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Short title: Environmental drivers of pigment concentrations

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Abstract

Carotenoids are important pigments producing integument coloration; however, their dietary availability may be limited in some environments. Many species produce yellow to red hues using a combination of carotenoids and self-synthesised pteridine pigments. A compelling hypothesis is that pteridines replace carotenoids in environments where carotenoid availability is limited. To test this hypothesis, we quantified concentrations of 5 carotenoid and 6 pteridine pigments in multiple skin colours and individuals from 27 species of agamid lizards. We show that environmental gradients predict the ratio of carotenoids to pteridines; carotenoid

concentrations are lower and pteridine concentrations higher in arid environments with low vegetation productivity. Both carotenoid and pteridine pigments were present in all species, but only pteridine concentrations explained colour variation among species and there were no correlations between carotenoid and pteridine pigments with similar hue. These results suggest that in arid environments where carotenoids are likely limited, species may compensate by synthesising more pteridines, but do not necessarily replace carotenoids with pteridines of similar hue.

INTRODUCTION

Understanding the environmental drivers of colour variation among species is a topic of enduring biological interest because colour is such a striking and variable trait. A textbook example is variation in carotenoid-based colour signals. In contrast to many other pigments that are synthesised by the body, carotenoids must be directly or indirectly acquired from the diet (McGraw 2005). Consequently, a longstanding question is whether colour expression is limited by the availability of carotenoids in the environment (Olson & Owens 1998; Moller *et al.* 2000). At the scale of individuals and populations within species, there is growing consensus that dietary availability of carotenoids in the wild does not often explain variation in the intensity of carotenoid colour signals (Moller *et al.* 2000; Hadfield & Owens 2006; Svensson & Wong 2011; Koch & Hill 2018). However, carotenoid availability may explain colour variation among species; for example, it predicts variation in carotenoid-based plumage colours, though not bare parts among species spanning 140 families of birds (Olson & Owens 2005). Currently, it is unclear whether environmental gradients indicative of carotenoid availability can explain inter-specific colour variation in other taxonomic groups (Svensson & Wong 2011).

In many species, yellow to red colours are not produced exclusively by carotenoids. Ectothermic vertebrates (fish, reptiles, amphibians), for example, often produce yellow to red colours using a combination of carotenoids and a biochemically distinct class of pigments called pteridines (Bagnara & Matsumoto 2006). In these species, pteridines are frequently found together with carotenoids in xanthophore pigment cells (Bagnara & Hadley 1973; Bagnara & Matsumoto 2006). Pteridines are synthesised *de novo* within these pigment cells from abundant purine molecules (Bracher *et al.* 1998; Ziegler 2003; Braasch *et al.* 2007) and vary in colour from yellow (e.g. sepiapterin, xanthopterin) to red (e.g. drosopterin, erythropterin) to 'colourless' (e.g. pterin, isoxanthopterin). These 'colourless' pteridines, which are found in large quantities within

xanthophores (Bagnara & Matsumoto 2006; McLean *et al.* 2017; McLean *et al.* 2019; Twomey *et al.* 2020b), may take crystalline form and thereby affect colour luminance and saturation (Oliphant & Hudon 1993; Palmer *et al.* 2018; Palmer *et al.* 2020) similar to colourless crystalline guanine within iridophores (Teyssier *et al.* 2015; Lewis *et al.* 2017; Nicolai *et al.* 2021). Given that carotenoids and pteridines are found within the same pigment cells and can produce a similar range of hues, a compelling but unsubstantiated hypothesis is that pteridine synthesis can compensate for limited carotenoid availability. Carotenoid limitation is expected to alter the relative cost of acquiring and sequestering carotenoids compared to synthesising pteridines. Specifically, when carotenoids are rare, it may be less metabolically costly to synthesise pteridines; whereas when carotenoids are abundant, it may be less metabolically costly to acquire, transport and sequester carotenoids (Grether *et al.* 2001; Grether *et al.* 2005). However, this hypothesis has yet to be tested at the interspecific level, even though the majority of vertebrates use a combination of carotenoids and pteridines to produce yellow to red integument colours (but see Grether *et al.* 1999; Grether *et al.* 2001; Grether *et al.* 2005 for seminal work on variation among populations of guppies).

