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Warning signal plasticity in hibiscus harlequin bugs

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Abstract

Color variation in aposematic (conspicuous and defended) prey should be suppressed by frequency-based selection by predators. However selection of color traits is confounded by the fact that coloration also plays an important role in many biological processes, and warning coloration may be constrained by biotic or abotic factors. Temperature, in particular the importance of thermoregulation, has been suggested as the source of much of the geographical variation in warning coloration we see in natural populations. Differential selection in different thermal environments may lead to developmentally canalized or 'fixed' differences between populations. Conversely, inter-population differences may be due to phenotypic plasticity, wherein trait expression is modified by environmental conditions. The hibiscus harlequin bug *Tectocoris diophthalmus* (Heteroptera: Scutelleridae), is a shieldback bug, with iridescent patches that show size variation between individuals, as well as inter-population variation with geographic patterning. This study aimed to identify environmental factors that drive the expression of this variable trait, using surveys, modeling, and experimental approaches. Surveys were taken at sites throughout Australia in three climate regions (tropical, subtropical, and temperate) at different time periods, and results were modeled with a multilevel ordinal regression. We tested for correlations between colouration and several biotic (density, host plant) and abiotic (temperature, rainfall) factors. We found strong phenotypic plasticity with respect to temperature and rainfall. Higher temperatures and increased rainfall were related to suppressed iridescence. A factorial experiment with tropical and temperate bugs in two climate-typical temperature regimes confirmed phenotypic plasticity in response to temperature, likely due to temperature sensitivity in melanin expression. Tropical and temperate populations showed striking differences between plasticity reaction norms, suggesting local evolution on the shape of phenotypic plasticity. We suggest that studying both biotic and abiotic selection pressures is important for understanding the causes of inter-population variation in aposematic signals.

Keywords Color variation · Phenotypic plasticity · Aposematic signals · Iridescence · Scutelleridae

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Introduction

Aposematic prey advertise unprofitability through signals that often utilize conspicuous coloration (reviewed in Ruxton et al. 2004). These signals are expected to be under strong purifying frequency-based selection by predators (Greenwood et al. 1989; Lindström et al. 2001). Nevertheless, it is common to find substantial intraspecific variation in color patterns or other visual displays (Stevens and Ruxton 2012). This variation may be the result of phenotypic plasticity, or it may be canalized at a population level. Signals are said to be 'canalized', if phenotypic expression is uniform despite environmental variation (Waddington 1940; Debat and David 2001). Canalization may come about from strong directional or stabilizing selection (Waddington 1942; Stearns 2002). Several aposematic species show evidence of this fixed colour variation, including wood tiger moths (Nokelainen et al. 2013) and *Heliconius* butterflies (Nadeau 2016). Conversely, if color traits (such as aposematic signals) vary with environmental conditions, then the trait is considered to be plastic (Whitman and Agrawal 2009; Fusco and Minelli 2010) and may vary between populations when conditions differ. Phenotypic plasticity in aposematic signals has been found in a number of insect taxa, including butterflies (Nice and Fordyce 2006; Lindstedt et al. 2011), lady beetles (Bezzerides et al. 2007), and true bugs (Johansen et al. 2010). The causes of this counter-intuitive variation, both fixed and plastic, have stimulated ongoing theoretical and empirical research.

Environmental clines, particularly in latitude and/or temperature influence coloration in a wide range of taxa (Rapoport 1969; David et al. 1985; Jablonski and Chaplin 2000). In some species these clinal difference are canalized by tight genetic controls (Dearn 1981; Telonis-Scott et al. 2011), but other species show strong interactions between temperature and color (Solensky and Larkin 2003; Otaki 2008). For example, plastic inverse relationships between temperature and melanization have been documented in butterflies (Solensky and Larkin 2003), beetles (Davis et al. 2008), grasshoppers (Oda and Ishii 1998), true bugs (Aldrich 1986), and flies (Heal 1989; Holloway et al. 1997). The genetic architecture underpinning this plasticity is well-understood in *Drosophila* flies (Gibert et al. 2007). A thermoregulatory benefit to temperature-dependent plasticity has been suggested for lepidopterans (Gunn 1998; Solensky and Larkin 2003) in which temperature-dependent plasticity may constrain aposematic signaling (Lindstedt et al. 2009). However, the benefit of thermoregulatory plasticity in other taxa is more equivocal and has been questioned (Umbers et al. 2013).

Population density may also influence local expression of aposematic signals. For example, melanin-based coloration in some insects is plastic in response to density (Gunn 1998; Barnes and Siva-Jothy 2000). This phenomenon is best known in phase-changing locusts, which are cryptic green and brown when solitary but turn conspicuously yellow with black markings in crowded conditions (Pener and Simpson 2009). This is thought to enable predator defense via aposematism when locus densities are high enough to encourage avoidance learning by predators (Sword 1999, 2002). Density-dependent melanism may also protect against pathogens (Barnes and Siva-Jothy 2000; Cotter et al. 2004). This is due in part to melanized cuticles being more resistant to fungal penetration (St. Leger et al. 1988), but also due to upregulation in the melanin synthesis enzymes, in particular phenoloxidase (Wilson et al. 2001). Melanin itself, and free radicals produced by phenoloxidase enzymes, are toxic to numerous pathogens and parasites (Wilson et al. 2001; Nappi and Christensen 2005). This creates a possibility for trade-offs between aposematic signal efficacy and immune defense (Friman et al. 2009).

