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 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 and in protecting against oxidative damage. We raised monarch butterflies (*Danaus plexippus*) on their milkweed host-plants (Apocynaceae) with increasing quantities of cardenolides to test whether (1) the sequestration of secondary defences is associated with costs in the form of oxidative lipid damage and reduced antioxidant defences; and (2) that reduced oxidative state can decrease the capacity of individuals to produce aposematic displays. In male monarchs conspicuousness was explained by an interaction between oxidative damage and sequestration: males with high levels of oxidative damage become less conspicuous with increased sequestration of cardenolides, whereas those with low oxidative damage become more conspicuous with increased levels of cardenolides. There was no significant effect of oxidative damage or concentration of sequestered cardenolides on female conspicuousness. Our results demonstrate a physiological linkage between the production of coloration and protection from autotoxicity, and differential costs of signalling in monarch butterflies.

Key-words: aposematism, cardenolides, cardiac glycosides, resource competition, oxidative lipid damage, *Danaus plexippus*, 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 some 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 some Dendrobatid poison frogs [14] including *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 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 (figure 1a, c). 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 higher chances of surviving attacks (figure 1b, d [16]). Directly controlling antioxidant availability is challenging experimentally, consequently it is unclear whether or not colour-toxin relationships are influenced by the resource state of prey [14, 15], and has not yet been examined experimentally [11, 12, 16, 17, 19].

We manipulated 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 achieved this by using a model aposematic system, the monarch butterfly (*Danaus plexippus*), that selectively metabolises cardenolides present in its host-plants and stores them in its body [20, 21]. We used 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 for an individual to sequester. We test whether (i) the quantity of sequestered secondary defence by caterpillars is associated with levels of oxidative lipid damage and antioxidant defences in adults; and (ii) 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

70 cardenolide sequestration [23] we used monarchs from a single population. We reared them
71 on four milkweed species (see table 1) that show within- and between-species, and among-
72 population variation in cardenolide concentration, diversity, and polarity [22, 24-26]. Based
73 on the published records of biological activity and chromatic quantification (e.g., HPLC and
74 thin layer chromatography) of cardenolides of milkweeds in different populations/latitudes
75 [27, 28], our four plant species were used to represent a gradient of low to high cardenolide
76 defences, with the aim to create different costs of sequestration. We predicted that
77 concentrations of cardenolides in the body and wings of newly eclosed adults would differ
78 according to the cardenolides of the host-plant on which they were reared (see methods): *A.*
79 *curassavica* (*Ac*) ≥ *A. syriaca* (*As*) > *A. incarnata* (*Ai*) ≥ *A. tuberosa* (*At*).

80 Because sequestration, modification, and storage of allelochemicals are believed to be
81 oxidatively stressful for many chemically defended organisms [18], we predicted that body
82 levels of oxidative lipid peroxidation would differ amongst individuals according to host-
83 plant, as follows: *Ac*≥*As*>*Ai*≥*At*. In turn, we predicted that survival to eclosion would differ
84 according to host-plant as follows: *At*≥*Ai*>*As*≥*Ac*, and that the redness, luminance, and
85 conspicuousness of the warning signals would differ: *Ac*≥*As*>*Ai*≥*At*. In addition to testing for
86 treatment effects, we examined associations between individual levels of cardiac glycosides
87 (CGs), oxidative state, and warning signal redness, luminance, and conspicuousness. We
88 predicted a positive association between levels of CGs and lipid peroxidation, a negative
89 association of CGs with antioxidant defences, and that highly toxic prey will be the most
90 conspicuous [16].

91 **Materials and methods**

92 ***(a) Milkweed plants***

93 Milkweeds (*A. incarnata, A. tuberosa, A. curassavica, and A. syriaca*) were locally sourced
94 in Amherst, Virginia, USA. We did not measure cardenolide content in the leaves of the
95 plants used in the experiment, but used published records about the spatial repeatability of
96 cardenolide defences that take into account latitudinal clines [27-29]. From these records we
97 predicted that for milkweeds grown in Mid-Atlantic and Southeastern regions of the United
98 States, total cardenolide content would vary in the order *A. curassavica* (*Ac*) ≥ *A. syriaca* (*As*)
99 > *A. incarnata* (*Ai*) ≥ *A. tuberosa* (*At*). The plants were grown without the use of pesticides as
100 described previously [30]. All plants were healthy with undamaged leaves at the start of the
101 experiment.

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

103 Eggs were obtained from wild caught Monarch females in Virginia (USA) that were kept in
104 captivity until they laid eggs following previous methods [31]. Larvae were reared by LPB on
105 single plants under the same conditions. All larvae had abundant food during the experiment.
106 Larvae were monitored daily for pupation starting at Day 12.

107 Upon eclosion, the date and the individual's sex were recorded. Adults were placed in
108 glassine envelopes and frozen on dry ice until dead, then stored in a freezer at -80ºC before
109 being shipped to the UK on dry ice. Adults were stored at -80ºC in JDB's laboratory until
110 they were analysed.