Within the broad classes of carotenoids and pteridines, specific pigments have different hues, are acquired or metabolised in different ways, and therefore have different costs and roles in colour production. Carotenoids are produced by plants and the most dominant carotenoids in angiosperms are yellow xanthophylls such as lutein (Heath *et al.* 2013). Insect herbivores generally sequester carotenoids in proportion to the concentration found in the diet (Heath *et al.* 2013). Red ketocarotenoids, such as astaxanthin and canthaxanthin are comparatively rare in terrestrial ecosystems (primarily produced by microalgae and yeast), but some animals, including birds and turtles, can metabolically convert dietary yellow carotenoids to red ketocarotenoids (Lopes *et al.* 2016; Mundy *et al.* 2016; Twyman *et al.* 2016). Due to the cost of metabolic conversion, ketocarotenoids are more strongly associated with measures of individual quality and sexual selection than dietary yellow carotenoids in birds (Weaver *et al.* 2018). The low dietary availability of ketocarotenoids in terrestrial environments may favour the use of red pteridines, particularly if metabolic conversion is costly or unavailable (i.e. in groups that lack the ketolation enzymes necessary for metabolic conversion).

Here, using an extensive dataset of concentrations of 5 carotenoid and 6 pteridine pigments, we test whether pigment concentrations are associated with environmental gradients indicative of carotenoid availability among 27 species of Australian agamid lizards (186 skin samples, 79 individuals, 28 populations with distinct coloration). We use highly accurate liquid chromatography-mass spectrometry to quantify pigment concentrations in skin tissues of agamid lizards (McLean *et al.* 2017; McLean *et al.* 2019). We tested whether environmental gradients predict the concentrations of total carotenoids and pteridines, the ratio between them, or the concentrations of five functionally different groups: dietary yellow and red ketocarotenoids; yellow, red and other 'colourless' pteridines. We refer to the latter category as 'other pteridines' for brevity and to prevent confusion because they affect colour expression. Next, we tested whether pteridines replace carotenoids with a similar hue (carotenoid mimicry hypothesis, Grether *et al.* 1999). The carotenoid mimicry hypothesis predicts a negative correlation between the concentrations of similarly coloured carotenoid and pteridine pigments (i.e. yellow carotenoids with yellow pteridines, red ketocarotenoids with red pteridines). Since concentrations of different pigment types may depend on the strength of sexual selection, we simultaneously tested for relationships between pigment concentrations and proxies for the strength of sexual selection (sexual dichromatism and sexual size dimorphism). Lastly, we evaluated how total carotenoid or pteridine concentrations, as well as each of the five functional groups, covary with skin colour (hue, saturation, luminance). We show that environmental gradients can predict the balance between environmentally acquired and self-synthesised pigments in vertebrates.

MATERIALS AND METHODS

Study Species and Sample Collection

We captured three to five individuals of each of 27 species of agamid lizards (28 populations, which included genetically differentiated populations of *Ctenophorus pictus* from Victoria and South Australia, 79 individuals, 186 tissue samples) from various locations in Victoria, South Australia and Western Australia between September 2015 and January 2016 (Figure 1; Table S6). All individuals were adult males for all but one species, *Ctenophorus maculosus*, for which we sampled a female because it is the only species in our dataset with reverse sexual dichromatism. All lizards were sampled during the spring-summer breeding season to record seasonal coloration in some species of the genus *Diporiphora*. Study species were selected

