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ORIGINAL PAPER

No honesty in warning signals across life stages in an aposematic bug

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Abstract
Theory predicts that warning signals should exhibit low variation to increase learning efficiency in predators. Many species, however, exhibit variation in warning colours within species and even within populations. An understudied example of within species variation is that between life stages, where animals change warning colouration throughout ontogeny. Understanding how warning signals change throughout life can help us identify the different ecological pressures that affect the evolution of warning signals. We used the Australasian cotton harlequin bug (*Tectocoris diophthalmus*) to explore how adults and nymphs differ in their defensive secretions and colouration. We performed spectrophotometric colour measurements and toxicity bioassays. Our results show, overall, no consistent association between colour and toxicity within species. There was no clear pattern for females and nymphs. Adult males, however, present the highest contrast against backgrounds, highest internal contrast, and highest toxicity. There was no association between colour and toxicity within males, nymphs or females. Our results suggest weak signal honesty in warning signals across life stages and sexes, and demonstrate that variation in colour within species is not necessarily linked to changes in toxicity.

Keywords Aposematism · Ontogeny · Toxicity · Predation · Colour

Introduction

Warning (or aposematic) colours are employed by many animals to advertise toxicity, unpalatability or general unprofitability to their predators (Poulton 1890). Predators learn to associate these colours with a negative stimulus, and subsequently avoid prey with similar colourations (Stevens and Ruxton 2012). In theory, warning colourations should exhibit low variation, because consistent signals are easily remembered by potential predators that

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learn to avoid these colour patterns (Joron and Mallet 1998). Field experiments have shown that local aposematic prey have lower attack rates than novel models, supporting a role for predator learning in the maintenance of warning signals (Lindström et al. 2001; Noonan and Comeault 2009; Chouteau and Angers 2011). Moreover, there are many examples of convergence in colouration (mimicry) between different toxic species (Mallet and Gilbert Jr 1995; Stuckert et al. 2014; Twomey et al. 2014; Amézquita et al. 2017), which also supports the idea that low variation and convergence in warning signals is beneficial for aposematic prey.

Aposematism is a positive frequency-dependent strategy, where uncommon phenotypes are disadvantaged because they are unprotected from potential predators trained with the most common signal (Endler 1988; Sword 1999). Field studies have confirmed the view that polymorphism in warning signals should be selected against (Chouteau et al. 2016). However, there is ample evidence of variation in warning signals in several aposematic taxa, such as frogs, ladybirds, moths and butterflies (Mallet and Joron 1999; Wang and Shaffer 2008; Medina et al. 2013; Rojas and Endler 2013; Hegna et al. 2015), which is puzzling and often considered a paradox (Briolat et al. 2019). In some of these cases, where different morphs are allopatric, colour polymorphisms can potentially be explained by differences in predation pressure. For instance, populations of the harlequin frog (*Oophaga histrionica*) are usually separated by several kilometers, and there is the possibility that different sets of predators maintain different morphs in each population (Medina et al. 2013). Nevertheless, in other cases, such as in the wood tiger moth (*Arctia plantaginis*) the same population can have both yellow and white hindwing aposematic colourations (Hegna et al. 2015), and the presence of polymorphism in the same population has been attributed to potential mimicry, differential survival against predators and mating success, or heterozygote advantage (Nokelainen et al. 2011; Galarza et al. 2014; Rönkä et al. 2018).

A common but poorly studied phenomenon is ontogenetic colour change in aposematic signals. Many insects exhibit differences in aposematic colouration through their life. They can exhibit warning colours at single or multiple life stages and at multiple life stages the signal may be the same or different (Booth 1990; Lindstedt et al. 2016). As seen earlier, changes in warning signals within a population are often considered paradoxical (Joron and Mallet 1998; Briolat et al. 2019), which makes differences in warning signals across an individual's life difficult to explain. Why would the same individual exhibit different warning signals when selection should favour convergence and uniformity in sympatric warning colours? There is very little data on how colour and toxicity vary through the life of aposematic species, although see (de Jong et al. 1991; Grant 2007; Song et al. 2018). For instance, we don't know if these signals are 'quantitatively honest', namely, if conspicuousness correlates positively with the levels of toxicity across life stages [sensu Speed et al. (2010)].