Host plant may also play a role in the phenotypic plasticity of colour. Differences may be a direct consequence of pigments ingested by herbivores (Burghardt et al. 2001), or allocation of limited dietary resources (Ojala et al. 2007). Alternatively, insect species may display phenotypic plasticity as a way of enhancing crypsis (Pellissier et al. 2011) or augmenting aposematism (Tullberg et al. 2008; Umbers et al. 2014) against different plant backdrops. Different host plants may also host different milieus of predators, and variation in the composition of predator communities has been suggested as a cause of divergent selection on the expression of aposematic signals (Endler and Mappes 2004; Mochida 2011). Therefore, inter-population variation and phenotypic plasticity may reflect a shifting balance between selection by plants, predators, pathogens, and the thermal environment.

Tectocoris diophthalmus Thunberg 1783 (Heteroptera: Scutelleridae) is an emerging model organism for the study of color variation in aposematism. This common and charismatic species of shieldback stinkbug is broadly distributed across the eastern coast of Australia and offshore Pacific islands (Cassis and Vanags 2006). It has been demonstrated to be well-defended against avian but not arthropod predators (Fabricant and Smith 2014). Unlike many aposematic insects that have red or yellow patterns with black markings (Thery and Gomez 2010; Stevens and Ruxton 2012), adult *T. diophthalmus* expresses iridescent blue-green patches on an orange pigment background (Fabricant et al. 2013). The hue of both the iridescent patches and orange background are variable between individuals. The size of iridescent patches also varies remarkably between individuals, ranging in size from covering the entire dorsal surface to not being present at all (Fabricant et al. 2013). In addition to sexual dichromatism (in which males have larger iridescent patches, on average, than females) and high intrapopulation variance (Ballard and Holdaway 1926), there is anecdotal evidence for inter-population variance in the form of a north–south cline in coloration (Ballard 1927). This level of variation is surprising given that colouration in this species has been shown to induce aposematic avoidance learning (Fabricant et al. 2014).

The color production mechanisms have been characterized for *T. diophthalmus*, which is important for understanding selection pressures acting upon them. The orange pigmentation is created by pterins, while the iridescent patches are produced by an epicuticular proteinaceous matrix interwoven with melanized layers (Fabricant et al. 2013). This dependence on melanin to create the iridescent structural color likely makes the patches sensitive to factors affecting cuticle melanization. The process of depositing melanin is particularly sensitive to temperature, as higher temperatures reduce melanization in other insect species (Gibert et al. 2007; Stoehr 2010). Thus, we can predict iridescent patch size to be a highly plastic trait, and that populations at higher ambient temperatures will express smaller iridescent patches. Furthermore, given the role of melanin in producing the iridescent colouration, we predict that denser populations will express larger iridescent patches as a result of density-dependent prophylactic melanism.

The aims of this study are as follows: (1) to quantify geographic variation in iridescent patch size in *T. diophthalmus*, (2) to determine if this variation is associated with particular biotic (host plant and density), or abiotic (temperature and rainfall) factors and (3) to test if a factor identified in objective 2 influenced colouration in the same way in different populations. We do this using ecological surveys and modeling, in combination with rearing experiments to allow us to experimentally manipulate key environmental factors.

Materials and methods

Surveys and modeling

Sampling of populations was undertaken in three regions spanning the entire continental latitudinal range of *T. diophthalmus*; a tropical region (Darwin, Northern Territory), a subtropical region (Brisbane, Queensland), and a temperate region (Sydney, New South Wales; Fig. 1). Within these three regions, 2–4 sites were selected by visiting locations where this species is known to occur in abundance, based on museum records. Each region was surveyed in April 2011, September 2011, January–February 2012, March–April 2012 and September 2012, although not every site could be visited in each window. The site locations, GPS coordinates, and number of times visited are listed in Table 1. Collection in the Northern Territory was undertaken under Parks and Wildlife Commission Permit #41069 and an entrance to Aboriginal land research permit from the Northern Land Council.

Surveys were performed along transects of trees, shrubs, or hedge rows, ranging between 60 and 200 m. Transects were broken into 1 m × 1 m plots, wherein all *T. diophthalmus* and predaceous arthropods were counted. Adult *T. diophthalmus* were sexed and had their dorsal iridescence scored on a 0–5 scale ranging from 'no iridescence' to 'maximal iridescence' (Fig. 2). Bug density was calculated as total number of bugs divided by transect length (minus gaps between plants). Arthropod predators within transects were noted, and the leaves and seed pods of host plants were collected for identification.