based on the presence or seasonal expression of yellow-red coloration (as described in literature; Cogger 2018; Melville 2019), and to encompass the range of phylogenetic diversity within Australian agamids from a broad geographic and climatic range. Lizards were humanely euthanised with an intraperitoneal injection of sodium pentabarbitone (150 mg/ kg) after the lizard was calm in a cloth bag, and within 48 hours of capture. Immediately post-mortem, we took standardised photos from which we extracted RGB values and derived measures of hue, saturation and luminance (full details in Supplementary Information). Although these photos do not capture ultraviolet wavelengths, spectral data show that integument colours of species in this study have very little UV reflectance (Supplementary Information, Figure S4). Additionally, lizard colours derived from RGB have been shown to have a statistically similar distribution to near-simultaneously collected spectral data mapped in avian and agamid lizard visual color space (Smith *et al.* 2016). We took skin samples for liquid chromatography-mass spectrometry (LC-MS) analysis and stored the samples in methanol at -20°C in foil-wrapped tubes.

Pigment concentrations

We extracted pigments in tissue samples (approx. 3 x 3 mm) from 1 to 5 body regions of each lizard depending on the colour pattern of the species (186 tissue samples in total). This included 150 tissue samples from body regions that had a component of yellow-red (i.e. including shades of brown) and 36 samples from body regions that were black, grey, white or cream. We used a sequential carotenoid and pteridine pigment extraction procedure which was previously developed for lizard skin (McLean *et al.* 2017, full details in Supplementary Information). In brief, samples were weighed and homogenised in methanol:ethylacetate using a TissueLyser II system (with two 3mm tungsten-carbide beads; Qiagen, Hilden, Germany), and the resulting carotenoid extract was collected following centrifugation. Pteridines were then extracted from the tissue pellet using 2% ammonium hydroxide. We quantified concentrations of 5 carotenoids (lutein/zeaxanthin, 3'-dehydrolutein, β-carotene, astaxanthin, canthaxanthin) and 6 pteridines (drosopterin, xanthopterin, pterin, 6-biopterin, isoxanthopterin, pterine-6-carboxylic acid) commonly found in reptile skin. The yellow carotenoid β-cryptoxanthin and very low levels of the yellow pteridine sepiapterin have also been identified in skin tissue of agamid lizards (McLean *et al.* 2017; McLean *et al.* 2019); however runs for these pigments were inconsistent, so they were not analysed further. Trans-β-apo-8'-carotenal and biopterin-d3 were used as

internal standards for carotenoids and pteridines, respectively. Carotenoids and pteridines were quantified in separate LC-MS analyses on an Agilent 6490 triple quadrupole MS system with a Jet Stream electrospray ionisation source coupled to an Agilent 1290 series LC system (Agilent Technologies Inc, Santa Clara, CA). Chromatographic separation of carotenoids was achieved on an Agilent Zorbax Bonus RP column (2.1 x 50 mm, 1.8 µm.). Chromatographic separation of pteridines was achieved on a Waters Acquity UPLC BEH C8 column (2.1 x 100 mm, 1.7 µm).

Data were analysed using Agilent MassHunter Workstation Software (version B.07.00). All peak assignments were matched against commercial or purified (drosopterin) standards, confirmed with a qualifier ion and quantified against the linear range from six-point calibration curves (all $R^2 > 0.95$). Lutein and zeaxanthin cannot be baseline separated by this method, thus concentrations of the isomer pair Lutein/Zeaxanthin were estimated from a zeaxanthin calibration curve. Final concentrations were normalised against tissue weight: i.e. 'pigment per gram of tissue' referred to as "concentration" throughout for brevity. Commercial standards were used for all pigments except drosopterin, which we extracted and purified from fruit flies, *Drosophila melanogaster* (per Wilson & Jacobson 1977). Consequently, we use the relative response for drosopterin, which we refer to as "level".

For subsequent analyses, we calculated the total concentration of carotenoids and pteridines, as well as the concentrations of 5 different functional subcategories: dietary yellow-orange carotenoids (β-carotene, lutein/zeaxanthin and their common metabolite 3'-dehydrolutein), red ketocarotenoids (astaxanthin, canthaxanthin), yellow pteridines (xanthopterin), red pteridines (drosopterin) and other pteridines (pterin, 6-biopterin, isoxanthopterin, pterine-6-carboxylic acid). Although 3'-dehydrolutein is not a dietary carotenoid, we included it in this category because it is a common metabolite of the dietary xanthophylls lutein and zeaxanthin and indicative of dietary carotenoid intake (Albert *et al.* 2008; Nagao *et al.* 2015).