One potential explanation for colour changes between life stages is that each stage occupies a different ecological niche. Species with complete metamorphosis (holometabolous), like tadpoles and frogs, or caterpillars and butterflies, are exposed to very different ecological pressures through their life, and variation in selective pressures on each life stage could explain why colour varies between adults and young (Booth 1990). However, other species exhibit less dramatic changes in ecology across life (e.g. hemimetabolous insects, such as bugs), which makes variation in warning signals across life stages more puzzling. For instance, jewel bugs (Scutelleridae) have adults and nymphs that resemble each other in shape, share the same microhabitat, and this clade also exhibits maternal care (Javahery et al. 2000). Nymphs and adults, however, often have different colours. One possibility is that such differences in colouration are quantitatively honest, and reflect actual differences

in toxicity. This positive correlation has been shown in different aposematic taxa (Speed et al. 2010; Winters et al. 2014; Arenas et al. 2015; Summers et al. 2015). Furthermore, this correlation is predicted by models as well; if displays and defences compete for a shared resource, or if there is little innate avoidance in predators, warning signals should be quantitively honest (Blount et al. 2008; Lee et al. 2011; Summers et al. 2015). In this scenario we would expect changes in toxicity at particular life stages to be accompanied by an increase in conspicuousness. For instance, fifth instar larvae of panic moth caterpillars, *Saucrobotys futilalis,* present a relative increase in toxic defences that is accompanied by an increase in conspicuousness compared to previous instars (Grant 2007).

In this study we use the model system of the cotton harlequin bug (*Tectocoris diophthalmus*, Scutelleridae), an Australasian jewel bug, to help understand why warning signals change through the ontogeny of many aposematic insects. Specifically, we test whether changes in warning colours and conspicuousness are associated with changes in toxicity. We use spectral colour measurements and toxicity bioassays with water fleas to characterize for the first time the ontogenetic change in aposematism in a jewel bug.

Methods

Study species

Jewel bugs (Hemiptera: Scutelleridae) are hemimetabolous insects (i.e. metamorphosis into the adult form is gradual—eggs hatch into nymphs which resemble adults except that they are smaller and lack fully developed wings). They are known as 'jewel bugs', because they exhibit bright and conspicuous coloration (Javahery et al. 2000). They feed on plant sap and are closely related to stink bugs. Like stink bugs, jewel bugs possess odour glands that confer protection from predators (Staddon et al. 1987; Wink et al. 2000). Many jewel bugs are considered to be aposematic, including the cotton harlequin bug (*T. diophthalmus*), a species widely distributed in Australia which has been shown to be avoided by avian predators (Fabricant and Smith 2014; Fabricant et al. 2018). Adult cotton harlequin bugs are bright red/orange with metallic blue markings and in some adults (mostly males) the metallic blue markings occupy most of the scutellum. First instar nymphs emerge as bright red and transition into a metallic blue pattern with red markings in later instars (Fig. 1a), similar to that of most adult males. Importantly, like other Scutelleridae, this species exhibits maternal care, and females often take care of their progeny for several weeks after the eggs hatch (Giffney and Kemp 2014). Usually, all life stages can be found on the same tree, and it is not rare to observe aggregations of both adults and nymphs together.

Study site and insect collection

We collected 4/5th instar nymphs (N = 33) and adults (Females = 23, Males = 16) from Narrabeen, NSW, Australia (33.72 S, 151.29 E) during four field trips, in January, February and December 2018. We chose later instars because these will be the most likely to be present at the same time as adults, although all instars can be found in the same tree and have similar colouration. During each trip we collected both nymphs and adult females and males. Insects were taken to the Australian National University and immediately used for colour and toxicity analyses.

