the
237 distribution of the residuals. We analysed the effect of foodplant on total CG content,
238 bodyCG and wingCG content using a GLM with a Gaussian distribution and identity link
239 function. We set *A. tuberosa* as the level with which we compared the other plants, because
240 of its low cardenolide content. Eclosion success or failure was analysed with a binomial
241 GLM. To analyse how cardenolides and oxidative stress affected colour metrics in the
242 butterflies we used two methods. First, we used the R drop1 function to remove single model
243 terms to find the minimal adequate model and compared models using AIC (see
244 supplementary tables s1-s11). We explored interactions between two continuous variables
245 using the coplot function in the R package car [49]. Second, we built a generalized linear
246 model including six covariates *a priori* expected to affect warning signals (three markers of
247 oxidative state and three measures of sequestration – body, wing, and total CG content). The
248 model response variables were conspicuousness, redness, or brightness. We standardized all

249 covariates to a mean of 0 and a standard deviation (SD) of 1 to facilitate comparisons
250 between models and variables. We ran all possible models containing each covariate (without
251 interactions) using the R package MuMIn [50] and summed the cumulative AIC support for
252 the models containing each covariate. MDA, body cardenolides, and total cardenolides had
253 AIC weights of 0.153, 0.081, and 0.075 (supplementary table s8). All data were analysed
254 using R version 4.0.4.

255 **Results**

256 *CG concentrations*

257 In support of our first hypothesis, concentrations of cardenolides in the body and wings of
258 newly eclosed adults differed according to which host-plant species they were reared on
259 (Figure 1a; $F_{(3,37)} = 8.262$, P = 0.0002). Monarchs reared on *Ac* and *As* sequestered
260 significantly more cardenolides in total than those reared on *At* (estimate = 822.9 ± 184.3, t =
261 4.47, P = 7.6e-05; estimate = 726.6 ± 187.1, t = 3.88, P =0.0004). Those reared on *Ai* did not
262 sequester significantly different total amounts of CGs to those on *At* (estimate = 213.9 ±
263 189.2, t = 1.13, P = 0.27). Males tended to sequester more cardenolides in total than females,
264 but this was not significant at the alpha 0.05 level (estimate = 243.5 ± 133.4, t = 1.825, P =
265 0.076).

266 Body CG concentration tended to differ between food plants, but this was not significant at
267 the alpha 0.05 level (Figure 1b; $F_{(4,37)}= 2.471$, P = 0.061). Wing CG concentration
268 significantly varied between host-plants (Figure 1c; $F_{(3,37)}= 26.23$, P <0.001 ; Fig. 1c). Wing
269 CGs were significantly higher in *A*c and *As* compared to At (estimate = 1.56 ± 0.27, t =
270 5.698, P = 1.6e-06; estimate = 1.77 ± 0.29, t = 6.120, P = <0.001), but did not differ
271 significantly between *At* and *Ai* (p=0.38), nor between *As* and *Ac* (p= 0.79).

272 *Survival to pupation and eclosion*

We predicted that sequestration of cardenolides would impose a cost that would be associated with reduced survivorship to eclosion. Successful eclosion was significantly higher in monarchs reared on *At* (89%) compared with those reared on *As* (20%; estimate = -2.06 ± 0.751, t = 2.75, P = 0.009), while there was no significant difference between *Ai* (70%) and *At* (89%, estimate = -0.696 ± 0.728, t = -0.956, P = 0.34) or *At* (89%) and *Ac* (62%; estimate = -0.927 ± 0.690, t = -1.344, P = 0.19). Survival to pupation did not differ significantly according to host-plant species (0% *At*, 23% *Ai*, 33% *As*, 27% *Ac*; Fisher's exact test P = 0.28).