Predictors: Environment, sexual size dimorphism and sexual dichromatism

Environmental information was extracted using the R package ALA4R (Newman *et al.* 2020), which is an R implementation of the Atlas of Living Australia spatial portal (Belbin 2011). We selected eight variables that together characterise the environment of the species studied. We focused on variables related to vegetation productivity, seasonality and climatic conditions

during the warmest quarter as these are most relevant to carotenoid availability, particularly during the breeding season (Austral Spring) when lizards were sampled. The eight variables were the annual mean growth index for C3 and C4 megatherm plants, annual mean aridity index (the monthly ratio of precipitation to potential evaporation and an indicator of dryness), radiation, temperature and precipitation of the warmest quarter (Bioclim variables 26, 10 and 18 respectively), and temperature and precipitation seasonality (Bioclim variables 04 and 15 respectively; details in supplementary material Table S7). These variables were used in a principal component analysis where the two first axes extracted explained 47.8% and 33.6% of the total variation. The first axis (PC1) was associated with growth index of C3 megatherm (tropical, broadleaved) plants, aridity, radiation and temperature of the warmest quarter (Figure S5; Table S7). In the figures we multiplied PC1 by -1 so that it can be interpreted as overall productivity with high values indicating environments that are more productive, less arid and with less extreme summer radiation and temperatures. The second axis (PC2) is highly related to growth index of C4 plants (mainly grasses), precipitation of the warmest quarter and precipitation seasonality, with higher values indicating wetter, seasonal grasslands (Figure S5; Table S7). The two first axes were used as predictors in posterior analyses.

Measures of sexual dichromatism and size dimorphism were derived from Chen et al. (2013). Briefly, sexual size dimorphism was calculated using the index of Lovich and Gibbons (1992), where sexual dimorphism index (SDI) = [(mean size of male)/(mean size of female)] - 1. Mean male and female size (snout-vent length, SVL) measures were derived from the literature and measured from museum specimens (Chen *et al.* 2012; Chen *et al.* 2013). The index of sexual dichromatism was derived from scores of sex differences in the hue or intensity of colour patterns for each of 9 body regions, with 0 = no difference; 1 = difference in colour intensity or pattern and 2 = entirely different colour or difference in both colour and pattern (Ostman & Stuart-Fox 2011; Chen *et al.* 2012). Colours that may be generated by the same mechanism (e.g. yellow, orange and red) or that may reflect differences in descriptors used in field guides (e.g. cream, white) were scored as differences in colour intensity (1). Scores for the nine body regions were summed to derive a measure of overall sexual dichromatism ranging from 0–18.

Phylogeny and comparative analyses

We built a supermatrix phylogeny of the Amphibolurine Agamidae based on 2 mitochondrial (ND2 and ND4) and 3 nuclear (BDNF, RAG-1 and BACH1) genes, built around a multi-locus nuclear gene backbone taken from the Zheng & Wiens supermatrix dataset (Pyron *et al.* 2013; Zheng & Wiens 2016). Full details of the supermatrix assembly, alignment and phylogenetic analysis are given in Supplementary Information (Supplementary methods, Table S8, Figure S6). We used a subset of 1300 post-burnin trees (subsampled using logcombiner (Bouckaert *et al.* 2019) and pruned off all non-focal taxa (Phytools R package; Revell 2012) in subsequent phylogenetic comparative analyses.

