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Conspicuousness and Toxicity in Milkweed Bugs

Bachelorarbeit

vorgelegt an der Ernst-Abbe-Hochschule Jena
Fachbereich Medizintechnik und Biotechnologie

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Contents

List of Tables

List of Figures

1 Introduction

Prey animals are under constant risk of predation, and have evolved a variety of strategies to combat the different stages of the predation sequence that was proposed by Endler [1]. Predation events typically follow a sequence of six stages: 1) Encounter; 2) Detection; 3) Identification; 4) Approach; 5) Subjugation; 6) Consumption [1]. The strategies that have evolved for use before the contact takes place are called primary defences. These include anchoresis (seeking refuge), transparency, disruptive camouflage or warning colours [2, p. 5]. Strategies that are deployed post-contact in the sequence of predation are called secondary defences. These include chemical defences, like olfactory deterrents or toxins, mechanical defences, like spikes or urticating hairs, and behavioural defences, like deimatism or social-defensive grouping [2, p. 72][3].

Animales from a variety of taxa use a combination of primary defence and secondary defences. One example are the dart-poison frogs (*Dendrobatids spp.*) from Central and South America. They are brightly coloured and contain alkaloids in their skin [4]. Likewise, monarch butterflies (*Danaus plexippus*), many tiger moths (*Arctiinae spp.*) and ladybugs, combine bright warning colours with a secondary defence. [5, 6]. Together this combination is termed aposematism [7].

Aposematism enhances avoidance learning of prey by predators, which has been shown in lab and fieldwork experiments [8–10]. Aposematism also results in longer memory times of avoiding the aposematic prey by predators [11]. Warning colours and patterns can also serve as a signal for mate selection, as shown in strawberry poison frogs [12]. The primary defences of aposematic animals are not only visual, they can be acoustic or olfactory, but are mostly visual [2, p. 84]. It is hypothesized that warning signals are often visual, because it is maybe more energetically efficient to build the visual pigment once, in contrast to an olfactory or acoustic stimulus, that had to be produced all the time, when giving an aposematic signal [2, p. 85].

In a variety of aposematic species, the conspicuousness of an individual's warning signal and the quantitity of its chemical defence are correlated [13]. Blount et al. proposed a theoretical framework to explain these correlations that involves colour expression and the storage of toxins competing for energy or some other shared

'currency' within the organism [14]. Because there is evidence that pigments can have anti-oxidant qualities, Blount et al. developed the idea that antioxidants are the limiting resource [14]. The role of antioxidant availability on aposematic warning signals has received limited empirical attention [15, 16]. But Blount et al. found that monarch butterflies (*Danaus plexippus*) that sequestered higher concentrations of chemical defense had higher levels of oxidative damage. Monarch butterflies with higher oxidative stress allocated less resources to colour and toxicity than monarchs with lower oxidative damage. Heyworth et al. reared milkweed bugs (*Oncopeltus fasciatus*) on artificial diets with increasing quantities of cardenolides and examined how this affected signal quality (brightness and chroma) across different instars [17]. Bugs that sequestered more cardenolides had lower levels of the antioxidant glutathione, and bugs with less total glutathione developed less luminant orange warnings signales and reduced chroma of their black pathes compared to bugs with more glutathione.

There is a growing amount of research with evidence that signals can be affected by the possession of chemical defences. For example, the wood tiger moth (*Parasemia plantaginis*) have less conspicuous warning colours as larvae and adults, when they have higher concentrations of sequestered iridoid glycosides [18, 19]. There is also increasing evidence for costs associated with sequestering defences. For example, Agrawal et al. found that *D. plexippus* (monarch butterflies) showed an inverse relationship between sequestered cardenolides and growth of [20]. Pokharel et al. measured developmental time (larvae to adult, adult to death) and fecundity (physiological potential to bear children) when they fed *O. fasciatus* with diets containing different amounts of cardenolides [21, 22]. *Oncopeltus fasciatus* fed on diets with high cardenolide concentration showed faster developmental times (larvae to adult, adult to death), but lower fecundity. Agrawal et al. suggests that seed cardenolides from *Asclepias syriaca* induce not nearer specified negative effects on male *O. fasciatus* [23].

This present study investigates the relationship between the aposematic warning signals and toxicity across generations and across diets varying in secondary defence concentration to determine whether there are costs of sequestration, that affect growth and the ability of insects to produce visual signals. For this, the Hemiptera (true bug) *O. fasciatus* was selected based on its conspicuously patterned orange and black hemyltra.

Oncopeltus fasciatus is a Hemiptera (true bug) with a distribution between North America and the Caribbean. Its usual host plants are from the family of *Asclepiadaceae*, which produce cardenolides, a form of cardiac glycosides, in various parts

of the plant. Different studies found that *O. fasciatus* can sequester cardenolides (table 1.1). *Oncopeltus fasciatus* is known for having only few predators, thanks to the sequestered cardenolides [24]. The amount of cardenolides differ between species of *Asclepias* [25]. Cardenolides are a class of steroid toxins that have the Na^+/K^+–ATPase pump as a target, an enzyme highly conserved among the taxa of animals [26]. The Na^+/K^+–ATPase pump has the function to maintain the Sodium and Potassium gradients between the cell membranes. The cardenolides inhibits the function of the Na^+/K^+–ATPase pump by binding to the extracellular surface of the enzyme [27, 28]. That is why cardenolides are highly active toxins for different taxa of animals [26]. Members of the Lygaeinae having the highest known resistances against cardenolides, thanks to a mutated Na^+/K^+–ATPase, lowering the affinity of cardenolides to the Na^+/K^+–ATPase [29].

Table 1.1: Comparison of sequestered cardenolides of **Oncopeltus fasciatus** in different studies

Study	Diet	Setup	Sequestered Cardenolides
Pokharel2021 [21]	–artificial diet with 2, 6 or 10 $mg\,g^{-1}$ cardenolide per gram dry weight –equimolar mixture of ouabain and digitoxin –concentration of cardenolides chosen after natural concentrations [30]	–3 O. fasciatus L2 larvae in Petri dish fed with artificial seeds –freeze-dried bugs extracted and analysed by HPLC	– in $\mu g\,mg^{-1}_{\text{bug dry weight}}$ $2\,mg\,g^{-1}_{\text{diet dry weight}}$: 6.23 ± 0.76 $6\,mg\,g^{-1}_{\text{diet dry weight}}$: 15.36 ± 3.39 $10\,mg\,g^{-1}_{\text{diet dry weight}}$: 26.43 ± 5.32
Isman 1977 [30]	–dry A. curassavica seeds –plastic culture tube filled with distilled water and plugged with cotton.	–10 L1 Instar put in styrofoam box with seeds –Fed 10 d to 14 d after molding into Adult.	– in $\mu g\,mg^{-1}_{\text{teneral wet weight}}$ males: 7.26 females: 10.96
Agrawal et al. 2022 [23]	– Artificial Diet – sunflower seed agar-based diet (SSABD) with ouabain octahydrate, or glycosylated aspecioside, or labriformin in equimolar doses –diet formulation based on [21]	–3 freshly molted L3 instars in 90 mm Petri dish	–in $\mu g\,mg^{-1}_{\text{bug dry weight}}$ Adults(♂and ♀): 11.33 ± 0.61

Continuation Table 1.1

Study	Diet	Setup	Sequestered Cardenolides
Duffey and Scudder 1974 [31]	–Asclepias syriaca seeds, husked sunflower seeds, dried leaves, seeds, or pods of other plants. – liquid from cotton wicks in Erlenmayer flasks consisted of diluted salt solution (KCl $3\,\mathrm{mg\,l^{-1}}$, NaCl $1\,\mathrm{mg\,l^{-1}}$, $CaCl_2$ $0.5\,\mathrm{mg\,l^{-1}}$) [32]	–reared in controlled environmental chamber at 27 °C [32] –thin layer chromatography and spectrophotometry used with ouabain, digitoxin and strophanthidin K as standards	–comparison with digitoxin standard in $\mu g\,mg^{-1}_{\text{bug wet weight}}$ –teneral (♂+ ♀): 4.4 ± 0.3 –adult 1-3 weeks (♂+ ♀): 5.4 ± 0.4 –male (1-3 weeks): 4.7 ± 0.4 –female (1-3 weeks): 5.7 ± 0.6

Oncopeltus fasciatus is known for having a distribution of orange colours on the wings. Rodríguez-Clark measured five different traits (wing colour, pronotum colour, wing length, early fecundity and later fecundity) on *O. fasciatus* over the course of three generations [33]. *Oncopeltus fasciatus* was in the first generation taken from different places in North America and the second and third generation was kept in the lab. There was no significant difference of wing colour between the generations. But in another study, Davis found a difference in colour between males and females, in contrast to the findings of Rodríguez-Clark [34].

The need for checking colour objectively is of great importance, because there is the importance to quantify the parameters of colour according to a relevant visual system [35]. This is, because how organisms perceive colour is a long researched field, showing that every visual system differs [36]. Visual percetion depend on the different types of rods and cones photoreceptors in the retina. Rods are highly light-sensitive photoreceptors that are active during night. Cones are active during daylight and are used for colour vision [37]. Depending on the types of cones an organism can see different colours. Each cone facilitates the perception of light in a particular bandwidth, with the possibility that bandwiths of different cones are intersecting. Usually, the cones are labelled after the wave peak of the transmission spectra of this cone (Long wave L; Medium wave M; Short wave S; Ultraviolet wave UV). Humans, as an example, have usually three types, making them trichromats, but there is growing evidence of four types of cones for some people, called tetrachromacy [38]. The blue tit (*Cyanistes caeruleus*) has four cones (L, M, S, UV) [39]. Most insects having two cones for colour vision [40, 41]. Visual systems are also encoded by a

process described by Hering, who found that it is not possible to see different pairs of colours (Red/Green and Yellow/Blue) at the same time [42]. This theory is called the colour opponency theory. The red-green opponency channel is a gradient of colours having its end points with the colours green and red. There is evidence suggesting a linear relationship in red-green opponency channel [43]. The term "Redness" describes the relationship between the input of the Medium- to the Long wave receptor [44].