111 For analysis, the gut was removed, and the head, thorax and abdomen (hereafter, 'body')
112 were bisected longitudinally. One half of the body was returned to storage at -80ºC for later
113 analysis of cardenolides. The other half of the body was immediately homogenised in ice-
114 cold phosphate-buffered saline (PBS; 5 % w/v), for analyses of oxidative stress. The wings
115 were removed using dissecting scissors and shipped on dry ice to HMR's laboratory for

116 analyses of colouration (see below). Wings were then returned (on dry ice) to JDB's
117 laboratory for cardenolide analysis.

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

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

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

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

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

136 TAC was assayed by measuring the capacity of tissue homogenate to inhibit the oxidation of
137 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (#709001; Cayman Chemical). The
138 homogenate was further diluted with sample buffer (1:5 v/v) to bring the absorbance values

139 within the range of the standard curve. Samples were assayed in duplicate, as per the kit
140 instructions. Data are reported as nmols of TAC activity (Trolox equivalents) per g tissue.

141 *(iii) Malondialdehyde (MDA)*

142 MDA was measured using HPLC with fluorescence detection as described previously for
143 tissue homogenates (e.g. [32]). All chemicals were HPLC grade, and chemical solutions were
144 prepared using ultra pure water (Milli-Q Synthesis; Millipore, Watford, UK). Samples were
145 derived in 2 mL capacity polypropylene screw-top microcentrifuge tubes. For tissue
146 homogenates and for the standard (1,1,3,3-tetraethoxypropane, TEP; see below) a 20 µl
147 aliquot was added to 20 µl butylated hydroxytoluene solution (BHT; 0.05% w/v in 95 %
148 ethanol), 160 µl phosphoric acid solution (H_3PO_4; 0.44 *M*), and 40 µl thiobarbituric acid
149 solution (TBA; 42 m*M*). Samples were capped, vortex mixed for 5 s, then heated at 100 ^{o}C
150 for 1 h in a dry bath incubator to allow formation of MDA-TBA adducts. Samples were then
151 cooled on ice for 5 min, before 80 µl *n*-butanol was added and tubes were vortex mixed for
152 20s. Tubes were centrifuged at 12,000 x g and 4 ^{o}C for 3 min, before the upper (*n*-butanol)
153 phase was collected and transferred to an HPLC vial for analysis. Samples (10 µl) were
154 injected into a Dionex HPLC system (Dionex Corporation, California, USA) fitted with a
155 5µm ODS guard column and a Hewlett-Packard Hypersil 5µ ODS 100 x 4.6 mm column
156 maintained at 37 ^{o}C. The mobile phase was methanol-buffer (40:60, v/v), the buffer being a
157 50 m*M* anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5 *M*
158 potassium hydroxide solution), running isocratically over 3.5 min at a flow rate of 1 ml min^{-1}.
159 Data were collected using a fluorescence detector (RF2000; Dionex) set at 515 nm
160 (excitation) and 553 nm (emission). For calibration a standard curve was prepared using a
161 TEP stock solution (5 m*M* in 40 % ethanol) serially diluted using 40 % ethanol. Data are
162 presented as nmols MDA per g tissue.

163 *(iv) Cardenolide analysis*

164 For cardenolide analysis, samples (individual wings and the remaining half of the body) were
165 dried in an oven at 60 °C for 16 h. The dried material was ground to a fine powder using a
166 pestle and mortar, and weighed to the nearest 0.0001 g using a GR-202 electronic balance
167 (A&D Instruments Ltd., Abington, UK). The samples were placed in screw-top
168 polypropylene tubes for de-fatting and cardenolide extraction. To remove fats, 2 ml hexane
169 was added and samples were incubated at 35°C for 30 min, before being centrifuged at
170 10,000 x g and 4ºC for 5 min; the supernatant was collected and discarded. Ethanol (1.9 ml)
171 containing 20 µg digitoxin (#D5878; Sigma-Aldrich, Dorset, UK) as an internal standard
172 were added to the pellet and it was briefly vortexed to extract cardenolides (Ritland 1994).
173 Samples were placed in an XUB5 ultrasonic bath (Grant Instruments, Shepreth, UK) at 60°C
174 for 60 min, followed by further centrifugation (9,600 x g and 4ºC for 5 min). The supernatant
175 was removed and evaporated to dryness using a Savant ISS 110 SpeedVac Concentrator at
176 room temperature (Thermo Scientific, Altrincham, UK). Samples were then dissolved in 0.5
177 ml methanol by vortexing, and 50 µl of the solution was injected into a Dionex HPLC system
178 fitted with a Waters Spherisorb 5µm ODS2 column (150 x 4.6 mm) maintained at 20°C. A
179 multistep gradient of acetonitrile (ACN) and ultra pure water was used as the mobile phase,
180 as follows: initial concentration 20 % ACN held for 5 minutes, ramp to 70 % ACN at 20 mins
181 and held until 25 minutes, ramp to 95 % ACN at 30 mins and held until 35 mins, returning to
182 20 % ACN at 40 mins, with an end time of 50 mins. The flow rate was 0.7 ml min^{-1}. Peaks
183 were collected using a PDA-100 photodiode array detector (Dionex) at an absorbance value
184 of 218 nm. Spectral data between 200-400 nm were collected, and cardenolides were
185 identified as peaks with a symmetrical absorbance band with a maximum absorbance
186 between 217-222 nm [33, 34]. Data are presented as µg total cardenolides per 0.1 g tissue.