Survey data were analyzed using multilevel ordinal regression modeling (Hedeker and Gibbons 1994) in the R package 'Ordinal' compiled under R version 2.15.2 (Christensen

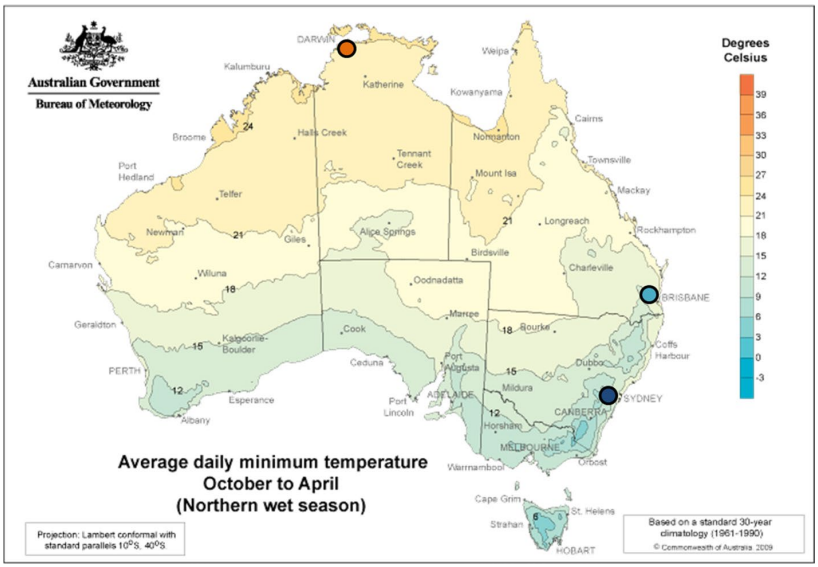

Fig. 1 Map of Australia showing sampling regions and average minimum temperature during the sampling period (taken from the Australian Bureau of Meteorology website). Orange = Tropical, Light blue = Subtropical, Dark blue = Temperate. (Color figure online)

Table 1 Survey sites and the number of times surveyed

Region	Site	Latitude	Longitude	No. of visits
Tropical	Casuarina	− 12.334761	130.8914	3
Tropical	Nhulunbuy	− 12.165625	136.7686	1
Subtropical	Uni of QLD	− 27.497128	153.0132	2
Subtropical	Glamorgan Vale	− 27.504161	152.6254	2
Subtropical	Sandgate	− 27.312566	153.0689	1
Subtropical	Gold Coast	− 28.030319	153.4353	1
Temperate	Narrabeen	− 33.722127	151.2971	3
Temperate	Maroubra	− 33.927694	151.2298	2

Fig. 2 Ordinal scale of iridescent patch size in *Tectocoris diophthalmus*. Each picture is an example bug typical to each pattern designation. Top row from left to right are scored 0, 1, 2, and bottom row left to right are 3, 4, 5

2010). This model assumes an underlying normal distribution to an ordinal response variable, and creates thresholds to partition the underlying distribution into these ordinal levels. Covariates and categorical predictors are used to create a linear model wherein the additive output is a value to be located between threshold values. The package 'Ordinal' uses maximum likelihood estimation using the Laplace approximation for models with random

effects. Candidate predictors of iridescence score included sex, local region, temperature, host plant genus, density of bugs, and average rainfall, all of which were treated as fixed factors. All bugs sampled in one survey share a survey number as their clustering variable, which was necessary for multilevel modeling. Survey number, as well as the sampling sites chosen within each region, were treated as random factors. Temperature at each site was characterized by the average over the prior 30 days at the nearest weather station; data were downloaded from the Australian Bureau of Meteorology website, rainfall was calculated in the same manner. A 30-day period was chosen based on the likelihood of capturing the molting conditions for the present cohort of adults (Ballard and Holdaway 1926). In a preliminary analysis, models fitted using alternative temperature metrics (overall average, average daily high, average daily low, average daily temperature range) were compared based on AIC score, and subsequently average minimum and average maximum temperature were selected as the most informative.

For a given model, significance of fixed factors was assessed using a Wald Z test. Overall fits of nested models were also compared using Likelihood Ratio Tests (LRT), whereas fits of non-nested models were compared using Akaike Information-Theoretic Criterion (AIC; Akaike 1974). Model building was done in a reverse stepwise manner, starting with a model including all factors. Factors with a non-significant Wald Z were removed one at a time, and the new model compared to the previous one with a likelihood ratio test. If this confirmed the removal of the factor did not reduce the power of the model, we proceeded with the new model. Given that temperature and rainfall were significantly correlated (Zuur et al. 2010), we deemed it inappropriate to jointly model them. Instead, competing models with average minimum temperature or average rainfall, including sex, host plant and survey as a random factor, were compared by AIC scores. This comparison reveals the relative explanatory power of each measure to explain local differences. For a full summary of the models tested, along with the AIC scores for each please see Supplementary Table 1.