With the widespread availability of digital cameras, there is now a suitable option for many researches to objectively quantify colour and to apply visual modelling to simulate how an organism sees its environment. In order to do this, the camera should be calibrated to measure reflectance from an animal. With the development of the micaToolbox for ImageJ, there is now a streamlined software to analyse pictures for colour measurement [45, 46].

In this study the following hypotheses were tested:

H1: *Oncopeltus fasciatus* shows a higher concentration of sequestered cardenolides when fed on a diet with a higher concentration of cardenolides.

H2: There is a positive relationship between the redness of measured orange wing parts from *O. fasciatus* and the sequestered concentration of cardenolides.

H3: *Oncopeltus fasciatus* sequesters a higher concentration of cardenolides in later generations.

H4: There is a positive relation between the generation and the weight of *O. fasciatus*.

To research these hypotheses, the following methods were applied. A combination of the longitudinal approach from Rodríguez-Clark, where *O. fasciatus* is kept for three generations and different traits will be measured. One is the colour of the wings, but with the theoretical framework from Stevens et al. and practically with usage of ImageJ and micaToolbox [35, 45, 46]. Furthermore, the weight and the gross cardenolide concentration of each individual bug will be measured, following a modified method for the cardenolide quantification from Pokharel et al. [21]. Additionally, a shift from a cardenolide free diet to a cardenolide containing diet is applied at the beginning of the experiment.

2 Methods

All chemicals were used in laboratory purity, if not else mentioned.

2.1 Animals and Husbandry

For the following experiments, a colony of *O. fasciatus*, originally sourced from the United States, were used. These insects were kept in plastic terrarium boxes (36 cm × 21 cm × 21 cm; Hagen Deutschland GmbH & Co., Holm, Germany). *Oncopeltus fasciatus* goes in its life-cycle through different stages. From an egg it developes in to a larva. The larvae have 5 different stages names from 1 till 5. From larvae stage 5 (L5) it moults into adults [47]. Twelve adults and twelve larvae of larvae stage (L2) were put in one container. A container got either ad libitum seeds from *Asclepias curassavica*, *Asclepias incarnata* or *Helianthus annuus*, also known as Sunflower. *Asclepias curassavica* has a higher concentration of cardenolides than *A. incarnata* [25]. Seeds of *H. annuus* contain no cardenolides [30, 48]. Additionally, cotton-plugged reaction vials (STARLAB, Hamburg, Germany 2 ml) filled with water were used as water supply. A piece of cotton functions a site for oviposition. Water filled reaction vials were changed weekly. The insects in the terrarium boxes got every week a new add-in of seeds, and a full change approximately once a month. These insects were at least for 3 generations on the same diet.

After approximately 30 d, 12 adults and 12 L2 larvae from each starting colony were distributed into a new terrarium box and maintained on the same diet treatment. Remaining insects were euthanized by freezing in a −20 °C or −80 °C freezer.

A sample of at least 53 adults per diet were selected (*A. curassavica* = 74, *A. incarnata* = 55, *H. annuus* = 53). The individual nett weight was measured. Additionally, they were individually photographed, and visual systems models were applied to the processed pictures (sections 2.3 and 2.4).

2.2 Study Design of the Growth Assay

To measure the effects of feeding cardenolides on growth, 10 larvae of stage 2 (L2), coming from the stock boxes, previously fed on *H. annuus* (section 2.1), were put into a vented Petri dish (Orange Scientific, Braine-l'Alleud, Belgium 100 mm x 20 mm vented or STARLAB, Hamburg, Germany 100 mm x 20 mm vented). They were randomly assigned to receive a diet of either *A. curassavica*, *A. incarnata* or *H. annuus* ad libitum, with two 2 ml cotton plugged reaction vials, filled with water. Water filled reaction vials were changed weekly and seeds biweekly. In the beginning of the experiment, twelve Petri dishes (3 x *H. annuus*, 5 x *A. incarnata*, 4 x *A. curassavica*) were created in the stated method. For better handling six Petri dishes were housed into a plastic container (28 cm × 20 cm × 9.5 cm, Rotho Kunststoff AG, Würeningen, Switzerland), from hereon called batch. These Petri dishes were randomly spatialized inside the container. It was ensured that the new Petri dishes stayed in the same batch, till all insects of that Petri dish died. Every day, the number of living individuals of *O. fasciatus* per Petri dish were recorded. Additionally, to the recorded number of surviving insects, it was marked how many adults were in the Petri dish. *Oncopeltus fasciatus* moults after it went through larvae stage 5 (L5). Freshly moulted adults of *O. fasciatus* show muted colours. *Oncopeltus fasciatus* were counted as an adult when it shows its known dorsal pattern of bright orange and black.

The weight of the insects was measured twice a week. For this, the instars and adults of one Petri dish were put into an empty tared Petri dish and weighted as a whole colony. Later the mean weight per insect were computed as the nett weight of the colony divided by the amount of insects per Petri dish (equation 2.1).

$$m_{mean} = \frac{m_{Insects}}{N_{Insects}} \tag{2.1}$$

When all insects in a Petri dish turned into adults, mated and laid eggs, 60 % of the adults from a Petri dish were photographed (sections 2.3 and 2.4) and kept for longevity. The remaining 40 % were also photographed (sections 2.3 and 2.4), euthanized and froze in liquid nitrogen and stored in −80 °C freezers for later cardenolide quantification (section 2.5). It was randomly decided if an adult was frozen or kept for longevity.

For the next generation, 20 L2 instar of each experimental Petri dish were distributed into two new Petri dishes, ten per Petri dish, and kept on the same diet as their parents. As part of the experiment, three generations (F0, F1, F2) of all three diets were kept, that stayed on the same diet during the experiment.

2.3 Photography

Pictures were taken with a modified Nikon D7000 digital SLR (Nikon, Tokyo, Japan), that allows the camera to capture pictures in the electromagnetic spectrum of 300 nm to 700 nm. The camera is connected on a rail to adjust the distance between the camera and the object. The lens is fitted with a custom-built ring illuminator, that illuminates the insects with LEDs emitting light between 380 nm to 780 nm, and houses a filter changer. The used filters were a Baader UV-IR blocking filter (Baader Planetarium, Mammendorf, Germany) to permit visible light from 420 nm to 680 nm and a Baader UV pass filter to pass UV light from 320 nm to 380 nm. Other used light sources included two flexible lamps on the top, as well as 3 panels with UV and VIS LED's, one on each side and one at the back.

Before taking pictures, the camera needed to be adjusted. The camera was moved vertically on the rail until the specimen could be fully seen in the display. After that, the picture was taken in focus. Before each photographing session, it was ensured that the camera had the right settings for optimal pictures. For all pictures a ISO-value of 320 and an Aperture of F0 was used. The Exposure times varied between pictures in the visible and ultraviolet range. For the visible range, exposure times of 0.001 25 s, 0.003 125 s, and 0.008 s were used. In the ultraviolet range, longer exposure times of 0.03 s, 0.076 92 s, and 0.2 s were used.

Insects were sedated and photographed on a custom-built carbon dioxide-plate. Each photograph included a 40 % Spectralon grey Standard (Labsphere Inc., North Sutton, NH, United States), a grey balance and a 10 mm scale. For every insect, three pictures were taken in the visible spectrum, as well as three pictures in the ultraviolet spectrum, each with different exposure time for post hoc selection of optimal exposure.

2.4 Picture Analysis and Cone Catch Models

To measure the reflectance of the orange part of the hemelytra from *O. fasciatus*, the program ImageJ was used in conjunction with the plugin micaToolbox v2.2.0 [45, 46]. The hemelytra are the forewings of Heteroptera (true bugs) [49]. Multispectral image stacks were created for each insect, one image in the human visual spectrum, as well as one in the ultraviolet spectrum [45]. For this, two regions of interest (ROI) on the hemelytra of *O. fasciatus* were chosen, two orange parts under the black head plate, as well as two black areas under the orange parts. Those regions of interest were selected in micaToolbox for each multispectral image stack using a Wacom

Cintic pen display (Wacom Co., Ltd., Kazo, Japan).

After the creation of the regions of interest of all photographed insects, batch processing scripts were conducted. Cone catch models of the Eurasian blue tit (*C. caeruleus*), the flap-necked chameleon (*Chamaeleo dilepis*) and the jumping spider *Habronattus pyrrithrix* were created for each photographed picture, to measure how the stated species perceives the black and orange wing segments of *O. fasciatus* [45]. As a luminance channel in *C. caeruleus* and *C. dilepis*, double cones were chosen [50, 51]. For *H. pyrrithrix*, the medium wave cones were assumed as the luminance channel [40, 41].

Of the orange wing parts, the red-green opponency channel, hereby called "redness", were calculated as described in methods from Higham et al. (equation 2.2) [44]. Values with a higher red-green opponency channel are likely perceived as more red [44].

$$r = \frac{LW - MW}{LW + MW} \tag{2.2}$$

2.5 Cardenolide Quantification

A modified method from Pokharel et al. was used to extract the sequestered cardenolides in *O. fasciatus* [21].

Before the extraction method could be used, it had to be validated. Firstly, it was checked if the used 5 ml reaction vials (Eppendorf SE, Hamburg, Germany) contain fat from the manufacturing process. For this, the reaction vials were rinsed with pure methanol that were collected into a cleaned glass vial. The methanol was evaporated under a N_2 stream, reconstituted and measured in NMR. No fat was found from the manufacturing process. One millilitre of the digitoxin standard was evaporated under a N_2 flow, reconstituted in 200 µl and measured in a Bruker Avance III HD 500 NMR (Bruker, Billerica, United States). After that, 1 ml of the digitoxin standard was used with the extraction method to see how good the recovery rate was. The following method showed a good compromise of low fat load and high recovery rate (figure 2.1).