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

188 The reflectance of the forewings of each butterfly, and of four leaves from *Asclepias syriaca*
189 (as a representative background that the butterflies can rest on. Obtained from A. Agrawal),
190 were measured using an Ocean Optics USB2000 spectrophotometer, with specimens
191 illuminated at 45º to normal by a DH1000 balanced halogen deuterium light source. The
192 measuring spot diameter was 3 mm, with spectra recorded at 0.34nm intervals from 300 to
193 700nm and measured relative to a WS-1 reflectance standard. For each wing, 3-4 non-
194 overlapping measurements were taken per orange segment of the wing (figure s1; total
195 number of measurement spots per forewing = 13-14). For the leaves we took eleven non-
196 overlapping measurements. Spectrophotometry data were recorded using Ocean Optics
197 OOBase32.

198 To determine differences in chroma between treatments, we modelled the predicted photon
199 catches of a generalized passerine bird (passerines being the main avian predators of
200 monarchs [35]) for each spectrum following the Vorobyev-Osorio model [36], using the R
201 package PAVO [37]. We used the cone types of a blue tit, *Cyanistes caeruleus* [38],
202 longwave, LWS, λmax 563nm; mediumwave, MWS, λmax 503 nm; shortwave, SWS, λmax
203 448 nm ; ultraviolet, UVS, λmax 371 nm; and double dorsal, DD λmax 563 nm]. We
204 calculated redness as the ratio of long wave to medium wave cone responses. We also
205 calculated a measure of chromatic conspicuousness as ΔS between the mean colour of the
206 forewings of the butterflies and mean colour of the *A. syriaca* leaves using the Vorobyev-
207 Osorio colour discrimination model, which is based on evidence that colour discrimination is
208 determined by noise arising in the photoreceptors and is independent of light intensity. We
209 used a Weber fraction value of 0.05 for the most abundant cone type [39]. We calculated
210 luminance as the response of the dorsal double cone [40].

211 ***(e) Data analyses***

Left- and right-wing cardenolide concentration (from here on CG) were significantly positively correlated ($r = 0.96$, $t = 20.75$, $df = 38$, $p = 2.2e-16$). There was no significant correlation between asymmetry in wing CG concentrations and the mean CG of the wings ($r = 0.10$, $t = 0.64$, $df = 38$, $P = 0.52$), and so for each individual we calculated a mean wing CG concentration. Left- and right-wing redness were significantly positive correlated ($r = 0.85$, $t = 10.27$, $df = 38$, $P < 0.0001$), as were left- and right-wing conspicuousness ($r = 0.86$, $t = 0.33$, $df = 38$, $P < 0.0001$), and left- and right-wing luminance ($r = 0.47$, $t = 3.32$, $df = 38$, $P = 0.002$). For each individual we calculated a mean forewing redness, conspicuousness, and luminance.

We log transformed malondialdehyde (MDA) and wing and body CG to normalise the distribution of the residuals. We analysed the effect of foodplant on total CG content, bodyCG and wingCG content using a GLM with a Gaussian distribution and identity link function. We set *A. tuberosa* as the level with which we compared the other plants, because of its low cardenolide content. Eclosion success or failure was analysed with a binomial GLM. To analyse how cardenolides and oxidative stress affected colour metrics in the butterflies we constructed a full model that included all pairwise interactions and a three-way interaction between MDA x total CG concentration x sex and compared this to a simple model that included only the fixed effects of MDA, total CG concentration and sex. We compared the reduced model to the full model by using information criteria (AIC; see supplementary tables s1-s13). We visualized the interaction between two continuous variables using the rsm package in the R [41]. All data were analysed using R version 4.0.4.

Results

CG concentrations

In support of our first hypothesis, concentrations of cardenolides in the body and wings of newly eclosed adults differed according to which host-plant species they were reared on (Figure 2a; $F_{(4,36)} = 7.42$, P = 0.0002). Monarchs reared on *Ac* and *As* sequestered significantly more cardenolides in total than those reared on *At* (*Ac vs At:* estimate = 822.9 ± 184.3, t = 4.47, P = 7.6e-05; *As* vs *At*: estimate = 726.6 ± 187.1, t = 3.88, P =0.0004). Those reared on *Ai* did not sequester significantly different total amounts of CGs to those on *At* (estimate = 213.9 ± 189.2, t = 1.13, P = 0.27). Males tended to sequester more cardenolides in total than females, but this was not significant at the alpha 0.05 level (estimate = 243.5 ± 133.4, t = 1.825, P = 0.076).