We tested whether variation in the concentration of carotenoids and pteridines was associated with environmental gradients of habitat productivity (indirect compensation) or indices of sexual selection. The response variables in these models were: 1) total carotenoids; 2) total pteridines; and 3) the ratio of carotenoids to pteridines. The predictor variables were environmental PC1 and PC2, sexual size dimorphism and sexual dichromatism (which are uncorrelated, $r^2 = 0.05$, Estimate = -0.003 – 0.009). Given that information on sexual selection indices only exists at the level of species rather than the individual, we also ran species-level models (27 species). We calculated total carotenoids, total pteridines and the ratio of carotenoids to pteridines based on average pigment concentration per species. We used these measures as the response variables and the two indices of sexual selection as predictors.

We next tested for associations between the concentrations of specific carotenoid and pteridine pigments (direct compensation). The variables in these models were the concentrations of: 1) dietary yellow-orange carotenoids (lutein/zeaxanthin, 3'-dehydrolutein, β-carotene); 2) red ketocarotenoids (astaxanthin, canthaxanthin); 3) yellow pteridines (xanthopterin); 4) red pteridines (drosopterin); and 5) other pteridines (pterin, 6-biopterin, isoxanthopterin, pterine-6-carboxylic acid).

Lastly, we tested whether the concentrations of pigments present in skin tissue were associated with its colour. The response variables were luminance, saturation (the intensity of the colour) or hue. For luminance and saturation, we ran two models with either total carotenoids or total pteridines as the predictor. For all colour variables (luminance, saturation and hue) we also ran two models with concentrations of pigment subcategories as predictors: 1) dietary carotenoids

(yellow-orange) and xanthopterin (yellow) and 2) ketocarotenoids (red), drosopterin (red) and other pteridines. These two models were built to avoid having highly correlated predictors in the same model. Using these same subcategories of pigments, we tested for concentration differences between tissues with a yellow to red component (150 tissues, including browns) and those without (36 tissues that were black, grey, white or cream; total 186 samples). Lastly, we tested for associations between colour (luminance, saturation and hue) and environmental gradients of habitat productivity (PC1 and PC2).

All models were run as phylogenetically controlled mixed models in the R package MCMCglmm (Hadfield 2010). We sampled 1300 phylogenies from the posterior distribution of possible phylogenies generated in the Bayesian phylogenetic analyses. The trees employed had 28 tips, which corresponded to the 27 species sampled and two tips from the two populations of *Ctenophorus pictus*. For all models we used phylogeny as a random factor to control for phylogenetic relatedness between species. Given that we had several individuals per species and all individuals had more than one tissue sampled, we also included as random effects the individual and species ID (except in species-level models). We followed Ross et al. (2013) and sampled a tree at iteration t, and ran the MCMC mixed model for 1500 iterations, saving the last sample. This process was repeated for 1300 iterations (one per tree), and the first 300 runs were discarded as burn-in. Inverse Wishart priors (weakly informative) were used for the covariances and we used parameter expanded priors for the random effects. We ensured that all effective sample sizes were above 1000 and visually assessed convergence in the models using the command plot(model). We used custom code to extract a statistic that quantifies the percentage of variance explained by the fixed factors in our models (equivalent to r^2). The graphs presented were generated using ggplot and the predicted fit lines were obtained from simplified mixed models (same as described above but only including significant variables). All pigment concentration variables were $\log_e$ transformed to facilitate convergence, for variables with concentrations of zero we added 0.1 to all samples to avoid infinite values. We present 95% confidence bounds from the posterior distribution of the estimate based on phylogenetic mixed models run on 1000 phylogenies, where cases in which the upper and lower confidence bounds do not overlap zero indicate a significant effect.