For the extraction method, each frozen adult (section 2.2) was freeze-dried, weighed and put into a fast prep matrix tube (MP Biomedicals Germany GmbH, Eschwege, Germany) with approx. 450 mg of ⌀ 2.3 mm zirconium/glass-pellets (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). One millilitre of a digitoxin standard ($c = 0.01\,mg\,ml^{-1}$), dissolved in methanol, was added to the matrix tube. The sample was homogenized in the FastPrep 24-5G Tissue Homogenizer (MP Biomedicals

Germany GmbH, Eschwege, Germany) at 6.5 m s^{-1} for 45 s for 2 cycles with a pause time of 100 s between cycles.

After the two extractions cycles, the samples were centrifuged at 16 000 RCF for 3 min and 700 µl of supernatant were collected. The procedure of homogenization and centrifugation were repeated 2 additional times with pure Methanol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) instead of the Digitoxin standard.

To diminish the amount of fat in the extracts, CHROMABOND HR-X SPE cartridges (3 ml; 200 mg; 85 µm Macherey-Nagel GmbH & Co. KG, Düren, Germany) were used with a SPE Manifold (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

The SPE cartridges were wetted with 2 ml of pure Methanol and compressed with a spatula. Most of the Methanol were released, till approximately 400 µl remained in the cartridge.

The supernatants were transferred into the SPE cartridge and collected in a 5 ml reaction tube (Eppendorf SE, Hamburg, Germany). Afterwards, 2 ml of Methanol were added for washing of the SPE cartridge. Under a N_2-flow, the purified extracts were boiled down. Next, they were dried in a desiccator for at least 12 h, weighted afterwards and stored in a −80 °C freezer until further usage.

For the LC-MS measurement, samples were reconstituted in 200 µl Methanol and resolved under help of an ultrasonic bath. These extracts were cleaned with Minisart SRP4 Syringe filters (pore size 0.45 µm; Sartorius Stedim Lab Ltd, Stonehouse, United Kingdom) and injected in conical N9 screw neck vials with solid glass bottom (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Samples were measured in a Agilent LC-MS with a Agilent InfinityLab Poroshell 120 EC-C18 2.7 µm column (Agilent Technologies, Inc., Santa Clara, United States) at a flow of 0.5 ml min^{-1}. The mobile phase consisted of Acetonitril spiked with formic acid (0.1 v/v%), and water spiked with formic acid (0.1 v/v%). Acetonitril-Water (ACN/H_2O) gradients were used as follows: Starting with a ACN-H_2O (5 %/95 %) gradient for 5 min. After that it was changed to a ACN-H_2O gradient to (95 %/5 %) over 25 min. Gradient stayed for this for 15 min, following a change back to a ACN-H_2O (5 %/95 %) gradient for 2 min with a total time of 47 min. A different injection volume per sample was used, based on the mass of the extract. This was to ensure that the peak of digitoxin has for all samples an approximate similar area.

Symmetrical peaks with a wavelength between 218 nm to 220 nm were assumed as Cardenolides [21, 52].

To do the quantifications, the peak area of the found cardenolides and the digitoxin standard were measured with the program Bruker Compass DataAnalysis 5.1 (Brukel

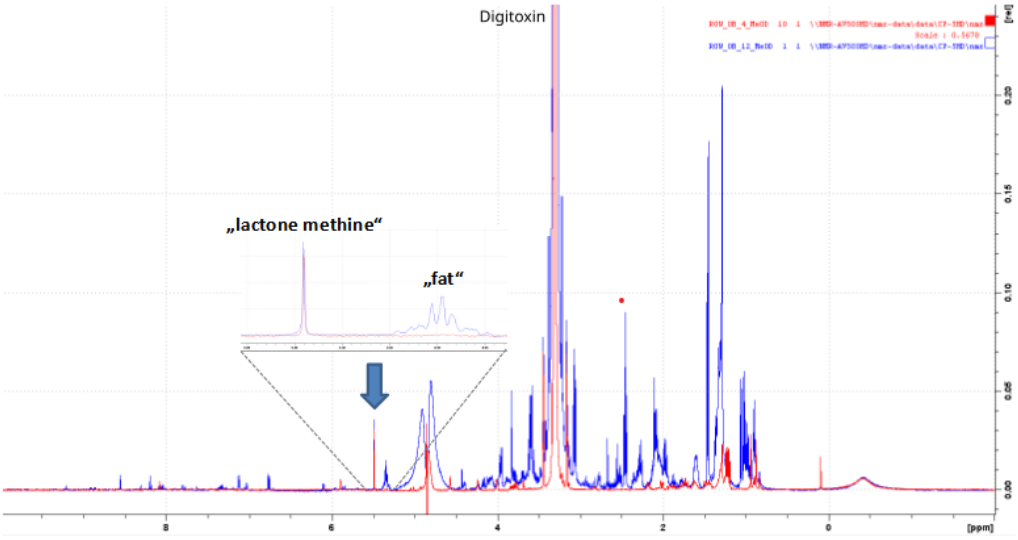

Figure 2.1: Comparison of internal digitoxin standard evaporated and measured (red) compared to the digitoxin standard extracted from **Oncopeltus fasciatus** and cleaned with SPE cartridge (blue). Both spectra show a high overlap in the Digitoxin peak. Both spectra were scaled to the same solvent peak intensity, measurement parameters were identical. Many thanks to Christian Paetz for compiling this figure.

Daltonik GmbH, Bremen, Germany). The mass of digitoxin in each injection was calculated with the used mass of the digitoxin standard, the used injection volume and the full volume of our sample (equation 2.3).

$$m_{mass\ digitoxin\ in\ injection} = \frac{V_{injection} \cdot m_{digitoxin}}{V_{sample}} \tag{2.3}$$

Next, the relative amount of cardenolides in the injection was calculated (equation 2.4). The cardenolides were put into relation of the digitoxin standard. These relations of cardenolides to the analytical standard were called cardenolide units (CU).

$$CU_{injection} = \frac{m_{mass\ digitoxin\ in\ injection} \cdot A_{cardenolides}}{A_{digitoxin}} \tag{2.4}$$

After the calculation of the cardenolide units in the used injection, the cardenolide units of the whole sample volume were calculated (equation 2.5).

$$CU_{sample} = \frac{CU_{injection} \cdot V_{sample}}{V_{injection}} \tag{2.5}$$

2.6 Statistic and Data Analysis

Python were used with the packages 'pandas' for data preparation and with 'seaborn' and 'matplotlib' for figures [53–55]. For statistical models and tests, the programming language R were used [56]. Mixed models were compiled with the R package 'lmertest', a variation of 'lmer4', to obtain p-values [57, 58].

For the comparisons of population (section 3.1), a linear model were used to predict the influence of the diet on the weight or redness (section 2.4) [59].

To analyse the relationship of diet and generation for the redness and luminance, linear mixed models were used. This was to ensure that the random effect of the Petri dishes was taken into account [60]. For the data of each of the three visual models, the redness or the luminance were used as the dependable variable. The diet and generation served as the independent variables.

Linear mixed models were also used for the relationship of colour and cardenolide content (section 3.3). A linear mixed model, from hereon called full model, was compiled, that predicted the cardenolide concentration with diet, sex and generation as fixed effects and with the Petri dish label as a random effect. A AICc model selection was used to distinguish between the used models. The AIC is a selection criterion to see which model from a dataset explains the data best [61]. The AICc criterion was used, which corrects for small sample sizes [61]. In general, lower AIC(c) values are favoured. The full model showed the lowest AICc values of the analysed mixed models, that is why it was chosen.

3 Results

3.1 Stock Boxes

Data was generated as stated in sections 2.1, 2.3 and 2.4.

3.1.1 Redness

The redness of *O. fasciatus* was analysed as described in the methods (section 2.4). Adults of *O. fasciatus* showed the highest redness with a diet of seeds from *A. curassavica*, followed by *A. incarnata* and *H. annuus* with the lowest redness, regardless of the studied visual system (figure 3.1).

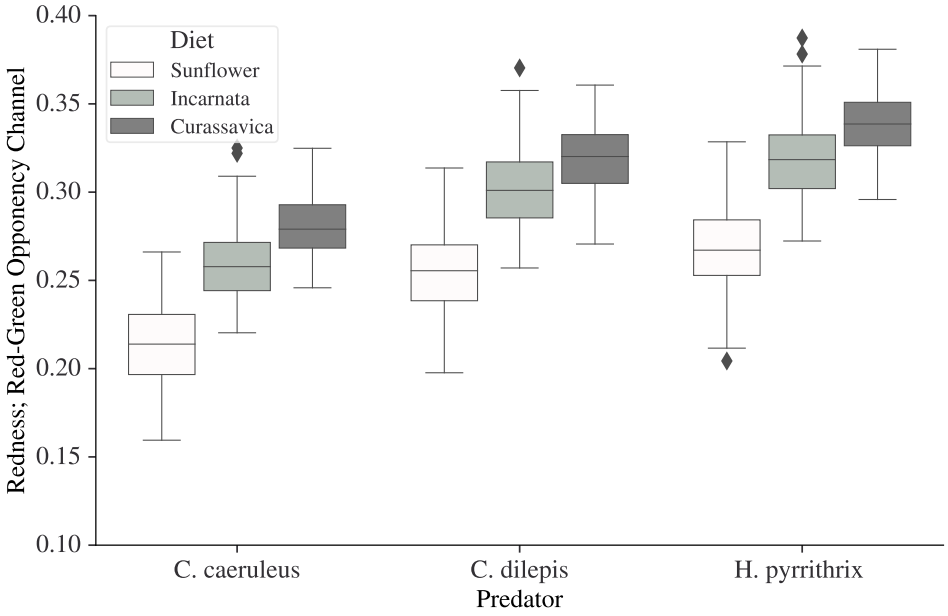

Figure 3.1: Redness is highest with the most toxic researched diet of **Asclepias curassavica**, regardless of predator visual system. Redness of orange wing part from **Oncopeltus fasciatus** stock box insects as seen by the visual model systems of the predators **Cyanistes caeruleus**, **Chamaeleo dilepis** and **Habronattus pyrrithrix**.