Body CG concentration tended to differ between food plants, but this was not significant at the alpha 0.05 level (Figure 2b; $F_{(4,37)}$= 2.471, P = 0.061). Wing CG concentration significantly varied between host-plants (Figure 1c; $F_{(4,36)}$= 19.25, P <0.001 ; Fig. 2c). Wing CGs were significantly higher in *A*c and *As* compared to At (*Ac* vs *At*: estimate = 1.59 ± 0.29, t = 5.48, P < 0.0001; *As* vs *At*: estimate = 1.78 ± 0.30, t = 6.05, P = <0.001), but did not differ significantly between *At* and *Ai* (estimate = -0.05 ± 0.29, τ = –0.18, p=0.38).

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 no significant effect of hostplant or sex on MDA (main effect $F_{4, 37} = 0.77$, $P = 0.55$). 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 (figure 3; main effect: $F_{2, 38} = 4.08$, $P = 0.025$; CG: 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)

There was no significant effect of foodplant or sex on SOD activity (main effect $F_{4, 37} = 0.28$, $P = 0.89$). We found no significant effect of total CG concentration or sex on SOD activity (main effect: $F_{2,38} = 0.52$, $P = 0.60$).

Total antioxidant capacity (TAC)

There was no significant effect of foodplant or sex on TAC (: $F_{(4,37)}= 0.42$, $P = 0.79$) or total CG concentration ($F_{2,38} = 0.19$, $P = 0.83$).

Sequestration, oxidative stress and warning signals

Because malondialdehyde was the only marker associated with individual levels of sequestration, we proceeded to analyse its association with warning signals, but did not conduct analyses on superoxide dismutase activity or total antioxidant capacity effects on warning signals. Monarchs varied significantly in conspicuousness (chroma (ΔS): $F_{3,35} =17.85$ $P < 0.0001$). Males were significantly more conspicuous in chroma (ΔS) than females (estimate = 5.30 ± 0.82, t = 6.49, P < 0.001), and both sexes decreased in conspicuousness

with increasing MDA, though this was not significant at the alpha 0.05 level (estimate = -1.26 ± 0.65, t = -1.95, p = 0.06). There was no significant effect of CG concentration on wing conspicuousness (estimate = -0.00002 ± 0.0008, t = -0.03, P = 0.98). Because of the significant sexual dimorphism in colour and sequestration, we separated the analysis by sex and found a significant two way-interaction between MDA and total CG concentration on the conspicuousness (chroma ΔS) of male forewings (main effect: $F_{3,21}$ = 8.54, P = 0.0007; MDA x CG: estimate = -0.003 ± 0.001, t = -3.24, P = 0.004) that is represented by a curved surface (Figure 4). Males with the highest levels of sequestered cardenolides had the most conspicuous warning signals when oxidative damage was lowest, and reduced their warning signal conspicuousness as oxidative damage increased, while those that sequestered the lowest levels of cardenolides showed no change in warning signal conspicuousness across all levels of oxidative damage. The conspicuousness of males warning signals decreased with increasing sequestration in males with high oxidative damage, whereas conspicuousness increased in males with low oxidative damage with increasing sequestration. There was no significant effect of oxidative damage or concentration of sequestered cardenolides on female forewing conspicuousness (F = $_{3,10}$ = 0.06, P = 0.9819).

Males also had significantly redder forewings than females (main effect: $F_{3,35}$ = 5.81, P = 0.002; sex estimate = 0.24 ± 0.06 , t = 3.82, P = 0.0005), but there was no significant effect of MDA or CG concentration on male and female redness (MDA: estimate = -0.04 ± 0.049, t = -0.86, p = 0.40; CG concentration: estimate = 0.00001 ± 0.00006, t = 0.16, P = 0.87). We found a significant difference in brightness of the monarchs ($F_{3,\,35}$ = 10.19, P = 0.00006). Males were significantly brighter than females (estimate = 0.03 ± 0.007, t = 4.37, p = 0.0001) and both sexes decreased in brightness with increasing MDA (estimate = -0.01 ± 0.005, t = -2.40, p = 0.02). There was no significant effect of CG concentration on wing brightness (estimate = 0.000003 ± 0.000007, t = 0.37, P = 0.70).

307 **Discussion**

308 We reared individual monarch caterpillars on whole plants from single plant populations of
309 four milkweed species with varying phytochemistry (*Asclepias tuberosa*, *A. incarnata*,
310 *A.syriaca*, and *A.curassavica*; [42]). Monarchs that sequestered higher concentrations of
311 cardenolides experienced higher levels of oxidative damage than those that sequestered lower
312 concentrations (measured as the concentration of malondialdehyde; MDA). Although there is
313 some evidence that cardenolides can be a burden for monarch caterpillars [22, 43], our results
314 are some of the first to show a potential physiological mechanism of oxidative damage as a
315 cost of sequestration for monarchs (see also [21, 29]). We found a trade-off between
316 sequestration and conspicuousness in male monarchs mediated by oxidative damage. Males
317 with high oxidative damage had decreasing conspicuousness with increasing sequestration,
318 whereas males with low oxidative damage showed increased conspicuousness with increasing
319 sequestration. This is the first evidence to support Blount et al's [16] resource allocation
320 trade-off model – prey with lower oxidative damage are able to allocate more resources to
321 colour and toxicity than prey with higher oxidative damage – and shows that an individual's
322 ability to deal with oxidative damage can result in colour-toxin correlations in monarch
323 butterflies.