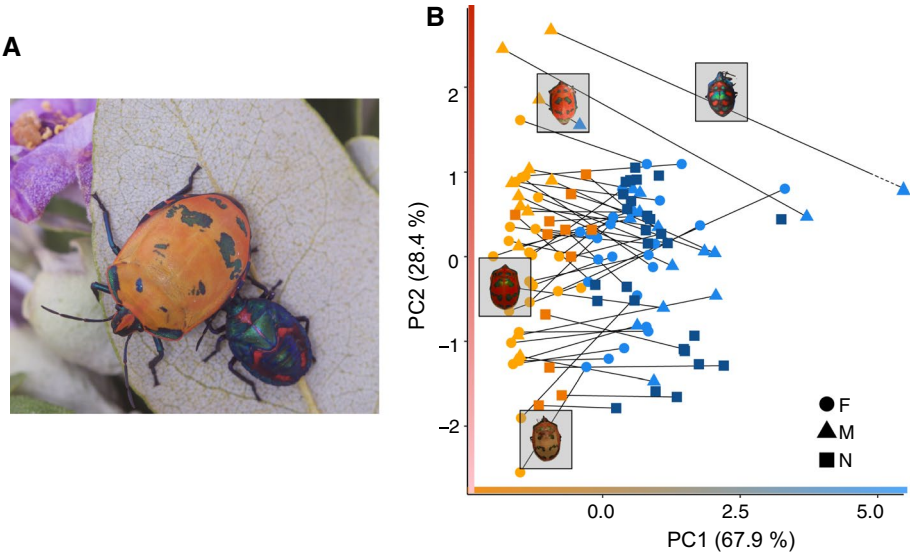

Fig. 1 **a** *Tectocoris diophthalmus* adult female (orange) and nymph (blue) on their host plant (*L. patersonia*). Males sometimes look like the females and in other instances they have a colouration more similar to the nymphs. **b** Colour space with all individuals for which colour was measured: triangles indicate males, circles indicate females and squares indicate nymphs. Blue colours represent the blue patches measured and orange indicate the red/orange patches in each individual. Black lines join colours measured in the same individual, therefore shorter lines represent lower internal contrast while long black lines show higher internal contrast

Colour measurements

We measured colour reflectance of nymphs (N = 33) and adults (F = 23, M = 16) using an Ocean Optics JAZ spectrophotometer (Pulsed Xenon lamp, 300–700 nm) and a custom made 2 mm probe extension with a 45° angle surface that was placed against the body of the insect. To euthanise the insects we put them in a container in a −20°C freezer for 15 min and then measured colouration on a background patch and one of the markings. We chose locations on the semi-elytra of the bug, close to the centre, where the body is relatively flat so the probe could cover any environmental light—in curved surfaces it is harder to prevent environmental light from entering the probe. In some cases, it was too difficult to measure the colour of the markings for the nymphs, because these were very small and the measures were not accurate, so many nymphs only have a background measurement (23 out of 33). We also measured the colour of five leaves and five fruits of the main plant where insects were found (*Lagunaria patersonia*), to provide information on the visual background where these bugs live. Measures were done with an automatic integration time and a boxcar width of 10. The spectra obtained was then processed in the R package pavo (Maia et al. 2013), where we used a smoothing parameter of 0.37, negative values were transformed to zero and only wavelengths from 300 to 700 nm were used.

Since different species present different visual sensitivities, we analysed colour using a visual model that takes into account the visual system of potential predators using the *vismodel* command in pavo. To analyse the colour of the bugs we used the visual system of an average passerine with UV cone type which is the visual system of most passerines

(analyses were repeated with only V cone type and they were qualitatively identical). For receptor stimulation we used the sensitivity data of a blue tit (*Cyanistes caeruleus)*. This was chosen because the most likely predators of these bugs are urban passerines such as magpies (*Gymnorhina tibicen*), noisy miners (*Manorina melanocephala*) and currawongs (*Strepera graculina*). We also chose a standard midday light illumination under a clear sky (D65) and a quantum catch metric that follows Fechner law, meaning that the signal of the receptor channel (from the blue tit in this case) is proportional to the logarithm of the quantum catch. To quantify the contrast (chromatic and achromatic) between the bug and the background we used the command *coldist* in the R package pavo (Maia et al. 2013) and used as background the average spectral measures of the leaves and fruits of *L. patersonia*. Noise was calculated using a neural receptor-noise model with Weber fraction of 0.1, which corresponds to the receptor noise of an insectivorous passerine (*Leiothrix lutea*). Since each individual had two colour patches, we calculated general contrast for each individual by adding the contrast of both colour patches against each background and weighting each patch relative to the average area occupied by each patch.