RESULTS

312 Skin tissues from all 27 species contained both carotenoid and pteridine pigments (Figure 1).
313 Among our samples, lutein/zeaxanthin (yellow) and β-carotene (orange) were the carotenoids
314 with the highest concentrations. Isoxanthopterin and pterin-6-carboxilic acid were the
315 pteridines with the highest concentrations but we also found substantial concentrations of
316 yellow xanthopterin and red drosopterin (Figure S1).
317
318 **Environmental gradients and pigment concentrations**
319 Total carotenoid concentration was significantly associated with environmental gradients.
320 Individuals living in arid environments with low vegetation productivity (and thus potential
321 carotenoid limitation; environmental PC1) had a lower concentration of total carotenoids
322 (Figure 2a, Table 1, r^2= 0.16). This association was driven by dietary yellow carotenoids (Table
323 S2) because they comprise the great bulk of total carotenoids; there was no association
324 between red ketocarotenoids and environmental PC1 (Table S2). Individuals in less productive
325 environments also had a higher concentration of total pteridines (Figure 2b, Table 1, r^2= 0.14),
326 though associations between environmental PC1 and specific categories of pteridines (yellow,
327 red or other) were non-significant (Table S2). There was a significantly lower ratio of carotenoids
328 to pteridines in more productive environments (Figure 2c, Table 1, r^2= 0.17).
329
330 To further explore the environmental drivers of pigment concentrations, we examined the
331 association between specific environmental variables and total carotenoids, total pteridines, or
332 their ratio (Table S3). The strongest drivers of pigment concentrations were aridity and radiation
333 of the warmest quarter. Species with low carotenoids, high pteridines and low ratio of
334 carotenoids to pteridines were found in arid environments with high summer solar radiation.
335
336 There was no significant association between sexual selection indices and pigment
337 concentrations in the whole dataset analysis (Table 1) or at the species-level (Table S1),
338 although there was a trend for higher total carotenoid concentration in species with higher
339 sexual size dimorphism (Figure 2D).
340
341 **Correlations between pigment categories**
342 To test whether pteridines replace carotenoids with a similar hue (carotenoid-mimicry
343 hypothesis; Grether *et al.* 1999), we examined the relationships between either red

ketocarotenoids and drosopterin, or yellow-orange dietary carotenoids and xanthopterin. Neither relationship was significant (Figure 3), although there were significant associations between other pigment categories. Specifically, there was a significant positive correlation between the concentration of dietary carotenoids and ketocarotenoids, and between other pteridines and xanthopterin, and a negative correlation between xanthopterin and ketocarotenoids (Figure 3).

Pigment concentrations and skin colour

Variation in skin colours was associated with the concentration of pteridines but not carotenoids (Figure 4). Specifically, redder skin hues (lower hue values) were associated with higher drosopterin concentrations, and more saturated colours were associated with higher xanthopterin, other pteridines and total pteridine concentrations (Table 2). Tissues with higher concentrations of other pteridines also had lower luminance (darker). Yellow-red tissues (including browns, N=150) had higher concentrations of drosopterin (Figure 4C), other pteridines, and ketocarotenoids compared to black/grey/white tissues (N=36; 186 tissue samples in total); whereas dietary carotenoid and xanthopterin concentrations were similar in all skin colours (Table S4, Figure S2). Additionally, skin luminance was associated with habitat productivity (PC1 95% CIs 0.716 – 8.943), with darker colours in more vegetated environments (Figure S3), but environmental PCs did not predict hue or saturation (Table S5).

DISCUSSION

Using a large interspecific dataset of pigment concentrations in coloured skin tissue of agamid lizards, we tested whether pigment concentrations are associated with environmental gradients indicative of carotenoid availability. We found that species in more arid environments with high summer temperatures and radiation and lower vegetation productivity had lower concentrations of total carotenoids, higher concentrations of total pteridines and consequently, a lower ratio of carotenoids to pteridines. Across all species, the concentrations of carotenoid and pteridine pigments with similar hue (red ketocarotenoids and drosopterin, yellow dietary carotenoids and xanthopterin), were uncorrelated, indicating that carotenoids are not simply replaced with pteridine pigments of a similar hue (carotenoid mimicry). Although the concentration of dietary carotenoids was of similar magnitude to the concentration of drosopterin or xanthopterin, only pteridine concentrations predicted colour variation among

species: redder hues were associated with higher concentrations of drosopterin, and more saturated colours were associated with higher concentrations of pteridines (xanthopterin, other and total). We found no relationship between carotenoid or pteridine concentrations and indices of sexual selection (sexual dichromatism and sexual size dimorphism), which is consistent with the lack of association between carotenoid concentration and skin colour. Taken together, these results suggest that environmental carotenoid availability may alter the balance between the cost of acquiring and sequestering carotenoids *vs* synthesising pteridines to generate yellow-red skin colours.