324 Some authors [44] have rejected the idea that there can be a mechanistic link between
325 warning signals and chemical defences that involve intrinsic costs of production or
326 maintenance of defences because there was no clear mediator. However, condition-dependent
327 expression of colourful signals can arise when the underlying physiological pathways that
328 produce colouration are dependent on the same core cellular processes [16, 45]. We found
329 stable levels of antioxidant defences (superoxidase dismutase and total antioxidant capacity)
330 but increased oxidative damage was associated with reduced warning signal conspicuousness,
331 which suggests that the monarchs that are better able to control pro-oxidant levels can

332 sequester more cardenolides and still invest in warning signals [46]. Similar patterns are
333 observed in *Aristolochia*-feeding pipevine swallowtails butterflies (*Battus philenor*) that store
334 nitrophenanthrenes and have significantly higher tissue levels of carotenoids than related
335 species which mimic them in coloration but do not store toxins [47]. Similarly, the rank order
336 of body concentrations of carotenoids in three species of lepidoptera matches the rank order
337 of dietary exposure to the prooxidant toxin furanocoumarin (8-MOP) [Papilio polyxenes >
338 Spodoptera eridania > Trichoplusia ni; [18]]. Antioxidants are involved in detoxification
339 processes [48] as well as pigment synthesis pathways directly [49], or as cofactors of
340 enzymes [50]. Our results allow us to speculate that antioxidant availability has a role in the
341 biochemistry underlying the variations in warning colouration and toxicity in aposematic
342 animals. However, warning colours are usually regulated by more than one mechanism, and
343 we suggest that this area of research warrants further biochemical study.

344 It is likely that oxidative state depends on the combination of genetic, environmental and
345 gene by environment (GxE) interactions that determine an individual's condition. Monarchs
346 show patterns of local adaptation to their hostplants (based on larval growth rate [29]), and
347 also show GxE interactions in sequestration ability [23] which may reflect either a lack of
348 evolutionary history with different species of *Asclepias*, or a physiological trade-off in
349 sequestration ability. Our results suggest that populations of monarchs that are sympatric with
350 high cardenolide milkweeds could be less subject to oxidative damage, either because they
351 have evolved higher antioxidant efficiency, or because they are able to accumulate more
352 antioxidants to provide enhanced antioxidant protection. Further research on this topic could
353 have interesting implications for understanding host shifts and range expansions as well as
354 our understanding of automimicry (the occurrence of palatable 'cheaters' in a chemically
355 defended population). Whether automimicry in monarchs arises directly from a deficiency in
356 an individual's capacity to cope with reactive oxygen species warrants further study [51].

The resource competition model does not investigate or predict sex differences in resource limitation. However, Blount et al [52] 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. Males also sequestered more cardenolides than females. This behavioural difference between the sexes 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 moderately conspicuous individuals, this could maintain the signal variability we observe in males and in other aposematic prey.

Our results depend, to some extent, on the measure of signal conspicuousness measured against the leaves of one of the four hostplants (*A. syriaca*). The differences in conspicuousness of the forewings to the hostplant leaves range from (ΔS) 14 to 29. These 'suprathreshold' colour differences are often assumed to scale linearly with colour distance, but this is not the case in some visual systems [53]. Future research could calculate conspicuousness against a range of hostplant species and plant parts, as well as considering predator visual acuity and the effects of increasing viewing distance on warning signal conspicuousness. Experiments testing predator responses to the colour differences and the consequences for prey survival will also be important for determining whether the levels of measured conspicuousness are also perceived as visually distinct at a distance. 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 [54, 55], and follows a standard approach to using the natural between species and population variability in plant toxicity to test for costs of sequestration [21, 27, 42]. However, it is methodologically difficult to separate the costs of sequestration from other potential effects of plant allelochemicals [56]. Future work could focus on comparative phytochemical profiles to understand resource availability, and experiments that control nutritional content while manipulating toxin content [57].

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 [51]. 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 [42, 58]). 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.

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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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606

607 **Figure 1.** The resource allocation trade off model by Blount et al (2009) predicts that
608 individual prey acquire antioxidant resources from their environment (represented by the
609 circle resource pool), which they divide via genetically or physiologically controlled flow
610 rates (*f*) between sequestering defensive toxins (red circle) and in using antioxidant to
611 producing pigments used for warning signals (orange circle). Each individual or population
612 has access to a given level of resources (represented by the size of the circle) that they
613 allocate to each defence type. In scenario (a) prey allocate resources equally to colour and
614 toxicity. Those with more resources are able to allocate more resources than prey with a
615 smaller resource pool which results in (c) a positive correlation between colour and toxicity,
616 with only the most resource rich prey having the most colourful and toxic defences. In
617 scenario (b) resource availability is very high for some prey (represented by the largest circle
618 resource pool) and, in this situation, those prey with excess resources invest more in toxins
619 than warning signals (represented by the larger *f* and red circle), and results in (d) a negative
620 correlation between colour and toxicity. Blount et al (2009) suggest that this is because
621 highly toxic prey can protect themselves sufficiently even with low levels of warning
622 colours.