We also calculated internal contrast for each individual, which could be interpreted as a measure of signal strength, if patterns with higher contrast are more easily learned by predators (Arenas et al. 2015). To do this we extracted the xyz coordinates of the avian tetrahedral colour space calculated above and then computed the Euclidean distance between the background point and spot point for each individual using the three coordinates. Therefore, this measure of internal contrast represents the distance in colour space between the spots and the background colour *inside* each insect.

Defensive secretion bioassays

We tested the strength (absolute and relative to size) of the defensive secretions of the cotton harlequin bugs collected using common water fleas (*Daphnia lineata*) as our test organism. There is evidence showing that the defensive secretions of cotton harlequin bugs are effective against avian predators (Fabricant and Smith 2014; Fabricant et al. 2018), however, it is challenging to use avian predators to compare the effect of these secretions between insect life stages, since the behavioural reaction is not expected to be large. Although *Daphnia* are not natural predators of these bugs they are ideal in this case because these have been shown to be excellent test subjects in ecotoxicology studies: they are highly sensitive, and have been used effectively in biotoxicity assays with other aposematic insects (Harmon and Mousseau 2007; Arenas et al. 2015). *Daphnia* were obtained locally from two different providers (University of Melbourne and aquaticlivelood.com.au). After being in the freezer for 15 min, each cotton harlequin bug was weighed to the nearest 0.01 g using a microbalance and colour measurements were taken (see above) before placing the bug in methanol. Extractions were done immediately after each trip, leading to four separate extractions, called 'trials'. To extract the toxins we followed the protocol described in Arenas et al. (2015) and macerated each individual in a 1.5 ml Eppendorf with a plastic pestle for 1 min. After maceration we added 1.5 ml Methanol 99% (EMSURE®) and vortexed the tube. We put the Eppendorf tube in a −4°C fridge overnight to allow further extraction of defensive secretions. The next day we centrifuged the tube at 13,000 rpm for ten minutes. The supernatant was extracted into a new Eppendorf tube using a micropipette and the pellet and previous tubes were discarded. To obtain the extracted toxins we evaporated the excess methanol using a Vacuum concentrator set at 45°C for 2 h, until there was no visible methanol in the sample. Once the methanol was evaporated, we added

1.5 ml ultrapure water and we homogenised the sample using a vortex. Initial pilot assays showed that a 100% concentration provided enough variation across samples to detect repeatable differences between individuals, so all subsequent assays were conducted at this concentration. To check for reproducibility, we repeated the bioassays in a subsample of 30 extracts and found high repeatability ($r^2 = 0.82$). We also prepared a methanol control using a 5% methanol solution to ensure that residual methanol was not affecting the results of the bioassay. This solution is an overestimation of the possible residual left from the process, since samples were left in the evaporator until no methanol was visibly detectable (less than 1% in the sample). Additionally, we had ultrapure water controls for each of the assays.

The effect of the extracts was tested by placing 10 adult *D. lineata* in 0.5 ml of a 100% solution in a glass test tube. We checked results after 1, 3 and 6 h. However, for two of the four trials it was necessary to use a different *Daphnia* supplier. Although all *Daphnia* were the same species (*D. lineata*), the individuals from our second supplier were more sensitive. Hence, for the third and fourth trials we checked the tubes after 30 min and 1 h. Trial number was included in the statistical analysis to control for differences between *Daphnia* batches and other conditions that might change with the date of the assays. Each trial included nymphs, males and females.