Controlled rearing experiment

During the September 2011 surveys, 80 late-stage (primarily 4th instar out of 5) juveniles were collected from sites at the extreme northern (Casuarina NT) and southern (Narrabeen NSW) edges of the continental range of the bugs. These bugs were reared to adulthood in a common garden experiment. Within each site, bugs were randomly sampled and assigned to either 'hot' (31C) or 'cold' (23C) temperature treatments (n = 40 for both treatments) with 12:12 h photoperiods and stable ambient humidity of approximately 30%. Each treatment group was further split in half, to be reared in separate mesh enclosures containing a potted *Hibiscus tileaceus* plant (approximately 1 m in height from soil to crown), and supplemented with *Hibiscus heterophyllus* seed pod cuttings replaced once a week. Splitting treatment groups onto two plants was a measure to prevent excessive competition and overstressing the host plant, and data within each treatment group was pooled in the final analysis.

Plants were checked once per day for new adults. Any freshly enclosed bugs that didn't appear to have finished drying were left until the next day, to make sure they were fully sclerotized. They were then killed by freezing and their iridescence was scored as above. Data were analyzed using the Ordinal package as in the previous experiment with iridescence score as the response variable, and treatment, region of origin, and their interaction as fixed factors. Only data from males were analyzed, while results for females are described, as female survivorship was quite poor.

Results

Surveys and modeling

In total, 1117 *T. diophthalmus* bugs were sampled from eight sites in three regions of Australia. The tropical sites had significantly repressed iridescent patches, and thus lower iridescence scores, compared to the subtropical site, while the temperate sites had only somewhat higher iridescent scores than subtropical sites (Table 2). The three regions also showed different frequencies of iridescent scores, with the tropical sites showing a strong bimodal skew toward the extreme ends of the scale, the subtropical sites being somewhat uniform in distribution, and temperate sites somewhat uniform with a skew towards more iridescent bugs (Fig. 3). The random factor of site did not improve the fit of the model, and thus each region can be considered a contiguous population (Table 2).

Among the candidate models, the one with average low temperature as the temperature metric had a greater explanatory power (AIC score: 2871.08), compared to average high (AIC score: 2974.72), and minimum temperature exhibited a significant negative relationship with dorsal coloration, as indexed by iridescence score (Table 2). Iridescent score was negatively correlated with minimum temperature over the 8-month period sampled (Wald $Z = -2.475$, $p = 0.013$, Fig. 4). However, the candidate model with the lowest AIC was the one containing average rainfall, and thus statistics given in the text refer to that model unless otherwise specified. Iridescence score was negatively correlated with average

Table 2 Results of multilevel ordinal regression modeling on data from survey, with average low temperature included as fixed factor

Effect	Coefficient	Standard Error	Wald Z	*p* value	In best fit model?[a]
Sex (male)	1.410	0.128	11.048	< 0.0001	Yes
Region—tropical	− 3.472	0.680	− 5.107	< 0.0001	Yes
Temperate	1.505	0.608	2.475	0.0133	Yes
Avg low temp	− 0.145	0.060	− 2.412	0.0159	Yes
Host plant	− 0.885	0.606	− 1.460	0.1442	Yes
Bug density	− 0.034	0.132	− 0.257	0.7975	No
Thresholds					
0|1	− 3.7829		1.0106		− 3.743
1|2	− 2.7381		1.0064		− 2.721
2|3	− 1.6178		1.0039		− 1.612
3|4	− 0.5587		1.0021		− 0.558
4|5	0.3119		1.0020		0.311
Random intercept	Standard deviation		Chi squared	*p* value	In best fit model?
Survey	0.649		$\chi 2 = 37.773$	< 0.0001	Yes
Site	< 0.0001		$\chi 2 = 0.002$	0.99	No

[a]Coefficients and *p* values for all fixed effects tested (excluding alternate temperature measures) are given. Coefficients and Wald Z/*p* values of best-fit included terms have been adjusted after exclusion of non-significant terms

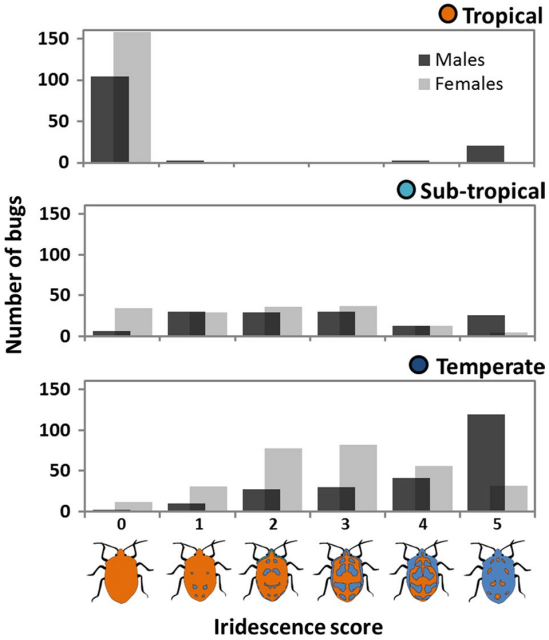

Fig. 3 Frequency histogram of iridescence scores sorted by sex and region. Total n = 1117