623 **Figure 2.** cardenolide sequestration by monarchs feeding on *Ascelpias tuberosa* (AT), *A.*
624 *Incarnata* (AI), *A. Syriaca* (AS), and *A. curassavica* (AC); a) total cardenolide (µg total
625 cardenolides per 0.1 g tissue) measured in the wings and bodies, b) body cardenolide content,
626 c) wing cardenolide content. Boxplots show the median, interquartile range, and the whiskers

627 represent the largest and smallest value within 1.5 times the 25th and 75th percentile. Outliers
628 are represented by small black dots. The mean cardenolide concentration is represented by
629 the larger black dot.

630 **Figure 3**. Total cardenolide concentration for males (filled triangle) and females (open circle)
631 and Malondialdehyde (MDA) concentration. Line is the smoothed conditional mean with
632 95% confidence intervals.

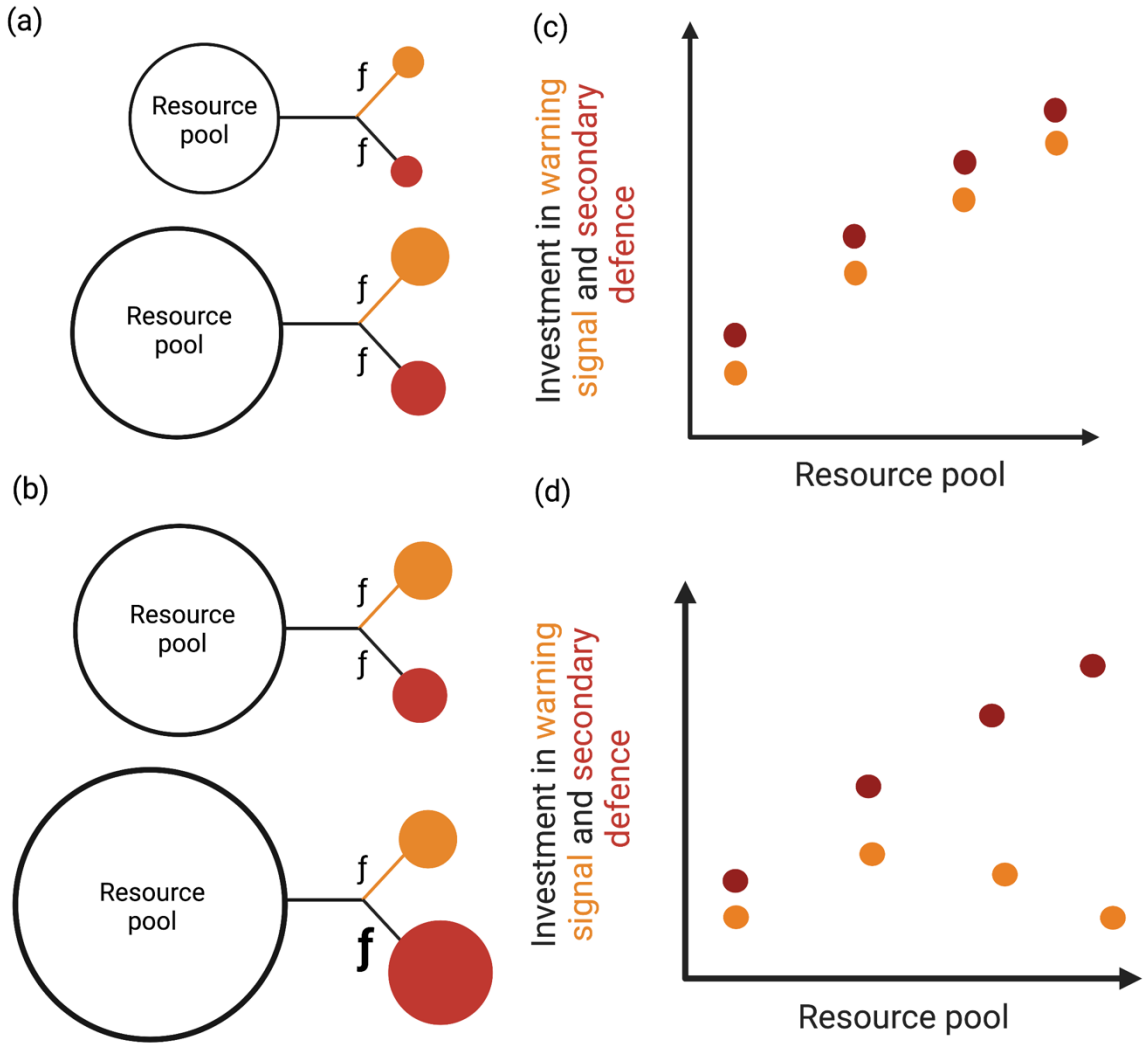
633 **Figure 4.** Three-dimensional surface (perspective) plot for the response surface predicting
634 the relationship between the conspicuousness of the male monarch's forewing, oxidative
635 damage measured as malondialdehyde (MDA), and total cardenolide concentration.