Statistical analyses

To test whether nymphs and adults present differences in contrast against different backgrounds we used linear models with five different response variables: chromatic contrast against leaves and fruits (dS leaves, dS fruits), achromatic contrast against leaves and fruits (dL leaves, dL fruits) and internal contrast. All these variables were log-transformed to increase normality. As the predictor variable we included the life stage and sex of the bug (as a single variable; adult female, adult male, or nymph). To visualise colour variation among individuals and life stages we performed a principal component analysis (scaled and centred) on the x, y, z coordinates obtained from the visual model analysis in pavo. These PCs were not used in any further analysis.

To quantify the differences in the strength of defensive secretions between nymphs and adults we used a linear mixed model (LMM) in the lme4 package (Bates et al. 2014). We used the number of dead *Daphnia* per assay as our response variable and we used the type of insect (adult female, adult male or nymph), the time interval (1st, 2nd or 3rd measure), the weight of the bug and the trial number—since experiments were separated in time and the *Daphnia* provider changed (see above)—as predictor variables. Trial number was included as a fixed effect instead of a random one due to the low number of levels and the expected differences in mean (Gelman and Hill 2006). We used bug ID as a random factor in the model to account for multiple measures for each individual insect extract. We used a logit transformation of the number of dead *Daphnia* to increase the normality of the residuals and model convergence. We also employed two alternative model families that are suitable for analysis of proportions (in this case the proportion of dead *Daphnia)*: beta regression and counts (Poisson) (Ferrari and Cribari-Neto 2004). These analyses were implemented in the R package glmmTMB (Magnusson et al. 2017) and model convergence was checked using the DHARma R package (Hartig 2019). To complement these analyses, we also conducted a separate model in which we used as response variable the number of dead *Daphnia* divided by the size of the bug (to obtain a measure of toxicity that takes size into account). The model was similar to our initial model described above except that it did

not include the weight of the bug as a predictor variable. We calculated *P* values from the models using the Anova command in R.

To explore whether differences in colouration could predict toxicity levels within nymphs or adults we tested across all data whether absolute and relative toxicity (i.e. toxicity divided by weight) could be predicted by either internal contrast or the contrast against backgrounds. We also included trial number as a predictor in the model. Since internal contrast and contrast against backgrounds were highly correlated (LM, $r^2 = 0.60$, $P < 0.001$), we ran separate models with internal contrast and background contrast as predictors. To test whether within life stage categories there was an association between colour and toxicity we ran a linear model similar to the one described above but including an interaction term for the colour trait and the life stage and sex.

Results

Colour differences between life stages

All stages were more contrasting against the fruits than the leaves of *L. patersonia* (Table 1, Figure S1, S2). Males and nymphs were in general significantly more contrasting against fruits and leaves than females (Table 1). Nymphs presented significantly lower internal contrast than males and females (Fig. 1b and Figure S3, t-value = 2.212, $P = 0.03$ and t-value = 3.244, $P = 0.002$), which had similar internal contrasts (t-value = 1.470, $P = 0.148$).

Table 1 Results of eight linear models to test for difference between life stages and sex (predictor) in chromatic and achromatic contrast against the colour of fruits and leaves (response), for the two different colours (red/blue) present in each bug. Each row shows a pairwise comparison from the same linear model

Predictor/response	Achromatic distance				Chromatic distance			
	Estimate	SD	t-value	*P* value	Estimate	SD	t-value	*P* value
Against fruit (red)								
Male versus Female	0.328	0.097	3.37	0.001	0.257	0.102	2.515	0.015
Female versus Nymph	0.342	0.102	3.342	0.001	0.152	0.107	1.413	0.164
Male versus Nymph	0.013	0.114	0.123	0.902	0.105	0.119	0.881	0.383
Against leaves (red)								
Male versus Female	0.135	0.28	0.485	0.63	0.135	0.101	1.347	0.184
Female versus Nymph	0.466	0.293	1.59	0.118	0.022	0.106	0.215	0.831
Male versus Nymph	0.331	0.326	1.015	0.315	0.113	0.117	0.958	0.343
Against fruit (blue)								
Male versus Female	0.159	0.074	2.141	0.0362	0.437	0.148	2.929	0.004
Female versus Nymph	0.167	0.063	2.636	0.01	0.373	0.126	2.958	0.004
Male versus Nymph	0.007	0.073	0.109	0.913	0.063	0.145	0.441	0.66
Against leaves (blue)								
Male versus Female	1.248	0.465	2.2684	0.009	0.341	0.152	2.236	0.028
Female versus Nymph	1.181	0.396	2.979	0.004	0.273	0.13	2.097	0.04
Male versus Nymph	0.067	0.455	0.147	0.883	0.068	0.149	0.459	0.648