In a series of pioneering studies, Grether and colleagues showed that genetically determined pteridine synthesis mirrors environmental carotenoid availability among populations of guppies (Grether *et al.* 1999; Grether *et al.* 2001; Grether *et al.* 2005). In this species, carotenoid and pteridine concentrations are positively correlated among populations to maintain a consistent ratio of tunaxanthin (yellow carotenoid) to drosopterin (red pteridine). This ratio produces the specific orange hue preferred by females (Grether *et al.* 1999; Grether *et al.* 2001; Deere *et al.* 2012). Stabilising selection acting on hue within guppies can explain the positive correlation between pigment types across streams. Across lizard species, however, hue varies greatly, and stabilising selection would not be expected as different species use different signalling colours. Our results instead suggest that carotenoid limitation in some environments may be counterbalanced by synthesis of pteridines, regardless of their hue. In arid environments with low vegetation productivity, where carotenoids are presumably scarce, it may be less costly to synthesise pteridines than to acquire and metabolise carotenoids and *vice versa* in carotenoid-rich environments. However, the specific combination of pigments and skin colour pattern of each species is likely to depend on local selective pressures.

The strongest drivers of the association between total carotenoid concentrations in coloured skin and environmental PC1 were aridity and summer radiation. Most species of agamid lizards occupy semi-arid to arid environments, often with very little vegetation. All species of agamid lizard in this study are insectivorous, though some occasionally eat plant material including flowers (Cogger 2018; Melville 2019). Insects sequester carotenoids in proportion to their dietary availability (Heath *et al.* 2013); thus carotenoid availability may well be limited for both

insects and their predators in arid environments. Limited dietary availability of carotenoids, however, does not necessarily mean that carotenoid availability is limiting for integument coloration. Available carotenoids may be sufficient to meet physiological and colour signalling requirements (Koch & Hill 2018). Furthermore, environmental availability can be compensated by more efficient carotenoid assimilation and transport (Craig & Foote 2001; Koch & Hill 2018). Indeed, the prevailing view is that carotenoid limitation, where it exists, is due more to physiology (internal factors) than environmental availability (McGraw *et al.* 2003; Hadfield & Owens 2006; Simons *et al.* 2014; Koch & Hill 2018). This view is primarily derived from the literature on birds, in which there is limited and inconsistent evidence for an association between feather carotenoid concentrations and diet (Mahler *et al.* 2003; McGraw *et al.* 2003; Olson & Owens 2005). However, selection on carotenoid metabolism may differ greatly for birds compared to poikilothermic vertebrates (fish, amphibians, reptiles) because birds have different colour producing mechanisms and do not use pteridines to colour feathers. Thus, carotenoid availability may well be limiting for skin coloration in lizard species occupying arid environments.

We found that in agamid lizards, concentrations of ketocarotenoids were generally low (particularly astaxanthin) relative to other carotenoids. Astaxanthin is produced by a number of bacteria, fungi and algae, and can also be found in large quantities in some red flower petals (Ohmiya 2011). Agamid lizards are known to seek out and eat flower petals so could potentially obtain astaxanthin from the diet; however, astaxanthin and other ketocarotenoids are generally rare in the diet of terrestrial animals (Svensson & Wong 2011; Heath *et al.* 2013; Koch & Hill 2018). Nevertheless, in some species, ketocarotenoids from dietary sources can accumulate when enzymes responsible for carotenoid breakdown, such as the β-carotene oxygenase enzymes BCMO1 and BCO2, are disrupted or deactivated (Twomey *et al.* 2020a). More commonly, ketocarotenoids are metabolically converted from dietary yellow xanthophylls through oxidation reactions catalysed by ketolation enzymes (ketolases; Lopes *et al.* 2016; Mundy *et al.* 2016; Twyman *et al.* 2016). Metabolic conversion of dietary yellow xanthophylls to red ketocarotenoids has not been demonstrated in lizards, and the CYP2J19 gene that encodes the primary ketolase in birds and turtles is absent in squamates, tuataras and crocodilians (Twyman *et al.* 2016). A similar P450 enzyme (encoded by the gene CYP3A80) may act as a ketolase in the dendrobatid poison frog *Ranitomeya sirensis* and possibly other amphibians (Twomey *et al.* 2020a) but whether this may be the case in reptiles is not currently known. In