rainfall (Table 3, Fig. 5). However this model was not significantly better than that containing average minimum temperature. Therefore we report the results of both. Even when controlling for rainfall and temperature, there were strong regional differences in the distribution of iridescence scores (Tables 2 and 3). Compared to the subtropical region, tropical bugs were much less iridescent (Wald $Z = -8.330$, $p < 0.001$), while temperate region bugs were modestly more iridescent (Wald $Z = 3.827$, $p = 0.00013$). Bug density was not a significant predictor of iridescence score and was removed from both the final models (Tables 2 and 3). Sex was a strong predictor, as males had higher iridescence scores, on average, in all three regions at any given temperature or level of rainfall (Wald $Z = 11.139$, $p < 0.0001$) (Table 2 and 3, Fig. 4 and 5). When average rainfall was used as a fixed factor, host plant was also a significant predictor of iridescence score (Table 3, Fig. 6) with lower iridescence scores associated with feeding on *Lagunaria patersonia*. However this was not significant when average minimum temperature was included as a fixed factor (Table 2). Finally, the survey clustering variable was highly significant (LRT $\chi 2 = 47.759$, $p < 0.0001$). Therefore, the best-fit models included sex, region, host plant, and average low temperature or average rainfall as fixed effects, and survey number as a random effect.

Within tropical sites the most common predators observed were Green Weaver ants, Giant rainforest mantids and Assasin bugs (likely *Pristhesancus plagipenni*), subtropical sites also contained Assasin bugs as well as jumping spiders (likely *Mopsus mormon*), while the most commonly-observed predators in temperate sites were lynx spiders and smaller mantid species such as *Pseudomantis albofimbriata* and *Orthodira ministralis*. Average predator density was higher in tropical sites (0.053 ± 0.101 predators per meter), intermediate in subtropical (0.0332 ± 0.030 predators per meter) and lowest (0.013 ± 0.012 predators per meter) in the temperate region. However there was a high degree of

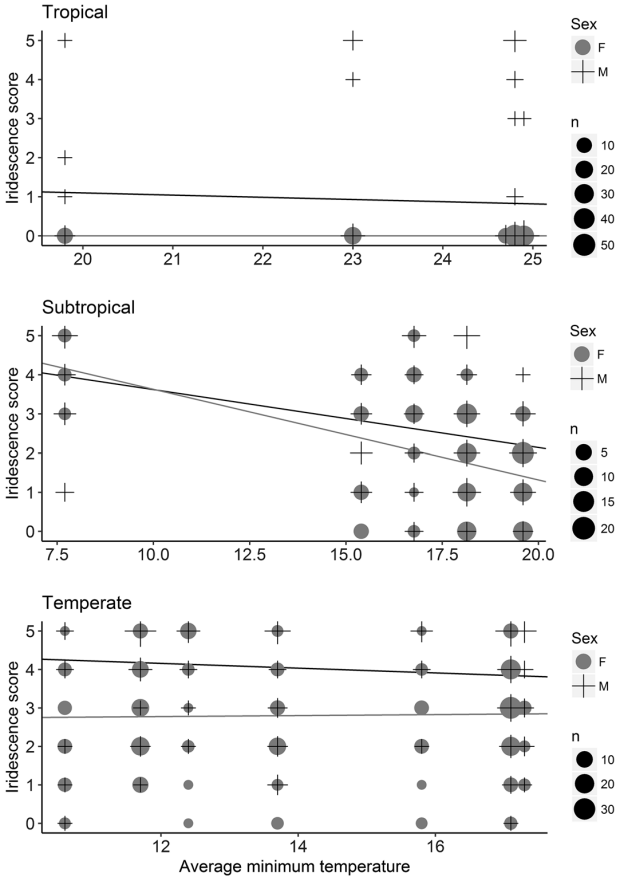

Fig. 4 Plot of iridescence scores against average low temperature for 30 days prior to survey. One plot for each region, with an independent regression line for each sex

variability between both sites and surveys, particularly in the tropics where predators were far more abundant during the rainy season than the dry season.

Controlled rearing experiment

In the rearing experiment male iridescence score was significantly influenced by temperature (Wald $Z = -2.50$, $p = 0.013$) as bugs from both regions of origins showed greater iridescence scores when reared at the lower temperature (Fig. 7). There was also a significant effect of the interaction between rearing temperature and region of origin (Fig. 7). Notably,

Table 3 Results of multilevel ordinal regression modeling on data from survey, with average rainfall included as fixed factor

Effect	Coefficient	Standard error	Wald Z	*p* value	In best fit model?[a]
Sex (male)	1.421	0.128	11.139	< 0.0001	Yes
Region—tropical	− 4.147	0.498	− 8.330	< 0.0001	Yes
Temperate	2.491	0.651	3.827	0.00013	Yes
Avg rainfall	− 0.155	0.051	− 3.020	0.00253	Yes
Host plant	− 1.291	0.612	− 2.110	0.03484	Yes
Bug density	0.006	0.129	0.048	0.961	No
Thresholds					
0|1	− 1.8254		0.3431		− 5.320
1|2	− 0.7828		0.3344		− 2.341
2|3	0.3322		0.3326		0.999
3|4	1.3859		0.3353		4.133
4|5	2.2538		0.3411		6.608
Random intercept	Standard deviation		Chi squared	*p* value	In best fit model?
Survey	0.6209		$\chi 2 = 47.759$	< 0.0001	Yes
Site	< 0.0001		$\chi 2 < 0.0001$	0.98	No

[a]Coefficients and p-values for all fixed effects tested (excluding alternate temperature measures) are given. Coefficients and Wald Z/*p* values of best-fit included terms have been adjusted after exclusion of non-significant terms

tropical bugs maintained their bimodal distribution in both treatments, merely shifting the proportion of high and low iridescence between temperature regimes (Fig. 7).