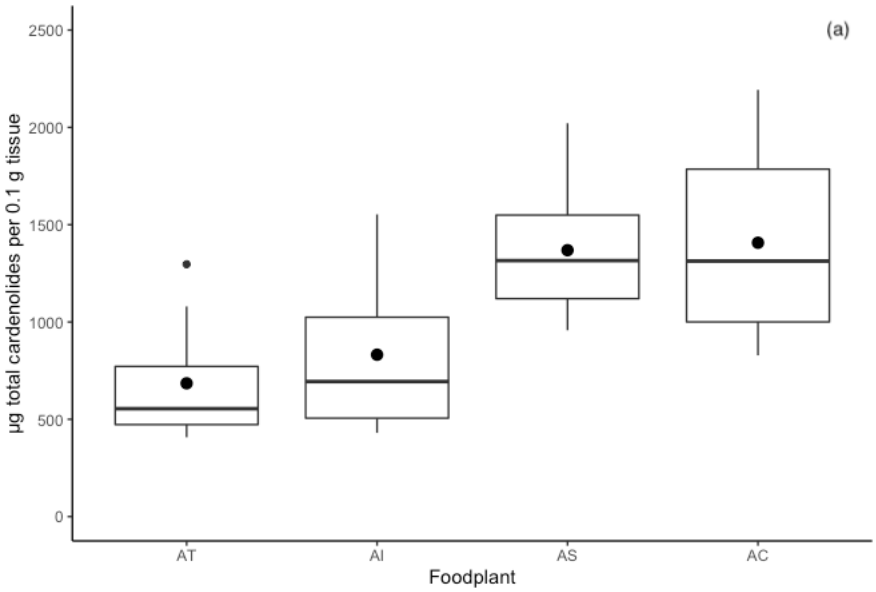
636 **Table 1** The four species of Asclepias used in thh rearing experiments with descriptions of
637 their use by monarchs and phytochemistry.

Ascelpias species	Description and cardenolide content
Ascelpias curassavica	A critical hostplant worldwide and contains a diversity of cardenolides, including apolar voruscharin which is detrimental to monarch performance [21, 59-62]
A. syriaca (common milkweed)	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 [63, 64]
A. incarnata (swamp milkweed)	Preferred hostplant in the Midwest USA and has low concentrations of cardenolides [58];

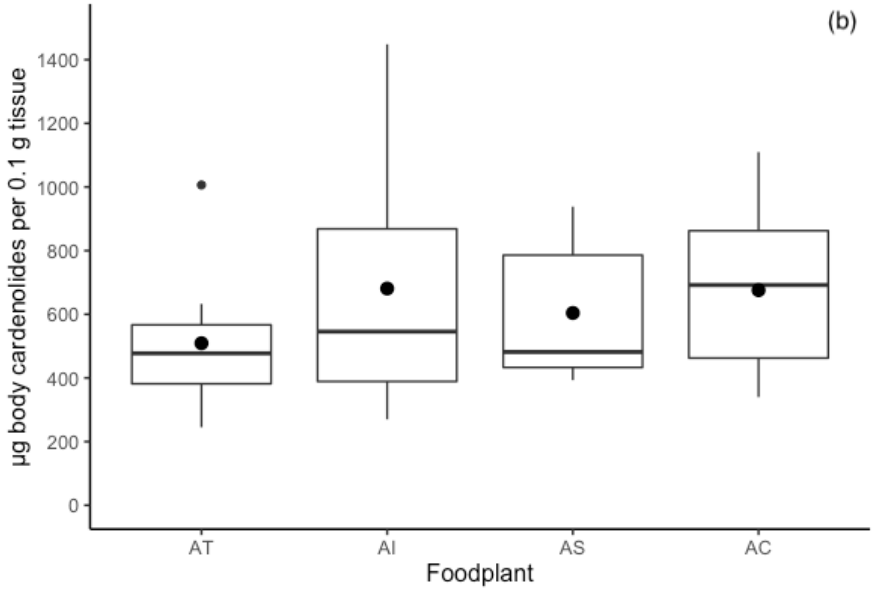
A. tuberosa (butterfly milkweed)	An occasional native foodplant in the wild with low concentrations of cardenolides [21, 65]