C. caeruleus

A diet of *A. incarnata* for *O. fasciatus*, compared to a diet of *H. annuus*, resulted in a significant increase in redness (estimate = 0.046 ± 0.003, $t = 15.30$, $p = <0.001$). *Oncopeltus fasciatus* fed on *A. curassavica*, compared when on a diet of *H. annuus*, showed also an significant increase in redness (estimate = 0.067 ± 0.003, $t = 21.02$, $p = <0.001$).

C. dilepis

Oncopeltus fasciatus fed on *A. incarnata*, compared when fed on *H. annuus*, resulted in a significant increase in redness (estimate = 0.049 ± 0.003, $t = 15.15$, $p = <0.001$). A diet of *A. curassavica* for *O. fasciatus*, compared to a diet of *H. annuus*, resulted also in a significant increase in redness (estimate = 0.066 ± 0.003, $t = 19.24$, $p = <0.001$).

H. pyrrithrix

For the visual data of the jumping spider *H. pyrrithrix*, a significant increase in redness with a diet of *A. incarnata* for *O. fasciatus* compared to *H. annuus* as a diet for *O. fasciatus* (estimate = 0.051 ± 0.003, $t = 15.71$, $p = <0.001$) and for *A. curassavica* compared to *H. annuus* (estimate = 0.071 ± 0.003, $t = 20.47$, $p = <0.001$) was measured.

Post-Hoc Tukey-Test

To see which colonies of *O. fasciatus*, fed on different diets (*A. curassavica*, *A. incarnata*, *H. annuus*), were significantly different between each other in redness, a post-hoc Tukey-test was applied. For the data for *C. caeruleus*, all diets significantly differ between each other (table 3.1). The visual data for *C. dilepis* showed a significant difference for all three diets compared to each other, (table 3.2), as well as for the visual system for *H. pyrrithrix* (table 3.3).

Table 3.1: Results of Tukey-Kramer Test for **Cyanistes caeruleus** visual system for stock boxes insects

Hypotheses	Estimate ± standard error	z value	Pr(>|z|)
Curassavica - Sunflower == 0	0.067 ± 0.003	21.021	$<1.00 \times 10^{-10}$
Incarnata - Sunflower == 0	0.046 ± 0.003	15.305	$<1.00 \times 10^{-10}$
Incarnata - Curassavica == 0	-0.021 ± 0.003	-6.721	$<1.00 \times 10^{-10}$

Table 3.2: Results of Tukey-Kramer Test for **Chamaeleo dilepis** visual system for stock boxes insects

Hypotheses	Estimate ± standard error	z value	Pr(>|z|)
Curassavica - Sunflower == 0	0.066 ± 0.003	19.236	$<1 \times 10^{-6}$
Incarnata - Sunflower == 0	0.049 ± 0.003	15.147	$<1 \times 10^{-6}$
Incarnata - Curassavica == 0	-0.017 ± 0.003	-5.076	1.08×10^{-6}

Table 3.3: Results of Tukey-Kramer Test for **Habronattus pyrrithrix** visual system for stock boxes insects

Hypotheses	Estimate ± standard error	z value	Pr(>|z|)
Curassavica - Sunflower == 0	0.071 ± 0.003	20.466	$<1.00 \times 10^{-8}$
Incarnata - Sunflower == 0	0.051 ± 0.003	15.709	$<1.00 \times 10^{-8}$
Incarnata - Curassavica == 0	-0.020 ± 0.003	-5.076	2.23×10^{-8}

3.1.2 Luminance

C. caeruleus

Oncopeltus fasciatus showed neither with a diet of seeds from *A. curassavica* (estimate = 0.002 ± 0.003, $t = 0.541$, $p = 0.589$) or *A. incarnata* (estimate = 0.001 ± 0.003, $t = 0.409$, $p = 0.683$) a significant difference in luminance, compared to *O. fasciatus* on a diet of seeds from *H. annuus*.

C. dilepis

Oncopeltus fasciatus fed on a diet from seeds from *A. curassavica* (estimate = 0.008 ± 0.004, $t = 2.064$, $p = 0.040$) was significantly brighter than sunflower, but not when fed on seeds of *A. incarnata* (estimate = 0.006 ± 0.004, $t = 1.596$, $p = 0.112$).

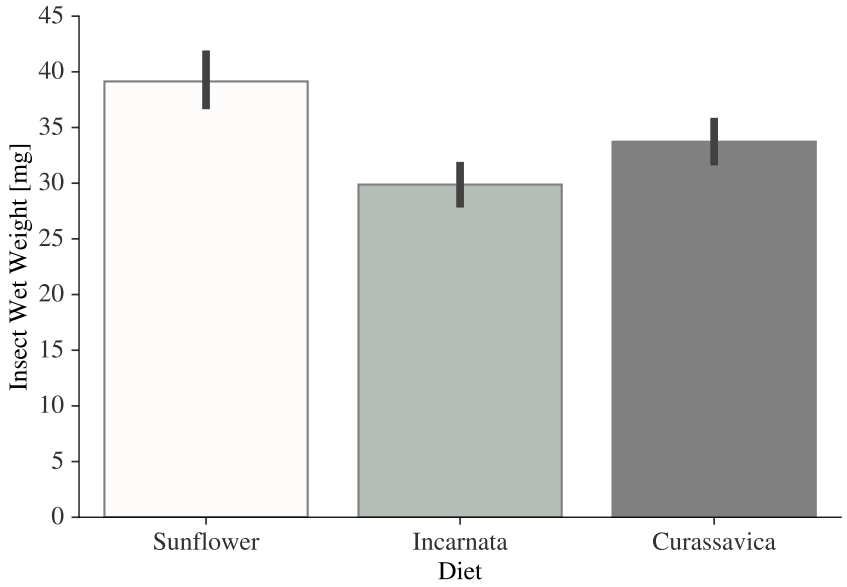

Figure 3.2: Insect wet weight of **Oncopeltus fasciatus** per diet from the stock boxes. Sample size per diet were Sunflower=53, Incarnata=55, Curassavica=74. Estimate is mean per diet with standard error.

H. pyrrithrix

Adults fed on a diet of seeds from *A. curassavica* were significantly less bright compared to sunflower (estimate $= -0.005 \pm 0.002$, $t = -2.065$, $p = 0.040$) but not when fed on *A. incarnata* (estimate $= -0.004 \pm 0.002$, $t = -1.776$, $p = 0.077$).

3.1.3 Weight

A linear model was applied to look on the effect of the different diets on the nett weight of adults from *O. fasciatus* (figure 3.2). A significant decrease in weight was found for *O. fasciatus*, when fed on a diet of seeds from *A. curassavica* compared to a diet with *H. annuus* seeds (estimate $= (-5.4 \pm 1.4)$ mg, $t = -3.905$, Pr $= <0.001$) as well as for an *A. incarnata* seed diet compared to a diet with *H. annuus* (estimate $= (-9.3 \pm 1.5)$ mg, $t = -6.248$, $p = <0.001$).

The linear model revealed that *O. fasciatus*, when fed on *A. curassavica* and *A. incarnata*, showed a significant different weight, compared to *O. fasciatus* which were fed on *H. annuus*. To compare the weights of the different diets, a Tukey-HSD test was applied. It showed that the weight of *O. fasciatus* on the different diets were significant different between each other (table 3.4).

Table 3.4: Results of Tukey-Kramer Test for weight for stock boxes insects

Hypotheses	Estimate	standard error	z value	Pr(>|z|)
Curassavica - Sunflower == 0	−5.413	1.386	−3.905	<0.001
Incarnata - Sunflower == 0	−9.263	1.483	−6.248	<0.001
Incarnata - Curassavica == 0	−3.850	1.371	−2.808	0.0138

3.2 Colour Relationship Growth assay

The relationship of diet and generation for redness and luminance was analysed in this part.

3.2.1 Redness and Luminance

Data was collected as stated in section 2.3 and section 2.4.

C. caeruleus

Redness Adults of *O. fasciatus* are showing a significant increase in redness when fed on a diet of *A. curassavica* compared to sunflower (estimate = 0.0252 ± 0.0076, $t = 3.317$, $p = <0.001$) as well with a diet of *A. incarnata* (estimate = 0.0187 ± 0.0075, $t = 2.485$, $p = 0.016$). With later generations, there is a significant decrease in redness between generation one and zero (estimate = -0.0171 ± 0.0064, $t = -2.673$, $p = 0.010$) as well as between generation two and zero (estimate = -0.0318 ± 0.0062, $t = -5.153$, $p = <0.001$)

Luminance Adults are significantly more bright in generation two compared to zero (estimate = 0.0123 ± 0.0037, $t = 3.340$, $p = 0.002$), but not between generation one and zero (estimate = 0.0027 ± 0.0038, $t = 0.703$, $p = 0.485$).

C. dilepis

Redness *Oncopeltus fasciatus* shows with a diet of *A. curassavica* a significantly higher redness, compared to *O. fasciatus* fed on a diet of *H. annuus* (estimate = 0.0305 ± 0.0070, $t = 4.364$, $p = <0.001$) as well as a diet of *A. incarnata* compared to Sunflower seeds (estimate = 0.0248 ± 0.0069, $t = 3.573$, $p = 0.001$).

With higher generations a significant decrease in redness is found. Once between generation zero and one (estimate = -0.0185 ± 0.0059, $t = -3.125$, $p = 0.003$) and between generation two to zero (estimate = -0.0298 ± 0.0057, $t = -5.264$, $p = <0.001$).