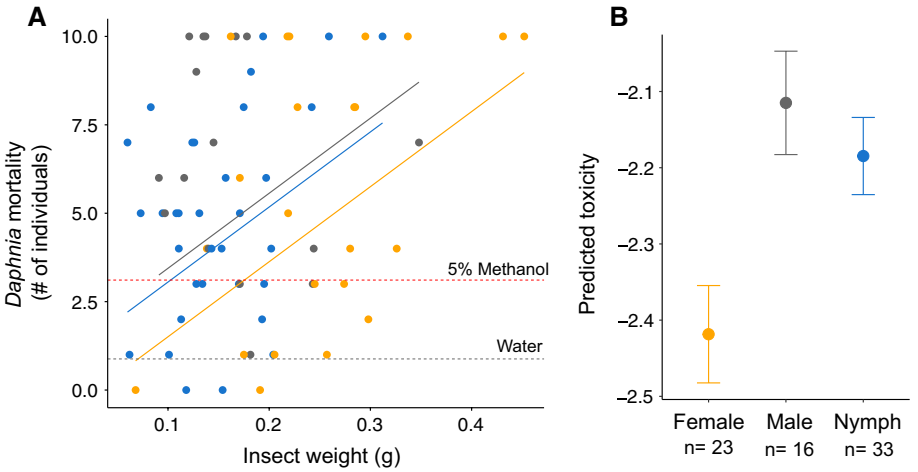

Fig. 2 Results of toxicity assays. **a** Association between insect size and absolute toxicity, lines represent predicted values from LMM (Table 2). **b** Predicted relative toxicity (logit *Daphnia* mortality) from model in Table 2a, controlling for the effect of size (mean size), trial number and hour measured (last measure). Circle shows mean and bars show 95% confidence interval of the mean. Colours in A and B represent different life stages and sex (orange: females, grey: males, blue: nymphs)

Table 2 Results of LMM testing differences in toxicity between life stages, taking into account hours of exposure and bug body weight

Response	(a) logit toxicity		(b) log(toxicity/weight)	
Predictors	Chi-square	*P* value	Chi-square	*P* value
Sex and stage	9.379	0.009	6.289	0.043
Weight	28.211	< 0.001	–	–
Trial number	85.068	< 0.001	42.96	< 0.001
Hour	171.72	< 0.001	110.20	< 0.001

(a) Results when using as response variable logit of dead *Daphnia* and (b) results when using the log of the ratio of number of dead (toxicity/weight)

Toxicity differences

Insect extracts killed on average more *Daphnia* than both water and methanol controls (mean ± SD; water: 1.09 ± 0.77, 5% methanol: 3.44 ± 3.04, insects: 5.73 ± 3.24). Larger individuals were more toxic than smaller individuals (Fig. 2a, Table 2). After controlling for the effect of weight (i.e. if weight was included in the model or toxicity was divided by weight), males were significantly more toxic than females and nymphs (Fig. 2b, Table 2 and S1). When using absolute toxicity values (without weight included in the model) males (which tend to be larger than nymphs) killed significantly more *Daphnia* than nymphs ($\chi^2 = 8.163$, $P = 0.004$), but there were no differences between males and females ($\chi^2 = 0.668$, $P = 0.414$) or nymphs and females ($\chi^2 = 0.672$, $P = 0.412$).

Association between colour and toxicity

Across all individuals there was no association between background contrast and absolute toxicity (Fig. 3a, b; Total background contrast against fruits: t-value = 0.262, $P = 0.794$, against leaves: t-value = 0.069, $P = 0.954$), or internal contrast and absolute toxicity (Internal contrast: t-value = 0.445, $P = 0.658$). There was no association either between colour and relative toxicity (Total background contrast fruits: t-value = 1.669, $P = 0.103$, against leaves: t-value = 1.192, $P = 0.240$, Internal contrast: t-value = 0.602, $P = 0.550$). There was also no association between colour related traits and toxicity when the analysis was done within females, males or nymphs (Table S2).