CG and oxidative stress

Malondialdehyde (MDA)

Contrary to our prediction that oxidative lipid damage would differ by hostplant, we found that the minimal adequate model did not include species of host-plant, and only included sex and the total concentration of cardenolide. In line with our prediction of a positive association between concentration of sequestered CGs and markers of oxidative damage, across all treatment groups individuals that sequestered higher total concentrations of CGs had higher levels of MDA (estimate = 0.0005 ± 0.0002, t = 2.51, P = 0.016). There was no significant difference between the sexes (estimate = -0.3097 ± 0.198, t = -1.57, P = 0.13).

Superoxide dismutase activity (SOD)

The minimal adequate model included only body CG concentration, which had no statistically significant association with SOD (estimate = 0.933 ± 0.616, t = 1.51, P = 0.14).

Total antioxidant capacity (TAC)

The minimal adequate model included foodplant, which did not significantly affect the concentrations of total antioxidant capacity (TAC: $F_{(3,38)}$= 0.3203, P = 0.81).

296 *Sequestration, oxidative stress and warning signals*

297 Because malondialdehyde was the only marker associated with individual levels of
298 sequestration, we proceeded to analyse its association with warning signals, but did not
299 conduct analyses on superoxide dismutase activity or total antioxidant capacity effects on
300 warning signals. We found a significant three-way interaction between MDA x total CG
301 concentration x sex on the conspicuousness (chroma ΔS) of monarch forewings (estimate = -
302 0.009 ± 0.005, t = -2.05, P = 0.049). Because males were more conspicuous than females, we
303 separated the analysis by sex. We found a significant two way-interaction between MDA and
304 total CG concentration on the conspicuousness (chroma ΔS) of male forewings (estimate = -
305 0.006 ± 0.002, t = -2.98, P = 0.007) that is represented by a curved surface (Figure 3). Males
306 with the highest levels of sequestered cardenolides had the most conspicuous warning signals
307 when oxidative damage was lowest, and reduced their warning signal conspicuousness as
308 oxidative damage increased, while those that sequestered the lowest levels of cardenolides
309 invested equally in warning signal conspicuous with increasing oxidative damage. There was
310 no significant effect of oxidative damage or concentration of sequestered cardenolides on
311 female forewing conspicuousness (MDA estimate = 0.320 ± 2.7859, t = 0.12, P = 0.911; CG
312 total estimate = -0.0005 ± 0.003, t = -0.14, P = 0.895). Males had significantly redder
313 forewings than females (estimate = 0.263 ± 0.063, t = 4.15, P = 0.0002), and as the
314 concentration of sequestered cardenolides increased the redness of the forewings increased
315 for both males and females, but this was not significant at the alpha 0.05 level (estimate =
316 0.0007 ± 0.0003, t = 1.95, P = 0.059). Monarchs with low levels of oxidative damage
317 produced redder warning signals with increasing concentrations of sequestered cardenolides,
318 whereas and monarchs with high levels of oxidative damage produced less red warning
319 signals with increasing concentrations of sequestered cardenolides, but this was also not
320 significant at the alpha 0.05 level (MDA x total CG interaction: estimate = -0.0002 ±

321 0.00009, t = -1.864, P = 0.071). Males had significantly brighter forewings than females
322 (estimate = 0.027 ± 0.007, t = 4.101, P = 0.0002). Both male and female monarchs with
323 higher levels of MDA had significantly less bright forewings (estimate = -0.01 ± 0.005, t =
324 2.04, P = 0.048).

325 **Discussion**

326 We reared individual monarch caterpillars on whole plants from single populations of four
327 milkweed species with varying phytochemistry [*Asclepias tuberosa*, *A. incarnata*, *A.syriaca*,
328 and *A.curassavica*; 51]. Monarchs that sequestered higher concentrations of cardenolides
329 experienced higher levels of oxidative damage than those that sequestered lower
330 concentrations (measured as the concentration of malondialdehyde; MDA). Although there is
331 some evidence that cardenolides can be a burden for monarch caterpillars [22, 52], our results
332 are some of the first to show physiological costs of sequestration for monarchs [see also 21,
333 39]. Male monarchs that sequestered the highest concentrations of cardenolides had the most
334 conspicuous warning signals when oxidative damage was lowest, and reduced their warning
335 signal conspicuousness as oxidative damage increased, while those that sequestered the
336 lowest levels of cardenolides invested equally in warning signal conspicuous with increasing
337 oxidative damage. Our results suggest that highly toxic prey reduce investment in signals
338 because they cannot bear the oxidative cost of investing highly in both traits. This is the first
339 evidence to support Blount et al's [16] resource allocation trade-off model, and shows that
340 variable access to resources can result in differential costs of signalling in monarch butterflies
341 [53].