Luminance Orange parts were brighter in generation two compared to zero (estimate = 0.0127 ± 0.0044, $t = 2.890$, $p = 0.006$). There was no significant difference in brightness between generation one and zero (estimate = 0.0018 ± 0.0046, $t = 0.398$, $p = 0.693$).

H. pyrrithrix

Redness Adults of *O. fasciatus* were significantly more red if fed on a diet of *A. curassavica* compared to *H. annuus* seeds (estimate = 0.0284 ± 0.0075, $t = 3.786$, $p = <0.001$) and on a diet of *A. incarnata* compared to *H. annuus* seeds (estimate = 0.0226 ± 0.0074, $t = 3.040$, $p = 0.003$).

With increasing generation occurs a significant decrease in redness. For generation two compared to generation zero (estimate = -0.0312 ± 0.0061, $t = -5.128$, $p = <0.001$) occurs a stronger decrease as for generation one compared to generation zero (estimate = -0.0178 ± 0.0063, $t = -2.811$, $p = 0.007$).

Luminance A diet of *A. curassavica* compared to sunflower results in significant less bright adults (estimate = -0.0086 ± 0.0026, $t = -3.303$, $p = 0.001$) as well as for *A. incarnata* compared to sunflower (estimate = -0.0088 ± 0.0026, $t = -3.400$, $p = <0.001$).

Adults compared from generation two to zero do show a significant increase in brightness (estimate = 0.0100 ± 0.0020, $t = 5.125$, $p = <0.001$) but not adults from generation one compared to generation zero (estimate = 0.0030 ± 0.0021, $t = 1.436$, $p = 0.153$).

3.3 Colour and Cardenolide Relationship

The cardenolide content was quantified as stated in section 2.5. All models with a cardenolide concentration are using the cardenolide concentration corrected by insect wet weight. Pictures were taken and analysed as in section 2.3 and section 2.4.

For the following analysis, all samples from the sunflower diet were excluded, due to the small sample size of one. One data point with an unusual high cardenolide concentration of ($15.35\,\mathrm{mg\,g^{-1}}$) with a diet of *A. incarnata* seeds was also excluded. In the figure 3.3 the outlier can clearly be seen.

3.3.1 Effect from Diet on Cardenolide sequestration

A significant difference in the sequestration of cardenolides of the bugs on the different diets was found. Additionally, a significant increase in cardenolide concentration

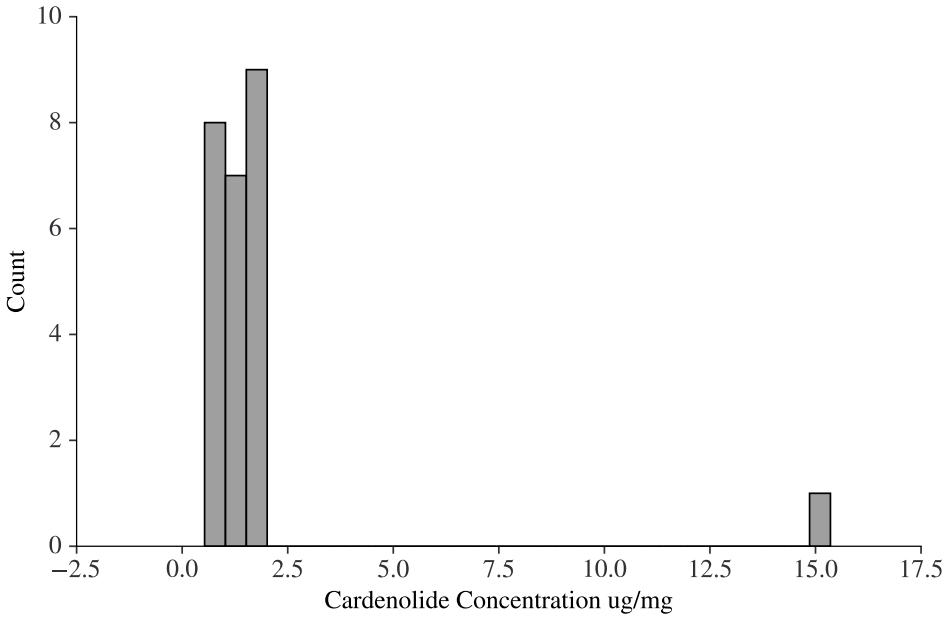

Figure 3.3: Histogram of the Cardenolide concentration, corrected by wet weight, for **Oncopeltus fasciatus** fed on a diet of **Asclepias incarnata** seeds. The outlier is clearly visible at the right.

with a diet of *A. curassavica* seeds compared to *A. incarnata* seeds (estimate = $(4.03 \pm 0.75)\,\mu g\,mg^{-1}$, $t = 5.364$, $p = <0.001$), and between generation one and zero (estimate = $(1.79 \pm 0.60)\,\mu g\,mg^{-1}$, $t = 2.976$, $p = 0.007$) was found. Females had a significantly higher concentration of cardenolides than males (estimate = $(0.97 \pm 0.42)\,\mu g\,mg^{-1}$, $t = 2.297$, $p = 0.026$).

3.3.2 Effects on Weight

To predict the influence of diet and generation for the weight, a linear mixed model was used. Females were significantly heavier than males (estimate = (16.710 ± 3.864) mg, $t = 4.324$, $p = <0.001$) and adults from generation two were lower in weight in comparison to generation zero (estimate = (-12.678 ± 5.376) mg, $t = -2.358$, $p = 0.026$). There was no significant difference in weight between *O. fasciatus* fed on seeds from *A. curassavica* compared to a diet of *A. incarnata* (estimate = (1.138 ± 3.625) mg, $t = 0.314$, $p = 0.757$). A significant interaction between sex of the bugs and generation on their wet weight was found (estimate = (-14.019 ± 5.247) mg, $t = -2.672$, $p = 0.013$).

The data of the analysis was separated by sex, and the models were run again. With that, females were significantly lighter in generation two compared to generation zero (estimate = (-29.698 ± 7.166) mg, $t = -4.144$, $p = 0.001$). Females were not significantly different in weight in generation one compared to generation zero

Table 3.5: Comparison of linear mixed models, regarding the colour and cardenolide relationship, for redness and luminance for different visual systems and their significant factors. Explanation of abbreviations and symbols: Pred : Predator; Fctr : Factor; Sign : Significance; Est : Estimate; Diet Cur. : Diet Curassavica; C. : Cardenolide Concentration SexM : Male Sex; Gen1 : Generation 1; Gen2 : Generation 2; / : P > 0.05; * : 0.05 > P > 0.01; ** : 0.01 > P > 0.001; *** : P< 0.001; + : positive estimate; - : negative estimate

Cardenolide subset; Redness Models with Diet												
Pred	Bluetit				Chameleon				Jumping Spider			
Fctr	Cur.	SexM	Gen1	Gen2	Cur.	SexM	Gen1	Gen2	Cur.	SexM	Gen1	Gen2
Sign	/	**	/	**	/	*	/	**	/	**	/	**
Est	+	+	-	-	+	+	-	-	+	+	-	-
Cardenolide subset; Redness Models with Cardenolide concentration												
Pred	Bluetit				Chameleon				Jumping Spider			
Fctr	C.	SexM	Gen1	Gen2	C.	SexM	Gen1	Gen2	C.	SexM	Gen1	Gen2
Sign	/	**	/	**	/	*	/	**	/	**	/	**
Est	-	+	-	-	-	+	-	-	-	+	-	-
Cardenolide subset; Luminance Models with Diet												
Pred	Bluetit				Chameleon				Jumping Spider			
Fctr	Cur.	SexM	Gen1	Gen2	Cur.	SexM	Gen1	Gen2	Cur.	SexM	Gen1	Gen2
Sign	/	/	/	/	/	*	/	/	/	/	/	*
Est	-	+	-	+	-	+	-	+	+	+	-	+
Cardenolide subset; Luminance Models with Cardenolide concentration												
Pred	Bluetit				Chameleon				Jumping Spider			
Fctr	C.	SexM	Gen1	Gen2	C.	SexM	Gen1	Gen2	C.	SexM	Gen1	Gen2
Sign	/	/	/	*	/	*	/	/	/	/	/	*
Est	+	+	-	+	+	+	-	+	+	+	-	+

(estimate = (-13.804 ± 7.678) mg, $t = -1.798$, $p = 0.097$). In contrast, males were significantly lighter in generation one compared to generation zero (estimate = (-9.935 ± 3.892) mg, $t = -2.553$, $p = 0.019$), and in generation two compared to generation zero (estimate = (-13.636 ± 4.353) mg, $t = -3.133$, $p = 0.005$).

3.3.3 Redness and Luminance

For each of the data from the visual systems (*Cyanistes caeruleus*, *Chamaeleo dilepis*, *Habronattus pyrrithrix*), the same two linear mixed models were applied. The first model used generation and diet (fixed effects) and the Petri dish label (random effect). The second model used the cardenolide concentration, corrected by wet weight, instead of diet, with otherwise the same formula as the first model. A summary of the results was added in table 3.5.

C. caeruleus

Redness Male adults of *O. fasciatus* were significantly more red than females (estimate $= 0.0145 \pm 0.0048$, $t = 3.010$, $p = 0.004$). Between generation two and zero was a significant decrease in redness (estimate $= -0.0266 \pm 0.0073$, $t = -3.629$, $p = 0.002$) but not compared from generation one to zero (estimate $= -0.0096 \pm 0.0067$, $t = -1.433$, $p = 0.170$). In the second model the cardenolide concentration corrected by insect wet weight was included, instead of the diet. This was to see how this factor varied the redness. No significant difference for cardenolide concentration was found (estimate $= -0.000\,31 \pm 0.000\,95$, $t = -0.327$, $p = 0.746$). But males were again more red than females (estimate $= 0.0137 \pm 0.0050$, $t = 2.745$, $p = 0.009$) and *O. fasciatus* from generation two was significantly different to *O. fasciatus* from generation zero (estimate $= -0.0279 \pm 0.0070$, $t = -4.001$, $p = 0.001$).