Figure 1

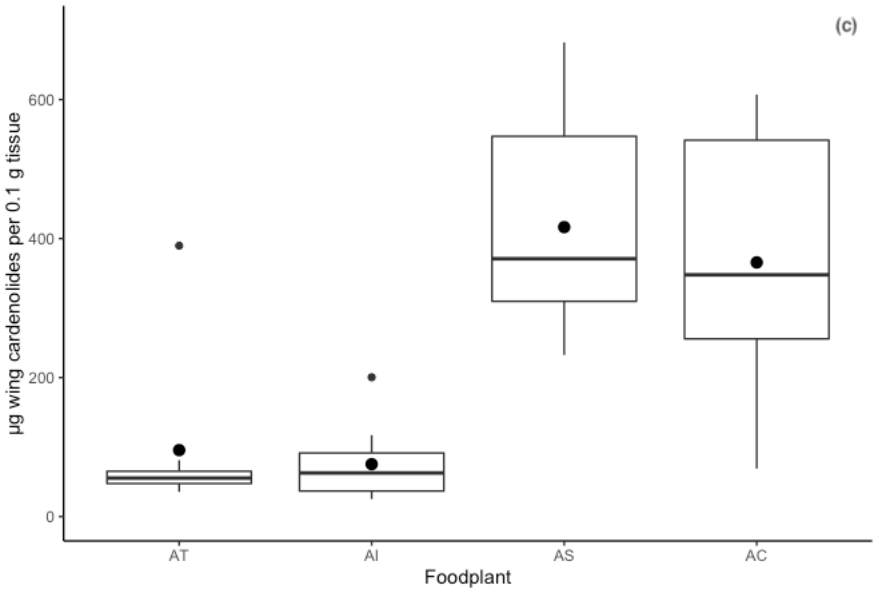



(a)
µg total cardenolides per 0.1 g tissue
2500
2000
1500
1000
500
0
AT
AI
AS
AC
Foodplant

641

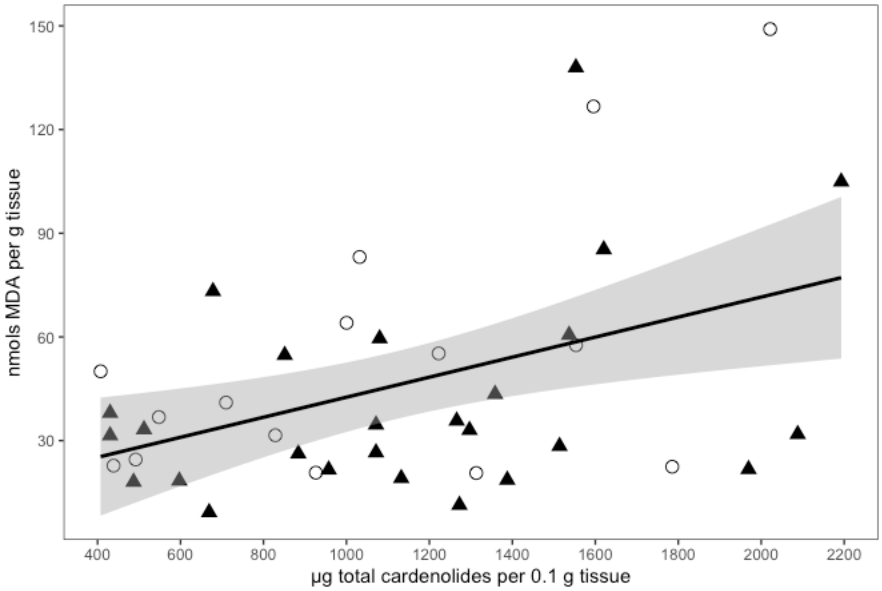

(b)
µg body cardenolides per 0.1 g tissue
1400
1200
1000
800
600
400
200
0
AT
AI
AS
AC
Foodplant

642



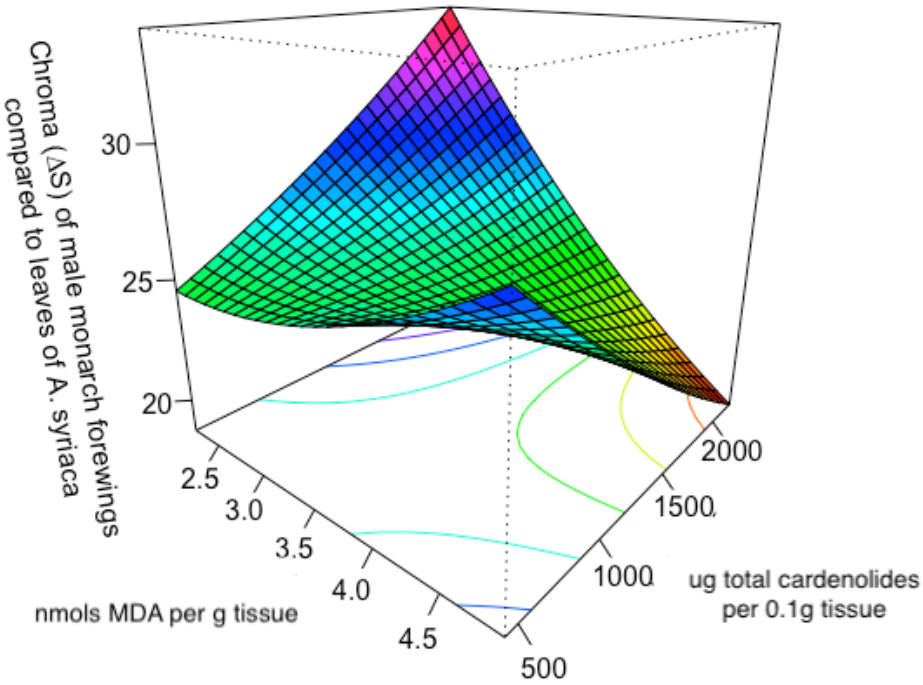
643

644 Figure 2a, b, c.



645

646 Figure 3



647

648 Figure 4

649

650