342 Some authors [54] have rejected the idea that there can be a mechanistic link between
343 warning signals and chemical defences that involve intrinsic costs of production or
344 maintenance. However, condition-dependent expression of colourful signals can arise when
345 the underlying physiological pathways that produce colouration are dependent on the same

core cellular processes [16, 55]. We found stable levels of antioxidant defences (superoxidase dismutase and total antioxidant capacity) but increased oxidative damage was associated with reduced warning signal conspicuousness, which suggests that the monarchs that are better able to control pro-oxidant levels can sequester more cardenolides and still invest in warning signals [56]. Similar patterns are observed in *Aristolochia*-feeding pipevine swallowtails butterflies (*Battus philenor*) that store nitrophenanthrenes and have significantly higher tissue levels of carotenoids than related species which mimic them in coloration but do not store toxins [57]. Similarly, the rank order of body concentrations of carotenoids in three species of lepidoptera matches the rank order of dietary exposure to the prooxidant toxin furanocoumarin (8-MOP) [Papilio polyxenes > Spodoptera eridania > Trichoplusia ni; 18]. Antioxidants are involved in detoxification processes [58] as well as pigment synthesis pathways directly [59], or as cofactors of enzymes [60]. Our results allow us to speculate that antioxidant availability has a role in the biochemistry underlying the variations in warning colouration and toxicity in aposematic animals. However, warning colours are usually regulated by more than one mechanism, and we suggest that this area of research warrants further biochemical study.

It is likely that oxidative state depends on the combination of genetic, environmental and gene by environment interactions that determine an individual's condition. Monarchs show patterns of local adaptation to their hostplants [based on larval growth rate 39], and also show GxE interactions in sequestration ability [23] which may reflect either a lack of evolutionary history with different species of *Asclepias*, or a physiological trade-off in sequestration ability. Our results suggest that populations of monarchs that are locally adapted to high cardenolide milkweeds could be less subject to oxidative damage, either because they have evolved higher antioxidant efficiency, or because they are able to accumulate more antioxidants to provide enhanced antioxidant protection. Further research on this topic could

have interesting implications for understanding host shifts and range expansions as well as our understanding of automimicry (the occurrence of palatable 'cheaters' in a chemically defended population). Whether automimicry in monarchs arises directly from a deficiency in an individual's capacity to cope with reactive oxygen species warrants further study [61].

The resource competition model [16] does not investigate or predict sex differences in resource limitation. However, Blount et al [62] found that ladybird females (the larger sex) are more susceptible to resource limitation than males, and hence more likely to signal honestly. In this study we found costs of signalling in male monarchs. Males are slightly larger than females and are the more active sex; this behavioural difference may increase their probability of detection and risk of predation, and also increase their sensitivity to oxidative damage. If predators are sensitive to the combined qualities of toxins and displays when they attack prey, this could mean they avoid highly conspicuous prey and reject highly defended but less conspicuous prey, and instead predate moderately defended and conspicuous individuals, this could maintain the signal variability we observe in males and in other aposematic prey. The relationship between visual signal strength, chemical defence, and predator response warrants further study.

Although we did not measure cardenolide content in milkweed plants, our results confirm previous research showing that the concentration of cardenolides in monarch butterflies varies depending on host-plant chemistry [63, 64], and follows a standard approach to using the natural between species and population variability in plant toxicity to test for costs of sequestration [21, 37, 51]. However, it is methodologically difficult to separate the costs of sequestration from other potential effects of plant allelochemicals [65]. Future work could focus on comparative phytochemical profiles to understand resource availability, and experiments that control nutritional content while manipulating toxin content.