Luminance Like for the redness, two analyses were done, one with the inclusion of the cardenolide concentration corrected by weight and one with the diet as a fixed effect. For the model without the cardenolide content, no significant difference was found.

The model with inclusion of the cardenolide content shows a significant increase in brightness between generation two and zero (estimate $= 0.0094 \pm 0.0042$, $t = 2.269$, $p = 0.036$) but not between generation one and zero (estimate $= -0.0044 \pm 0.0040$, $t = -1.114$, $p = 0.279$).

C. dilepis

Redness First, the model without the cardenolide concentration was conducted. Male adults were significantly more red than females (estimate $= 0.0139 \pm 0.0049$, $t = 2.832$, $p = 0.007$). Between generation two and zero were the adults significantly less red (estimate $= -0.0266 \pm 0.0073$, $t = -3.629$, $p = 0.002$). In contrast, no significance was found between generation one and zero (estimate $= -0.0096 \pm 0.0067$, $t = -1.433$, $p = 0.170$).

For the model with cardenolide concentration, a similar pattern as for the first model was found. Males are significantly more red (estimate $= 0.0137 \pm 0.0050$, $t = 2.745$, $p = 0.009$) and adults from generation two are less red than adults from generation zero (estimate $= -0.0279 \pm 0.0070$, $t = -4.001$, $p = 0.001$) but not between generation one and zero (estimate $= -0.0095 \pm 0.0068$, $t = -1.400$, $p = 0.179$).

Luminance For the model without cardenolide concentration, no factor had a significant influence on the brightness. For the cardenolide model, there was a significant increase in brightness from generation two to zero (estimate $= 0.0094 \pm 0.0042$, $t = 2.269$, $p = 0.0362$).

H. pyrrithrix

Redness The model with diet as a fixed effect shows that males are significantly more red than females (estimate $= 0.0139 \pm 0.0049$, $t = 2.832$, $p = 0.007$) as well as adults from generation two, compared to generation zero (estimate $= -0.0266 \pm 0.0073$, $t = -3.629$, $p = 0.002$). The cardenolide concentration model shows a similar pattern with more red males (estimate $= 0.0137 \pm 0.0050$, $t = 2.745$, $p = 0.009$) and a significant decrease between generation two and zero (estimate $= -0.0279 \pm 0.0070$, $t = -4.001$, $p = 0.001$).

Luminance For the model with diet as a fixed effect, no factor was significantly different to the intercept. In the cardenolide model a significant increase between generation two and zero was found (estimate $= 0.0094 \pm 0.0042$, $t = 2.269$, $p = 0.036$).

4 Discussion

This present study investigated the relation between aposematic colour properties and sequestered cardenolides in *O. fasciatus*, to examine the potential costs of sequestration on the growth of insects and the development of warning signals. First, populations of *O. fasciatus*, that had been maintained for more than three generation on diets that varied in cardenolide concentration, were analysed for redness (section 3.1.1) and luminance (section 3.1.2) according to three different visual systems, and weight (section 3.1.3) [44]. Adults of *O. fasciatus* showed the highest redness on a diet of seeds from *A. curassavica*, followed by *A. incarnata* and *H. annuus* with the lowest redness, regardless of the studied visual system. No clear pattern between diet and luminance was found (section 3.1.2). The diets containing cardenolides showed lower weight of adults of *O. fasciatus*, compared to *O. fasciatus* fed on *H. annuus* (section 3.1.3).

This is some of the first quantitative evidence for an effect of foodplant properties on visually perceivable differences in warning signal expression in milkweed bugs. To understand the differences seen in the populations that had been established for a number of generations on the different diets, the relationship between colour and generation was explored by rearing experimental groups on different diets and measuring changes in redness and luminance over time, and by quantifying cardenolide sequestration. For this, the same visual models were used as described in section 2.4. Regardless of diet, a significant decrease in redness was observed between generation one and zero as well between generation two and zero. *Oncopeltus fasciatus* fed on the cardenolide containing diets of *A. curassavica* or *A. incarnata* however, were consistently higher in redness, compared to a diet of *H. annuus* (section 3.2.1). This pattern was detectable for all three visual models.

The luminance were for all three visual systems significantly higher in generation two, compared to generation zero. Additionally, for the visual model of *H. pyrrithrix*, *O. fasciatus* fed on *A. curassavica* showed a significantly lower luminance, compared to *O. fasciatus* fed on *H. annuus*.

Oncopeltus fasciatus showed increased sequestration of cardenolides with a diet of *A. curassavica* seeds compared to *A. incarnata* seeds, and between generation one and

zero. This is in line with sequestration of different cardenolide concentration, based on the amount of cardenolides in the diet, that are in accordance to other studies [21, 30, 31]. Females also had significantly higher concentration of cardenolides than males. No significant effect of cardenolide concentration, however, was found on redness or luminance in any generation. This leaves an open question of what drives variation in warning signals that are observed in the wild, and have quantified experimentally in this study.

4.1 Cardenolide Sequestration

The data supported the hypothesis that *O. fasciatus* contains higher concentrations of sequestered cardenolides, when fed on a diet with a higher cardenolide concentration.

Other research shows that *O. fasciatus* sequestered more cardenolide with diets with a higher cardenolide concentration, either with fresh plant material or with artificial diets [21, 23, 30, 31]. A summary of the findings for cardenolide sequestering in *O. fasciatus* is included in table 1.1. Even though the methods of the stated studies are different there are still similarities.

Pokharel et al. kept *O. fasciatus* in Petri dishes and fed them with an artificial diet with three different cardenolide concentrations [21]. They found that *O. fasciatus* fed on diets with higher cardenolide concentration led to higher concentrations of sequestered cardenolides in *O. fasciatus*. These findings are in agreement with the present research (section 3.3.1). One difference is that Pokharel et al. used the concentration of cardenolides corrected by the dry weight, whereas the present research used the cardenolide concentration corrected by wet weight. This present study used the wet weight, cause not all samples were freeze-dried. Further experiments should use the dry weight instead of the wet weight. This is to exclude a potential source of error, because bugs could drink right before they were euthanized. This can result in a biased weight, whereas the dry weight shows the amount of weight that the insects gained through food intake alone.

Another difference of this and the other studies are the used group sizes of *O. fasciatus*. Pokharel et al. used group sizes of three whereas this study used ten L2 larvae. Gamberale and Tullberg found that chicks showed greater aversion on feeding the aposematic bug *Tropidothorax leucopterus* with greater group size [62]. Both *O. fasciatus* and *T. leucopterus* are aposematic bugs from the taxonomic family of the Lygaeidae [24, 62]. It could be that colonies with greater group size have individuals that sequester more and some that sequester less according to the potential predation risk that varies with group size. This warrants further study.

This present study found that females sequestered more cardenolides, corrected by wet weight. Isman found a difference in cardenolide sequestration for females and males. Females had a higher concentration of sequestered cardenolides, corrected by teneral wet weight [30]. The results of the present study research confirm this phenomenon (section 3.3.1). Duffey and Scudder fed *O. fasciatus* on *A. syriaca* and found that females sequestered more cardenolides than males, just like in the work of Isman and research of this report (section 3.3.1) [30, 31].

This present study suggest that there is a significant difference between generation one and generation zero in the sequestration of cardenolides for *O. fasciatus* but not between generation two and zero. Not many studies took the influence of generation into account. Rodríguez-Clark measured traits, like colour of wings of *O. fasciatus* for three generations [33], and she found no difference in colour between generations in contrast to the present study (section 4.3). Further studies should try to incorporate a longitudinal design, because it can gain insight about how *O. fasciatus* changes traits in shifting environments and diets, and give information on phenotypic plasticity.

4.2 Weight

For the insects kept in stock boxes (section 2.1), the diet had a significant influence on weight between the populations (section 3.1.3). A post-hoc tukey comparison showed a significant difference between all comparisons (table 3.4).

For the growth assay, the diet of *A. curassavica* were not significant different compared to *A. incarnata* (section 3.3.2). Females were significant heavier and bugs were significantly lighter in further generations (section 3.3.2). Furthermore, a significant interaction between sex and generation two was found. After separating the data between sexes, males showed a significant decrease in every generation (1st, 2nd), whereas females showed only a significant decrease in weight in the last (2nd) generation.

It was expected that the diet was influential for the weight in the growth assay, like it was for the data of the stock boxes, but it was not. An explanation for this difference could be the two different datasets. Whereas the dataset for the stock boxes used data from all three used diets, but in the cardenolide subset was only weight data from *O. fasciatus* on diets of *A. curassavica* and *A. incarnata*. This could mean that *O. fasciatus* of on a diet of seeds from *H. annuus* was significant different in weight, compared to the toxic diets (*A. incarnata* and *A. curassavica*). But for the data from the growth assay, *A. incarnata* was used as the intercept, due to no data for *H. annuus* was available.

Isman, and also Duffey and Scudder found that females were heavier than males regardless of diet [30, 31]. These findings are in agreement with the results in this present study (section 3.3.2). Isman found no correlation of wet weight and sequestered cardenolides [30], like in this present study (section 3.3.2).

In contrast to own findings, Isman observed that tenerals of *O. fasciatus* fed on a diet of *A. curassavica* have a higher wet weight than fed on *H. annuus*, whereas the results of this study found that *O. fasciatus* on a diet of *H. annuus* had the highest weight. One explanation for this difference could be the time *O. fasciatus* where kept on the same diet, leading to an adaptation to this diet. Gordon and Gordon found that during the first seven generations, survival and reproduction were marginal for new mutant strains of *O. fasciatus* [63].

To conclude, the diet was a significant factor for *O. fasciatus* kept for longer generations in the stock boxes, but not during the growth assay. *Oncopeltus fasciatus* was heavier with a diet of *H. annuus*. These finding are in contrast to other work about *O. fasciatus* [30]. Colonies of *O. fasciatus* became lighter with higher generation in the growth assay.