Female survival was low, particularly for temperate region bugs (cold n = 4, hot n = 2). The results of the temperate region females generally aligned with that of the males, displaying moderate iridescence in the cold regime and reduced iridescence in the hot regime. Tropical females reared in the hot temperature regime (n = 5) all lacked iridescent markings entirely. However, of the tropical females reared in a cold regime (n = 8), four displayed moderate amounts of iridescence (with scores of 1–4). In comparison, no tropical females with iridescent markings were observed during field surveys.

Discussion

As predicted, we found correlative and experimental evidence for phenotypic plasticity in the expression of iridescent patches in adult *T. diophthalmus* bugs. Survey data suggested a role of both temperature and rainfall in driving variation in iridescence in this species, although given the high level of correlation between the two measures it was impossible to separate their relative effects using observational data. In particular, tropical populations experienced both the highest temperatures and rainfall, and showed the lowest

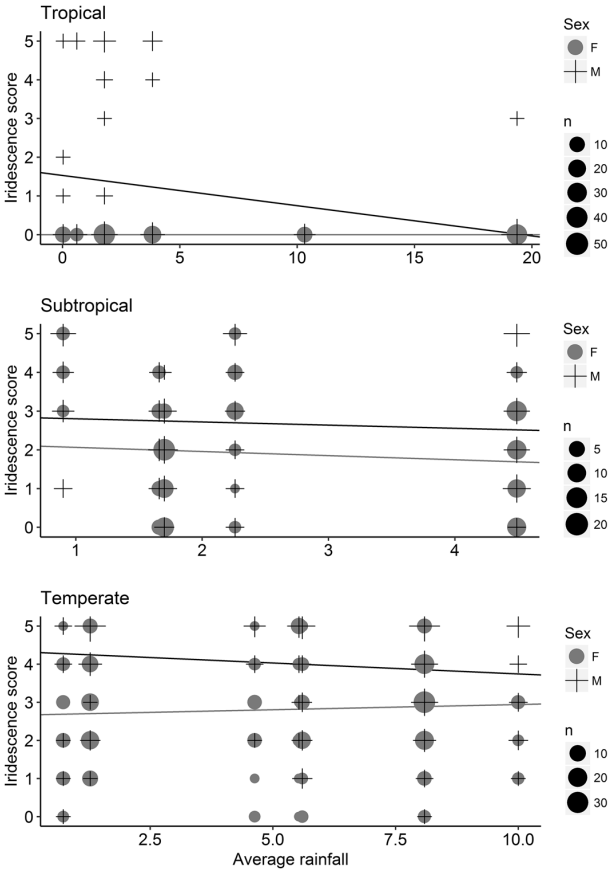

Fig. 5 Plot of iridescence scores against average rainfall for 30 days prior to survey. One plot for each region, with an independent regression line for each sex

iridescence scores. Nevertheless our experimental data show a clear effect of temperature on iridescence.

Particularly within the temperate population, which has both the broadest temperature range and greatest survey coverage, iridescent patch size increased with decreasing temperature (Fig. 4). Given the integral role of melanin in the production of iridescent patches (Fabricant et al. 2013), the etiology of this temperature plasticity is likely similar to mechanism underlying melanin plasticity (Gibert et al. 2007). It is unlikely that these structural blue patches would have a large impact on body temperature as previous studies have failed to find clear evidence of an effect of iridescent colouration on thermoregulation (Schultz and Hadley 1987; Doucet and Meadows 2009; Umbers et al. 2013), although this has yet to be explicitly tested in this species. Aldrich (1986) found similar temperature-dependent plasticity of melanic markings on the dorsal surface under the wings of a pentatomid bug,

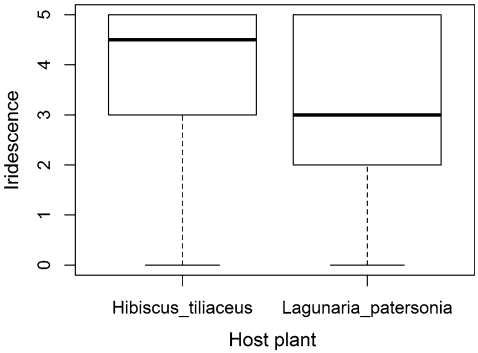

Fig. 6 Average iridescence scores of bugs from the Temperate region when found on *Hibiscus tiliaceus* and *Lagunaria patersonia*