In conclusion, our results support the proposal that oxidative state can be a key physiological mechanism that links warning colours to sequestration costs [16], and that the contribution of pigments to antioxidant activity for maintaining redox homeostasis is important [61]. Our results show that specialist herbivores must balance the benefits of plant secondary compounds for sequestration with the burden that these same compounds impose [though see 51, 66]. Future research should examine the possibly of natural selection for oxidative capacity, and whether aposematism evolves in species that have high antioxidant capacity. These studies could provide new theories for understanding the evolution of aposematism when coloration and toxicity do not coevolve. Documenting the costs associated with using secondary defences in natural systems is important for our understanding of the ecology and evolution of aposematism.

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Christopher Mitchell investigation, data curation.

Michael P. Speed conceptualization, methodology, writing - review & editing.

Graeme D. Ruxton conceptualization, methodology, writing - review & editing.

John A. Endler conceptualization, writing - review & editing, visualization.

Lincoln P. Brower conceptualization, methodology, investigation, resources.

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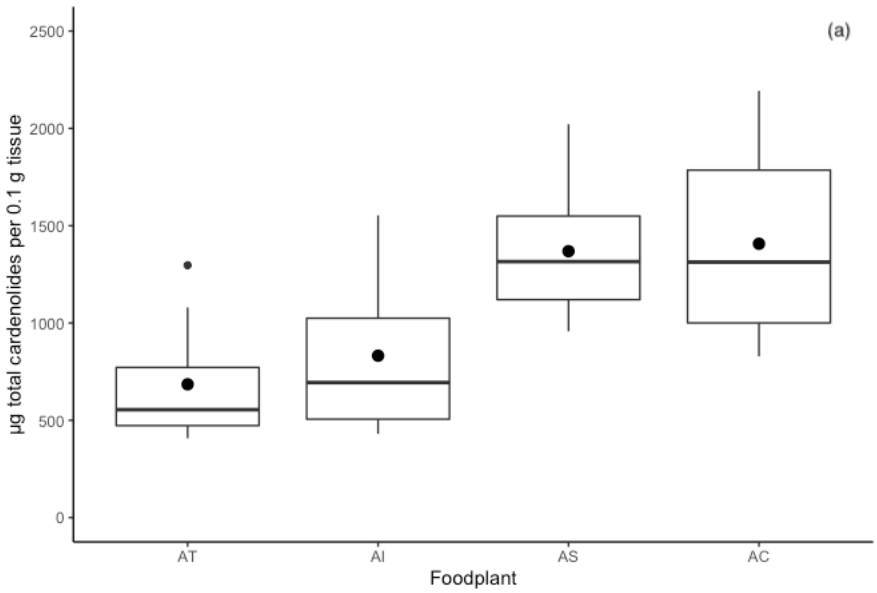
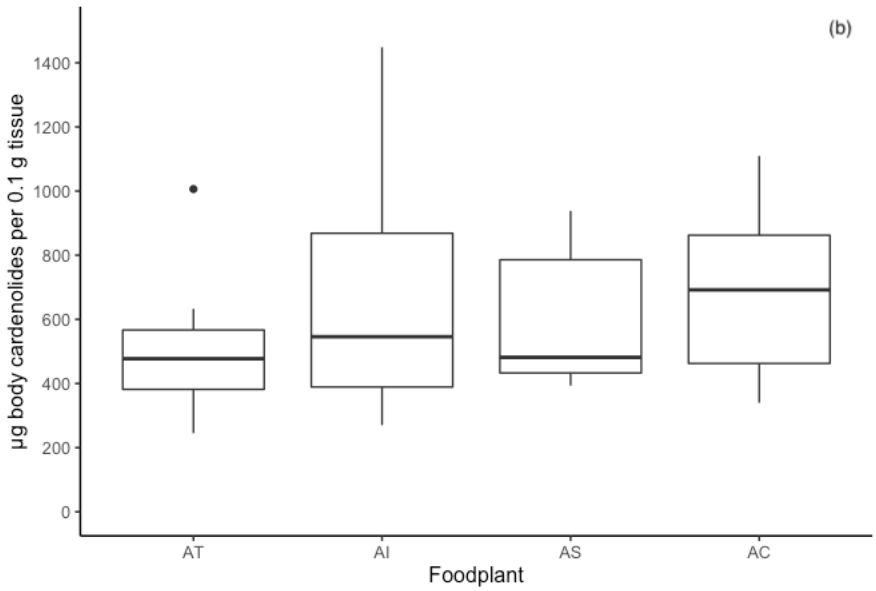
Figure 1. cardenolide sequestration by monarchs feeding on *Ascelpias tuberosa* (AT), *A. Incarnata* (AI), *A. Syriaca* (AS), and *A. curassavica* (AC); a) total cardenolide (µg total cardenolides per 0.1 g tissue) measured in the wings and bodies, b) body cardenolide content, c) wing cardenolide content. Boxplots show the median, interquartile range, and the whiskers represent the largest and smallest value within 1.5 times the 25th and 75th percentile. Outliers are represented by small black dots. The mean cardenolide concentration is represented by the larger black dot.