4.3 Redness, Luminance and Colour-Toxicity Relation

For the population boxes (section 2.1), a diet of *A. curassavica* or *A. incarnata* showed a significant increase in redness, compared to a diet of *H. annuus*, for all visual systems (section 3.1.1). For the luminance, toxic diets (*A. curassavica*: high cardenolide concentration; *A. incarnata*: low cardenolide concentration) diets were significant for different visual systems (*C. caeruleus*: none; *C. dilepis*: *A. curassavica*; *H. pyrrithrix*: *A. curassavica*). This is partially like in Heyworths work who reported that *O. fasciatus* kept on *H. annuus* showed the highest values of luminance between the diets of *H. annuus*, *A. incarnata* and *A. curassavica* [64, p. 94]. Although the method of photographing *O. fasciatus* is similar in this study, the study design of breeding is different. Heyworth used *O. fasciatus* that were kept for many generations on the same diet, whereas the present study looked at the shift from the diet of *H. annuus*, to one of the two toxic diets from *A. curassavica* or *A. incarnata*. The data in this present study suggested that diet has a significant effect on the expression of warning signals to different potential predators.

In the second part of the study colour was related to growth and sequestration across generations. when reared on a diet of But colonies from the succeeding generations (one and two) showed significance decrease in redness, regardless of the investigated visual system. It was hypothesized in this present study, that the

concentration of sequestered cardenolides enhances the aposematic qualities of an insect, based on the findings from Blount et al. [14]. The decrease in colour, together with the increased sequestration across generations suggest the opposite to Blount et al. But this can only be discussed tentatively, because the small sample size of *H. annuus* was excluded, and so there is no comparison of the non-toxic control diet, with the two toxic diet of seeds from *A. curassavica* and *A. incarnata*. Furthermore, when individual levels of sequestration were measured, there was no significant difference for cardenolide concentration found on colour. However, the results of the present study are like the work from Heyworth et al. who measured the colour with a similar setup as this present study and found that luminance differences in the bugs was explained by significantly lower levels of the antioxidant glutathione [17]. Bugs that sequestered more cardenolides had lower levels of glutathione. Bugs in this study, that sequestered more cardenolides, also had reduced red-green chroma of their black patches, that was unrelated to oxidative state [64]. The colour of the black patches was not analysed in this present study and could be focused on in future work.

In the present study, males had a higher red-green opponency channel (redness) compared to females, regardless of the visual system. Females had a significantly higher concentration of cardenolides than males, which could be evidence that sex differences in sequestration affects warning signal development. This requires further study. This result of the present experiment, are different to the work of Rodríguez-Clark, who was one of the first to check the parameters of colour of the wings of *O. fasciatus*, with the use of an artifical scale [33, 34]. Rodríguez-Clark kept and measured different traits longitudinal across three generations, similar as this experiment, and found no significance in wing colour between sexes. On the other hand, Davis measured the colour of *O. fasciatus* with the help of a vertical scanner and image analysis software and found a difference in wing colour between females and males [34].

4.4 Implications, Limits of this study and further outlook

One limitation of this study is the missing samples from the diet of seeds from *H. annuus* (section 3.3). This was due to lower survival of the insects on this diet. This is an interesting finding, given that bugs on *H. annuus* are heavier. Answering questions regarding survivability of *O. fasciatus* reared on different diets with the help of survival analysis warrants further study. Not many studies have measured

the brightness, or luminance and the colour in *O. fasciatus* together [34, 64]. This study shows that measuring them according to different visual systems is important to understand warning signal difference. Further studies in the same design that include measurement of oxidative state could provide more insight into the different effects of diet on the warning signal expression found in the present study. While there is no direct link between individual sequestration and warning colours, there is a tentative evidence for differences between diets that may affect other physiological processes that indirectly effect warning signal expression.

5 Summary

Predator-prey relationships have long served as models for the investigation of adaptation and fitness in natural environments. The type of defences that prey have evolved to avoid attack by predators include primary defences such as crypsis, masquerade, and disruptive colouration, and secondary defences like noxious chemicals, urticating hairs, and spines. Aposematism describes the combination of a primary defence of conspicuous warning colour and a secondary chemical defence.

This present study used multi spectral imaging and psychophysical visual modeling in conjunction with high performance liquid chromatography to investigate wheter the aposematic insect, the large milkweed bug *Oncopeltus fasciatus*, bears a cost of sequestering cardenolides chemical defences from its milkweed host plants, that are visible in the development of its warning colours.

Firstly, populations of *O. fasciatus* that were maintained on diets with varying quantities of cardenolides were measured for colour differences. Insects reared on the diet with the most cardenolides had redder warning colours than those reared on lower cardenolide or no cardenolide diets. The insects were redder when analysed for three different visual systems of potential predators (blue tits, jumping spiders, and lizards). This is some of the first quantitative evidence for an effect of foodplant properties on visually perceivable differences in warning signal expression in milkweed bugs.

Secondly, to understand how toxins in the diet can lead to the development of different visual signals, bugs from a toxin-free diet were shifted to either a low (*Asclepias incarnata*) or high (*Asclepias curassavica*) toxin diet, or remained on the no-toxin diet, and were followed for three generations, with the weight and colour analysed. *Oncopeltus fasciatus* fed on the toxic diet of *A. curassavica* or *A. incarnata* had significantly higher redness, compared to bugs fed on *H. annuus*, which was detectable by three visual systems of potential predators. *Oncopeltus fasciatus* sequestered higher amounts of cardenolides when reared on a diet with a higher cardenolide concentration, and females had a significantly higher concentration of cardenolides than males. The concentration of sequestered cardenolides per individual did not have a significant influence on the redness or luminance of the bugs. This

leaves an open question of what mediates the different diets, and in bugs that vary in colouration in the wild. The spatial and temporal variation in biotic and abiotic factors are likely to explain chemical defence and signal expression variability.

Bibliography

1. Endler, J. A. *Interactions between predators and prey* in *Behavioural Ecology : An Evolutionary Approach* (eds Krebs, J. R. & Davies, N. B.) 3rd ed. (Oxford, UK: Blackwell Scientific Publications, 1991).
2. Ruxton, G. D., Allen, W. L., Sherratt, T. N. & Speed, M. P. *Avoiding Attack* 2nd ed. (Oxford University Press, 2018).
3. Umbers, K. D. L., Bona, S. D., White, T. E., Lehtonen, J., Mappes, J. & Endler, J. A. Deimatism: a neglected component of antipredator defence. *Biol. Lett.* **13** (2017).
4. Saporito, R. A., Donnelly, M. A., Spande, T. F. & Garraffo, H. M. A review of chemical ecology in poison frogs. *Chemoecology* **22,** 159–168 (2012).
5. Malcom, S. B. Chemical defence in chewing and sucking insect herbivores: Plant-derived cardenolides in the monarch butterfly and oleander aphid. *Chemoecology* **1** (1990).
6. Jones, P. L., Petschenka, G., Fracht, L. & Agrawal, A. A. Cardenolide Intake, Sequestration, and Excretion by the Monarch Butterfly along Gradients of Plant Toxicity and Larval Ontogeny. *J. Chem. Ecol.* **45,** 264–277 (2019).
7. Poulton, E. B. *The colours of animals: their meaning and use, especially considered in the case of insects* (D. Appleton, 1890).
8. Exnerová, A., Landová, E., Štys, P., Fuchs, R., Prokopová, M. & Cehláriková, P. Reactions of passerine birds to aposematic and non-aposematic firebugs (**Pyrrhocoris apterus**; Heteroptera). *Biol. J. Linn. Soc.* **78,** 517–525 (2003).
9. Exnerová, A., Štys, P., Fučíková, E., Veselá, S., Svádová, K., Prokopová, M., Jarošík, V., Fuchs, R. & Landová, E. Avoidance of aposematic prey in European tits (Paridae): learned or innate? *Behav. Ecol.* **18,** 148–156 (Oct. 2006).
10. Borer, M., Van Noort, T., Rahier, M. & Naisbit, R. E. Positive Frequence-dependent Selection on Warning Color in Alpine Leaf Beetles. *Evolution* **64,** 3629–3633 (Dec. 2010).

11. Svádová, K., Exnerová, A., Štys, P., Landová, E., Valenta, J., Fučikova, A. & Socha, R. Role of different colours of aposematic insects in learning, memory and generalization of naive bird predators. *Animal Behav.* **77** (2009).

12. Cummings, M. E. & Crothers, L. R. Interacting selection diversifies warning signals in a polytypic frog: an examination with the strawberry poison frog. *Evol. Ecol.* **27,** 693–710 (2013).

13. Summers, K., Speed, M. P., Blount, J. D. & Stuckert, A. M. M. Are aposematic signals honest? A review. *J. Evol. Biol.* **28,** 1583–1599 (2015).

14. Blount, J. D., Speed, M. P., Ruxton, G. D. & Stephens, P. A. Warning displays may function as honest signals of toxicity. *Proc. Royal Soc. B: Biol. Sci.* **276,** 871–877 (2009).

15. Ojala, K., Julkunen-Tiitto, R., Lindström, L. & Mappes, J. Diet affects the immune defence and life-history traits of an Arctiid moth Parasemia plantaginis. *Evol. Ecol. Res.* **7,** 1153–1170 (2005).

16. Sandre, S.-L., Tammaru, T., Esperk, T., Julkunen-Tiitto, R. & Mappes, J. Carotenoid-based colour polyphenism in a moth species: search for fitness correlates. *Entomol. Exp. et Appl.* **124,** 269–277 (2007).

17. Heyworth, H. C., Pokharel, P., Blount, J. D., Mitchell, C., Petschenka, G. & Rowland, H. M. Antioxidant availability trades off with warning signals and toxin sequestration in the large milkweed bug (Oncopeltus fasciatus). *Ecol. Evol.* **13,** e9971 (2023).

18. Reudler, J. H., Lindstedt, C., Pakkanen, H., Lehtinen, I. & Mappes, J. Costs and benefits of plant allelochemicals in herbivore diet in a multi enemy world. *Oecologia* (2015).