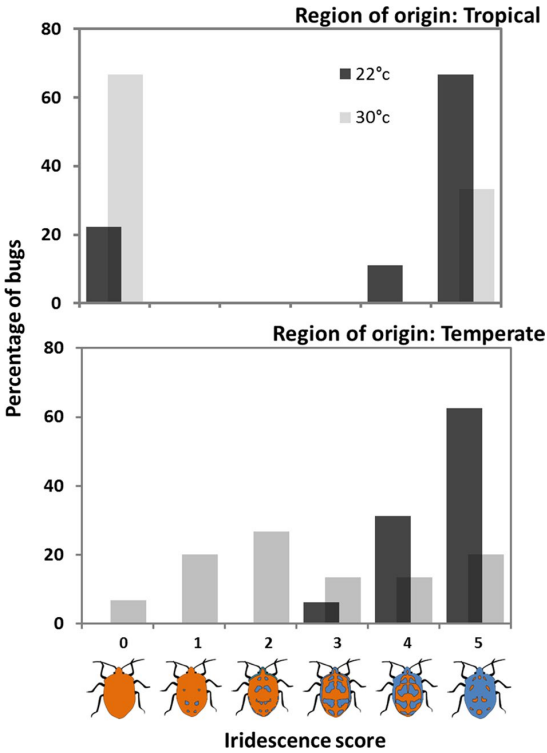

Fig. 7 Histogram of iridescent scores from rearing experiment. Top: Juveniles from Casuarina NT (tropical) raised in hot (n = 12) and cold (n = 10) conditions. Bottom: Juveniles from Narrabeen NSW (temperate) raised in hot (31C; n = 13) and cold (23C; n = 16) conditions

and concluded that its hidden nature made it unlikely that the plasticity had thermoregulatory or signaling benefit in that species. It has been speculated then that temperature-dependent melanism in insects may be a pleotropic effect of a conserved enzymatic pathway (Gibert et al. 2007).

Temperature strongly influences humidity, and environmental moisture has been shown to influence the development of iridescent markings in some insects (Seago et al. 2009). We found that average rainfall correlated significantly with the expression of the iridescence patches. This suggests that humidity-related factors may also drive phenotypic plasticity in this species. However, experimental work manipulating humidity but not temperature is needed in order to confirm this. In addition, it cannot be ruled out that evolutionarily canalized adaptations to climate contribute to the persistent bimodal skew seen in tropical bugs (Fig. 7).

Host plant was found to correlate with iridescence score only in the model containing average rainfall. Given that the bugs were found on only two host plant species, *Hibiscus tiliaceus* and *Lagunaria patersonia* (Malvaceae), across the whole survey, and *L. patersonia* (commonly known as the Norfolk Island hibiscus) was only observed to host the bugs in the Temperate region, this finding must be interpreted with caution. Within the Temperate region feeding on *L. patersonia* appears to be associated with lower iridescence scores (Fig. 7). It should be noted that while differences in host plant species can affect coloration when pigments are sequestered from the diet (Burghardt et al. 2001), *T. diophthalmus* generates its color patterns from endogenously-produced pigments (Fabricant et al. 2013). Furthermore, *H. tiliaceus* and *L. patersonia,* do not differ greatly in the reflectance spectra of their leaves and seed pods (Fabricant, unpublished data), so there is no evidence for divergent selection for maximal conspicuousness against local backgrounds (sensu Boughman 2002).

Finally, despite finding considerable variation in bug densities over the course of the survey (from an average of less than one bug every 10 meters to almost 6 bugs per meter at some sites) population density did not have a significant effect on patch size in *T. diophthalmus* (Tables 2 and 3), ruling out density-dependent prophylaxis via melanism and its disease-resisting consequences (Cotter et al. 2004). Therefore, the adaptive benefit (if any) of phenotypic plasticity in *T. diophthalmus* coloration remains uncertain. Density was highly seasonal, with higher densities recorded in tropical sites during the summer compared to the winter, whereas the opposite was true for temperate sites, as here the bugs would cluster into small areas during the winter.

Our results also confirm the previously observed pattern of sexual dichromatism in this species (Ballard and Holdaway 1926), as the average iridescence score for males was higher than that of females. The effect of sex was approximately equivalent to an 8 degree Celcius difference in rearing temperature (Table 4). The cause of this dichromatism is currently unknown, studies of courtship behavior in this species found no evidence to suggest females evaluate male colour during encounters (Keller 2012). Body size could also play a role, as females tend to be larger than males (Ballard and Holdaway 1926), or behavior, as females guard their eggs (Giffney and Kemp 2014). The colour differences were perhaps most clear in tropical regions, where the majority of females were entirely orange, while subtropical and temperate populations showed less extreme differences in the distribution of iridescence scores between the two sexes (Fig. 3). Interestingly this suggests a greater sensitivity to temperature in females than males in the tropical populations. However the low female survival during the rearing experiment means we were unable to test this. Sex differences in the degree of temperature-linked colour plasticity have been found in the hoverfly *Eristalis arbustorum*. However in this species it was males that showed greater plasticity (Ottenheim et al. 1996).