Discussion

There is mixed evidence that warning colouration provides quantitatively honest signals of toxicity, and that there is a concomitant evolution of toxicity and colouration (Summers et al. 2015). Ladybird adults and eggs present positive correlations between colour (contrast, saturation) and *Daphnia* mortality or coccinelline toxin concentration across species (Winters et al. 2014; Arenas et al. 2015). Nudibranchs and poison frogs present significant associations between colour brightness and toxicity against brine shrimp and mice, respectively (Summers and Clough 2001; Cortesi and Cheney 2010). However, in other cases, there is no quantitative signal honesty, for example there is no consistent association between colour and toxicity across burnet moth species (Briolat et al.

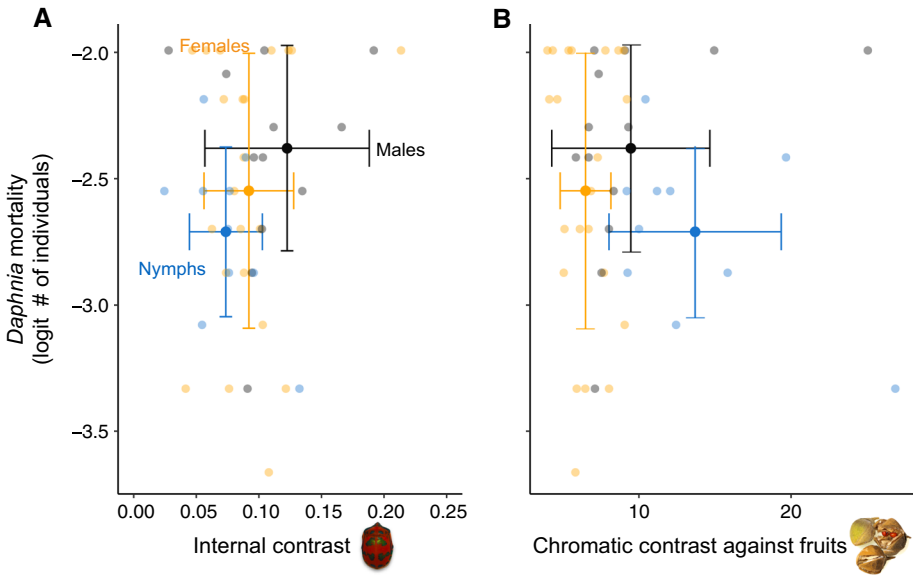

Fig. 3 Association between average colour and average absolute toxicity (on the last measure) across life stages. Lines represent standard deviations and transparent points show raw data. **a** Internal contrast calculated as the colour distance between the two colours present in each individual. **b** Chromatic contrast against fruits, calculated as the total contrast of an insect against the colour of the fruits of *L. patersonia*

2018). Within species there is no evidence either of an association between toxicity and colouration in poison frogs and terrestrial salamanders (Mochida et al. 2013; Stuckert et al. 2018; Sanchez et al. 2019), but there is an association within seven-spot ladybird beetles (*Coccinella septempunctata*) eggs (Winters et al. 2014) and in burying beetles *Nicrophorus vespilloides* (Lindstedt et al. 2019). Several studies have explored quantitative signal honesty, but very few have studied variation throughout ontogeny. These few examples have shown, however, that colour and toxicity can be associated though ontogeny. For instance, in panic moth caterpillars (*Saucrobotys futilalis)* changes in colour are associated with changes in predator deterrence in later larval stages (Grant 2007). Likewise, in spotted lanternflies (*Lycorma delicatula*) a transition to a conspicuous colouration was associated with a change in diet and toxicity (Song et al. 2018). Our results suggest that cotton harlequin bug males are both the most toxic (controlling for weight) and the most contrasting compared to nymphs and females. Males are also more toxic than nymphs—but not females—if not controlling for weight, since they are smaller than females. Given that we ignore if predators consume whole bugs or just sample them, it is hard to know which measure of toxicity (controlling or not for weight) is biologically more relevant. In any case, nymphs and females, present no consistent association between colour traits and toxicity. Moreover, within morphs (nymphs/males/females), there is no association between colour traits and toxicity, suggesting that quantitative signal honesty is absent in this species.