619 **Figure 2**. Total cardenolide concentration for males (filled triangle) and females (open circle)
620 and Malondialdehyde (MDA) concentration. Line is the smoothed conditional mean with
621 95% confidence intervals.

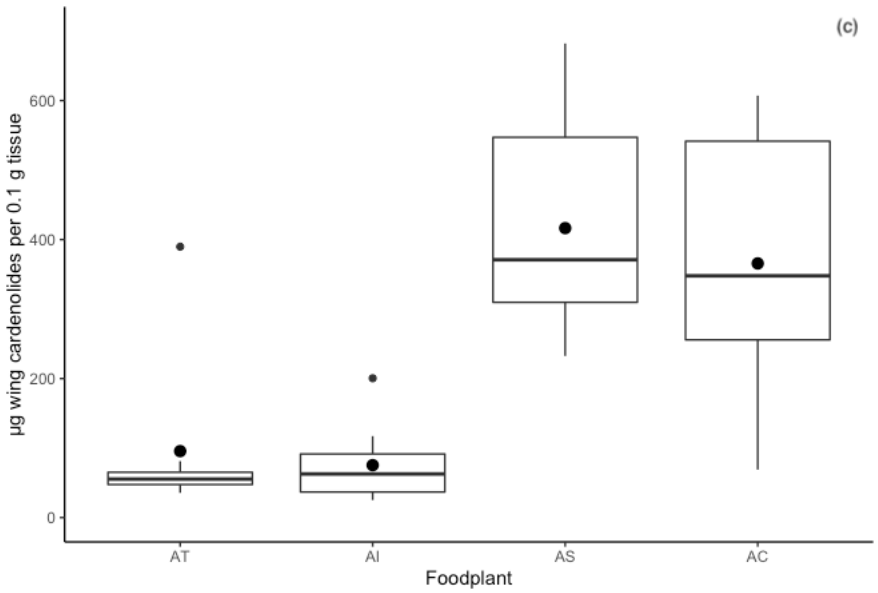
622 **Figure 3.** Three-dimensional surface (perspective) plot for the response surface predicting
623 the relationship between the conspicuousness of the male monarch's forewing, oxidative
624 damage measured as malondialdehyde (MDA), and total cardenolide concentration.