While temperature, and potentially rainfall, clearly had an effect on colouration in this species, the final models of the survey results also contained significant region effects. There were clear differences in iridescence between temperate and subtropical bugs

(Tables 2 and 3), and this difference was even stronger for tropical bugs, with the addition of a strong bimodal skew (Fig. 3). This means that there are other factors beyond environmentally-dependent plasticity that are influencing inter-population differences. This is also supported by the rearing experiment. Both rearing temperature and the interaction between rearing temperature and region of origin influenced adult coloration, confirming that both temperature-dependent plasticity and local adaptation were real effects in this system (Table 4). Iridescence is reduced for both populations in high compared to low rearing temperature. However, the two populations had very different distributions regardless of experimental temperature regime (Fig. 5). The selective agent causing this difference requires further exploration.

The difference between the tropical and temperate populations in the rearing experiment comes down to differences in their reaction norms, a term to describe how a trait varies along the range of environmental factor such as temperature (reviewed in Whitman and Agrawal 2009). For pigmentation traits, genotypes from tropical populations can have relatively uniform expression (Roskam and Brakefield 1996), while genotypes from the temperate population appear to have a steeper slope of phenotypic expression depending on environmental conditions (David et al. 1990). In our study, temperate bugs responded as expected, but in tropical bugs, the flat reaction norm was punctuated by a sudden transition between extreme states of iridescent patch size. However, detailed analysis of reaction norm shape is limited by the coarse nature of the temperature gradient in our study, and requires more fine-grained temperature intervals. Further investigation would also benefit from a quantitative genetic approach to isolate the responses of specific genotypes, as well as a better understanding of the genetic differentiation between populations in different regions in this species. It may be that tropical populations, which show less seasonal thermal variation, have evolved reduced plasticity and different reaction norms due to more uniform environmental selection regimes, compared to the temporarily fluctuating selection in temperate populations (Gavrilets and Scheiner 1993a; Liefting et al. 2009; Molina-Montenegro and Naya 2012; but see Azevedo et al. 1998; Jenkins and Hoffmann 2000). It may only be possible to disentangle the two scenarios with experimental evolutionary approaches.

Table 4 Results of ordinal regression modeling on data from rearing experiment

Effect	Coefficient	Standard Error	Wald Z	*p* value	In best fit model?[a]
Temperature (30 °C)	−1.707	0.684	−2.496	0.01257	Yes
Region (tropical)	−0.0874	0.863	−0.101	0.919	Yes
Temperature*region	−4.056	1.502	−2.701	0.007	Yes
Thresholds					
0|1	−3.5428		0.7765		−4.563
1|2	−2.7589		0.6518		−4.233
2|3	−1.8960		0.5582		−3.396
3|4	−1.4713		0.5182		−2.839
4|5	−0.5297		0.4685		−1.131

[a]Coefficients and *p* values for all fixed effects tested (excluding alternate temperature measures) are given

Finally, it is worth noting that the survey clustering variable random effect was significant and of sizable magnitude, while site was not (Table 4). This means that there are other factors influencing the distribution of colour morphs or their survivorship that are not accounted by our tested fixed factors, nor explained by site-specific differences. We can only speculate on these possible factors. Perhaps variations in temperature and rainfall matter in critical moments of development, and these effects are not captured in the 30 day average.

Another uncaptured factor is selection by predators. It was noted that while arthropod predator density (i.e. the number of predators observed divided by the length of the transect) was generally low across sites, there was a seeming abundance of large praying mantids during rainy season surveys of the tropical sites (Fabricant, unpublished data). While invertebrate predators such as assassin bugs have been observed to feed on *T. diophthalmus*, their attacks appear to be confined to nymphs, and jumping spiders also seem to avoid attacking adult bugs (Fabricant, personal observation, see also Fabricant and Smith 2014). In contrast, it has previously been demonstrated that praying mantids are insensitive to the defenses of *T. diophthalmus* (Fabricant and Smith 2014), but respond differently to bugs with larger iridescent patches, locating and attacking them more readily than those that are predominantly orange (Fabricant and Herberstein 2014). Thus, arthropod predators, in the form of mantids, may be inducing selection in the tropical region for having reduced iridescence, which could influence local reaction norms and counteract selection for phenotypic plasticity (Falconer 1990; Gavrilets and Scheiner 1993b). Further research quantifying the relative influences of bird and mantid predation across the range of *T. diophthalmus* would be highly beneficial in elucidating this potentially important mechanism.

In conclusion, predator selection on aposematic signals does not exist in a vacuum. Other selective forces including (but not limited to) climate (Lindstedt et al. 2009), parasites (Friman et al. 2009), and sexual selection (Maan and Cummings 2009) may influence the evolutionary trajectory of aposematic patterns. For widespread and variable species, explicit consideration of phenotypic plasticity (Johansen et al. 2010) and composition of local predator communities (Endler and Mappes 2004) is essential for a fuller understanding of the selective forces shaping signal variation and clines. Such a multivariate approach may also help solve other remaining uncertainties in the evolution and maintenance of aposematic signals (Stevens and Ruxton 2012).

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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