Colour contrast for all stages was higher against fruits than leaves. Cotton harlequin bugs spend a lot of time feeding on the fruits of *L. pattersonia*, so having higher contrast against fruits could be beneficial for the bugs, since predators are less likely to attack and more easily learn to avoid colours that are more contrasting against their background (Aronsson and Gamberale-Stille 2009; Arenas et al. 2015). Differences in luminosity (achromatic contrast) between insects and background and differences in hue (chromatic contrast) were significantly higher in males and nymphs, compared to females.

Males had higher internal contrast, because usually the red and blue colours in males were more saturated than in nymphs or in females. There is some evidence suggesting that high internal contrast could be beneficial in predator learning (Barnett et al. 2016), although other studies have found no effect (Aronsson and Gamberale-Stille 2008, 2009). In combination, our results suggest that the colour signal is stronger in males than in the other two categories. Iridescent patches in the cotton harlequin bug are more common in males, and are suggested to offer a benefit in predator avoidance (Fabricant et al. 2014). These patches are unlikely to play a role in mating, since there is no evidence that scutellerid bugs use colour as sexual signals and, in general, vision is not central in sexual selection in non-predatory heteropterans that live in dense vegetation, instead, these use body size and vibrational cues in courtship (McLain 1998; Čokl 2008).

Bioassays of defensive secretions revealed that extracts of both males and nymphs were stronger than female bug extracts, after considering body size. This suggests that nymphs and males are more toxic per unit of weight, because they are smaller than females. Our findings support earlier evidence showing that the number of epicuticular ductules and chemical compounds is higher in nymphs than in adults of *T. diophthalmus* (Staddon et al. 1987). It also confirms what has been found in other true bugs, where chemicals such as 3-methyl pentanol and hexane are found in higher concentrations in nymphs than in adults (Prudic et al. 2008; Abad et al. 2012). Prudic et al. (2008) found that individuals of the giant mesquite bug (*Thasus neocalifornicus*) that had a higher concentration of chemicals were also better at deterring invertebrate predators. Contrary to what occurs in larvae of coleoptera, diptera, and lepidoptera where aposematism is suggested to be less common

in larval stages (Heinrich 1993), heteropteran larvae show the opposite pattern, where nymphs are more chemically defended than adults.

Both colour and toxicity change significantly between life stages in the cotton harlequin bug, but in general these do not change in the same direction (i.e. increase in contrast is not linked to increase in toxicity) and there are significant sex effects. Males seem to be more protected than females and nymphs. One possibility is that males are more active or disperse longer distances than females and nymphs, and selection could favour a stronger and more effective aposematic signal in these. Males of other members of Pentatomorpha disperse significantly larger distances than females (English-Loeb and Collier 1987; Lee and Leskey 2015). Moreover, females of the cotton harlequin bug exhibit maternal care, which may constrain dispersal at least in comparison with males. Further experiments could explore this interesting possibility. Another explanation for our results could be that selection is not acting on males to be more aposematic, but rather that there are selective pressures to decrease toxicity in females. Maternal care or other physiological traits affected by maternity could potentially affect toxicity, especially if toxicity is related to the diet and females reduce intake while taking care of the eggs, although there is no evidence suggesting the latter occurs (Giffney and Kemp 2014).

Females and nymphs of *T. diophthalmus* look very different (Fig. 1), but the absolute toxicity levels are similar (i.e. without controlling for weight). The presence of such variation could mean that both phenotypes are protected and can deter predators equally effectively. This leaves open the question of why selection has favoured the evolution of such different colouration within the same species, if these changes are not linked to changes in aposematism. Future studies could explore in detail the drivers of colour change during ontogeny and the effect of changes in colour in predator learning processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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