19. Lindstedt, C., Talsma, J. H. R., Ihalainen, E. & Lindström, L. Diet Quality Affects Warning Coloration Indirectly: Excretion Costs in a Generalist Herbivore. *Evolution* (2009).

20. Agrawal, A. A., Böröczky, K., Haribal, M., Hastings, A. P., White, R. A., Jiang, R.-W. & Duplais, C. Cardenolides, toxicity, and the costs of sequestration in the coevolutionary interaction between monarchs and milkweeds. *Proc. Natl. Acad. Sci.* **118,** e2024463118 (2021).

21. Pokharel, P., Steppuhn, A. & Petschenka, G. Dietary cardenolides enhance growth and change the direction of the fecundity-longevity trade-off in milkweed bugs (Heteroptera: Lygaeinae). *Ecol. Evol.* **11,** 18042–18054 (2021).

22. Shenk, M. K. *Fertility and fecundity* in *The International Encyclopedia of Human Sexuality* 369–426 (John Wiley & Sons, Ltd, 2015).

23. Agrawal, A. A., del Alba, L. E., López-Goldar, X., Hastings, A. P., White, R. A., Halitschke, R., Dobler, S., Petschenka, G. & Duplais, C. Functional evidence supports adaptive plant chemical defense along a geographical cline. *Proc. Natl. Acad. Sci.* **119,** e2205073119 (2022).

24. Feir, D. **Oncopeltus fasciatus**: a research animal. *Annu. Rev. Entomol.* **19,** 81–96 (1974).

25. Petschenka, G. & Agrawal, A. A. Milkweed butterfly resistance to plant toxins is linked to sequestration, not coping with a toxic diet. *Proc. Royal Soc. B: Biol. Sci.* **282,** 20151865 (2015).

26. Dobler, S., Dalla, S., Wagschal, V. & Agrawal, A. A. Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase. *Proc. Natl. Acad. Sci.* **109,** 13040–13045 (2012).

27. Zhang, R.-R., Tian, H.-Y., Tan, Y.-F., Chung, T.-Y., Sun, X.-H., Xia, X., Ye, W.-C., Middleton, D. A., Fedosova, N., Esmann, M., Tzen, J. T. C. & Jiang, R.-W. Structures, chemotaxonomic significance, cytotoxic and Na+,K+-ATPase inhibitory activities of new cardenolides from Asclepias curassavica. *Org. & Biomol. Chem.* **12** (2014).

28. Yatime, L., Laursen, M., Morth, J. P., Esmann, M., Nissen, P. & Fedosova, N. U. Structural insights into the high affinity binding of cardiotonic steroids to the Na+,K+-ATPase. *J. Struct. Biol.* **174,** 296–306 (2011).

29. Lohr, J. N., Meinzer, F., Dalla, S., Romey-Glüsing, R. & Dobler, S. The function and evolutionary significance of a triplicated Na, K-ATPase gene in a toxin-specialized insect. *BMC Evol. Biol.* **17,** 1–10 (2017).

30. Isman, M. B. Dietary influence of cardenolides on larval growth and development of the milkweed bug **Oncopeltus fasciatus**. *J. Insect Physiol.* **23,** 1183–1187 (1977).

31. Duffey, S. S. & Scudder, G. G. E. Cardiac glycosides in **Oncopeltus fasciatus** (Dallas)(Hemiptera: Lygaeidae). I. The uptake and distribution of natural cardenolides in the body. *Can. J. Zool.* **52,** 283–290 (1974).

32. Duffey, S. S. & Scudder, G. G. E. Cardiac glycosides in North American Asclepiadaceae, a basis for unpalatability in brightly coloured Hemiptera and Coleoptera. *J. Insect Physiol.* **18,** 63–78 (1972).

33. Rodríguez-Clark, K. M. Effect of captivity on genetic variance for five traits in the large milkweed bug (**Oncopeltus fasciatus**). *Heredity* **51–61** (2004).

34. Davis, A. K. Gender- and Size-based Variation in Wing Color in Large Milkweed Bugs (**Oncopeltus fasciatus**) in Georgia. *Southeast. Nat.* **8,** 723–732 (2009).

35. Stevens, M., Párraga, A. C., Cuthill, I. C., Partridge, J. C. & Troscianko, T. S. Using digital photography to study animal coloration. *Biol. J. Linn. Soc.* **90,** 211–237 (Jan. 2007).

36. Conway, B. R. *A history of color research* in *neural mechanisms of Color Vision* (Springer, Boston, MA, 2002).

37. Kawamura, S. & Tachibanaki, S. Explaining the functional differences of rods versus cones. *Wiley Interdiscip. Rev. Membr. Transp. Signal.* **1,** 675–683 (2012).

38. Jordan, G. & Mollon, J. Tetrachromacy: the mysterious case of extra-ordinary color vision. *Curr. Opin. Behav. Sci.* **30,** 130–134 (Dec. 2019).

39. Hart, N. S., Partridge, J. C., Cuthill, I. C. & Bennett, A. T. Visual pigments, oil droplets, ocular media and cone photoreceptor distribution in two species of passerine bird: the blue tit (**Parus caeruleus L.**) and the blackbird (**Turdus merula L.**) *J. Comp. Physiol. A* **186** (2000).

40. Rao, D., Long, S. M., Tapia-McClung, H., Salgado-Espinosa, K., Narendra, A., Aguilar-Arguello, S., Robledo-Ospina, L., Rodriguez-Morales, D. & Jakob, E. M. *Deconstructing a visual signal: the role of motion and colour in predator deterrence* preprint on webpage at `https://www.biorxiv.org/content/10.1101/2022.03.01.482534v1.full.pdf`.

41. Cronin, T. W., Johnsen, S., Marshall, N. J. & Warrant, E. J. *Visual Ecology* (Princeton University Press, 2014).

42. Hering, E. *Grundzüge der Lehre vom Lichtsinn* (Julius Springer, 1920).

43. Larimer, J., Krantz, D. H. & Cicerone, C. M. Opponent-Process Additivity-I:Red/Green Equilibria. *Vis. Res.* **14,** 1127–1140 (11 1974).

44. Higham, J. P., Kimock, C. M., Mandalaywala, T. M., Heistermann, M., Cascio, J., Petersdorf, M., Winters, S., Allen, W. L. & Dubuc, C. Female ornaments: is red skin color attractive to males and related to condition in rhesus macaques? *Behav. Ecol.* **32,** 236–247 (2021).

45. Troscianko, J. & Stevens, M. Image calibration and analysis toolbox - a free software suite for objectively measuring reflectance, colour and pattern. *Methods Ecol. Evol.* **6,** 1320–1331 (2015).

46. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9** (2021).

47. Niswander, R. E. Life History and Respiration of the Milkweed Bug Oncopeltus fasciatus (Dallas). *Ohio J. Sci.* (1951).

48. Nishida, R. Sequestration of Defensive Substances from Plants by Lepidoptera. *Annu. Rev. Entomol.* **47,** 57–92 (2002).

49. Wootton, R. J. *Chapter 268 - Wings* in *Encyclopedia of Insects (Second Edition)* (eds Resh, V. H. & Cardé, R. T.) 2nd ed., 1055–1061 (Academic Press, San Diego, 2009).

50. Bowmaker, J. K., Loew, E. R. & Ott, M. The cone photoreceptors and visual pigments of chameleons. *J. Comp. Physiol. A* **191,** 925 (2005).

51. Stuart-Fox, D., Moussalli, A. & Whiting, M. J. Natural Selection on Social Signals: Signal Efficacy and the Evolution of Chameleon Display Coloration. *The Am. Nat.* **170,** 916–930 (2007).

52. Malcolm, S. B. & Zalucki, M. P. *Milkweed latex and cardenolide induction may resolve the lethal plant defence paradox* in *Proceedings of the 9th International Symposium on Insect-Plant Relationships* (eds Städler, E., Rowell-Rahier, M. & Bauer, R.) 193–196 (Springer Netherlands, Dordrecht, 1996).

53. Pandas development team, T. *pandas-dev/pandas: Pandas* version 1.5.3. Jan. 2023.

54. Waskom, M. L. seaborn: statistical data visualization. *J. Open Source Softw.* **6,** 3021 (2021).

55. Hunter, J. D. Matplotlib: A 2D graphics environment. *Comput. Sci. & Eng.* **9,** 90–95 (2007).

56. R Core Team. *R: A Language and Environment for Statistical Computing* R Foundation for Statistical Computing (Vienna, Austria, 2022).

57. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67,** 1–48 (2015).

58. Kuznetsova, A., Brockhoff, P. B. & Christensen, R. H. B. lmerTest Package: Tests in Linear Mixed Effects Models. *J. Stat. Softw.* **82,** 1–26 (2017).

59. Galton, F. Regression Towards Mediocrity in Hereditary Stature. *The J. Anthropol. Inst. Gt. Br. Irel.* **15,** 246–263 (1886).

60. Fisher, R. A. XV.—The correlation between relatives on the supposition of Mendelian inheritance. *Earth Environ. Sci. Trans.. Royal Soc. Edinb.* **52,** 399–433 (1919).

61. Claeskens, G. & Hjort, N. L. *Akaike's information criterion* in *Model Selection and Model Averaging* 22–69 (Cambridge University Press, 2008).

62. Gamberale, G. & Tullberg, B. S. Aposematism and gregariousness: the combined effect of group size and coloration on signal repellence. *Proc. Royal Soc. London. Ser. B: Biol. Sci.* **265,** 889–894 (1998).

63. Gordon, H. T. & Gordon, B. R. Milkweed bug mutants on sunflower seed: viability and eye-color interactions. *J. Hered.* **62,** 315–& (1971).

64. Heyworth, H. C. *How Bright and How Nasty: The Economics of Variable Aposematic Traits* MA thesis (University of Exeter, 2022).

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