(a)
µg total cardenolides per 0.1 g tissue
2500
2000
1500
1000
500
0
AT
AI
AS
AC
Foodplant

625

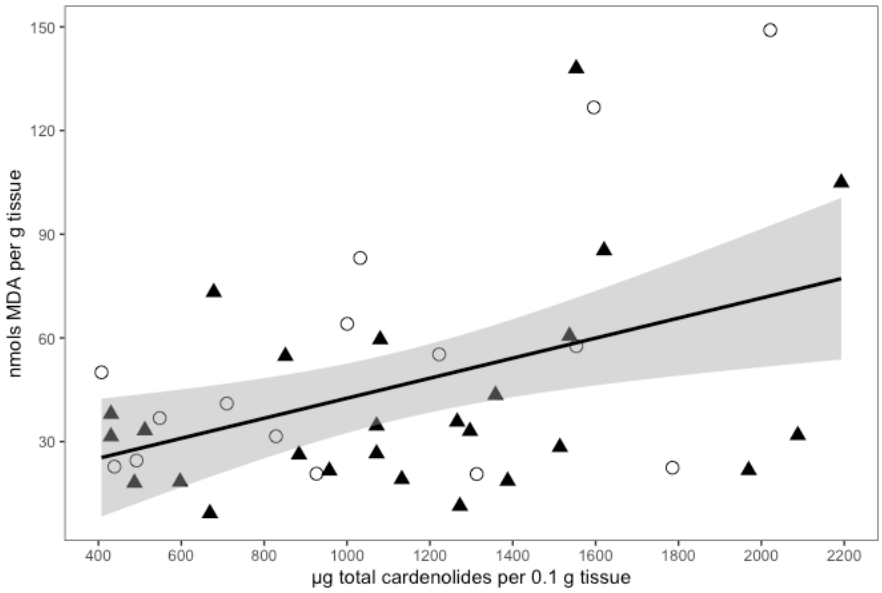

(b)
µg body cardenolides per 0.1 g tissue
1400
1200
1000
800
600
400
200
0
AT
AI
AS
AC
Foodplant

626



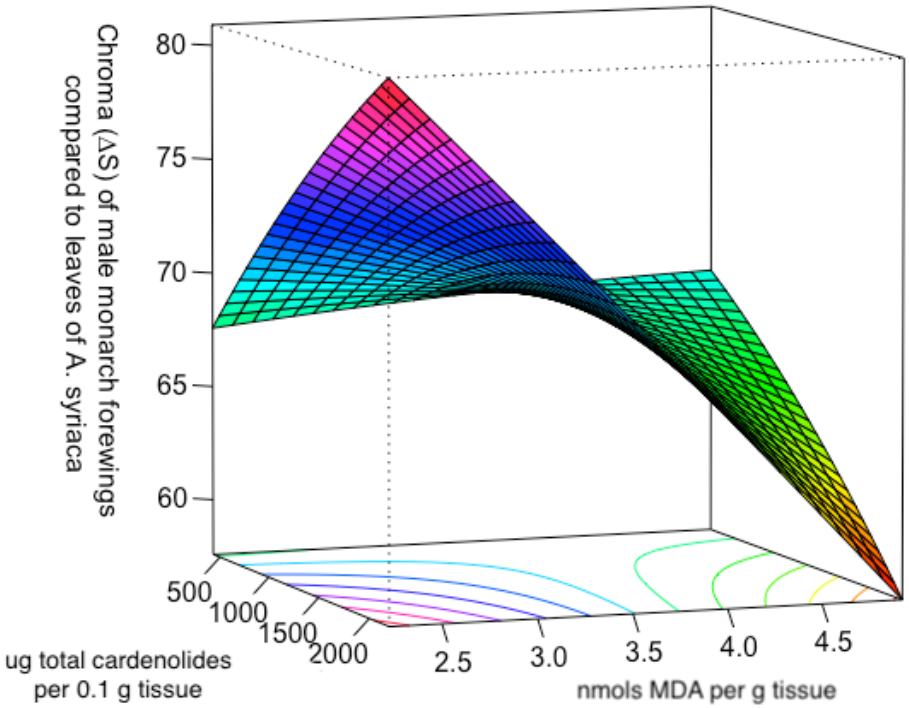
627

628 Figure 1a, b, c.



629

630 Figure 2



631

632 Figure 3