this species of frog, the carotenoid cleavage enzyme BCO2 is also disrupted, possibly facilitating accumulation of ketocarotenoids and their dietary precursors (Twomey *et al.* 2020a). BCO2 is associated with yellow coloration in the wall lizard *Podarcis muralis*, but not other polymorphic lacertids (Andrade *et al.* 2019). Therefore, it is unclear whether agamid lizards have evolved mechanisms to enhance assimilation or enable conversion of dietary carotenoids to ketocarotenoids. The positive association we identified between the concentration of dietary carotenoids and ketocarotenoids could indicate increased ketocarotenoid conversion when dietary carotenoid availability is high, or that ketocarotenoids are similarly more available through diet. An absence of a mechanism for ketocarotenoid conversion may explain the prevalence of drosopterin to produce orange and red hues in lizards and some other groups of poikilothermic vertebrates. However, neither ketocarotenoid nor drosopterin concentrations were associated with environmental gradients in our dataset.

Among the 28 taxa in our dataset, skin colour was associated with the concentration of pteridines rather than carotenoids and there was no correlation between the two. In most other lizards, yellow is produced by high relative concentrations of dietary carotenoids and orange-red is produced by a high relative proportion of red pteridines (usually drosopterin; Ortiz *et al.* 1963; Ortiz & Maldonado 1966; Macedonia *et al.* 2000; Steffen & McGraw 2009; Weiss *et al.* 2012; Haisten *et al.* 2015; McLean *et al.* 2017; Andrade *et al.* 2019). Although carotenoids contribute to skin coloration, carotenoid concentrations are often uncorrelated with hue, saturation or luminance (Steffen *et al.* 2010; Weiss *et al.* 2012). Instead, hue frequently corresponds to the concentration of red pteridines, particularly drosopterin (Steffen *et al.* 2010; Weiss *et al.* 2012; Andrade *et al.* 2019). Our data are consistent with these studies and suggest that the intensity of yellow-red coloration is seldom a reliable indicator of carotenoid content in lizards. This suggests in turn that expression of yellow-red signalling colours in lizards is unlikely to convey information on individual quality through mechanisms of honest carotenoid signalling such as resource trade-offs or indicator mechanisms (Koch *et al.* 2017; Koch & Hill 2018). Instead, the honesty of these colour signals may be maintained by other costs such as predation risk associated with conspicuous coloration (Stuart-Fox *et al.* 2003; Amdekar & Thaker 2019). More generally, honest carotenoid signalling may not apply to the many species of poikilothermic vertebrates that use a combination of pteridine and carotenoid pigments to generate yellow-red hues and have complex colour generation mechanisms.

Our comparative analysis uncovered broad patterns in pigment concentrations; however, mechanisms underlying skin colour in reptiles are complex and influenced by structural components. In ectothermic vertebrates, colour is produced by the combination of chromatophore cells containing different pigment types or crystalline structures and structural components of the dermis (e.g. collagen and connective tissue). Xanthophores containing yellow to red carotenoid and/or pteridine pigments comprise the upper layer of chromatophores and may be underlain by iridophores containing periodically arranged guanine crystals, and melanophores containing melanin pigments (reviewed in Grether *et al.* 2004; Bagnara & Matsumoto 2006; Olsson *et al.* 2013; Ligon & McCartney 2016). The extraordinary diversity of integument colours in reptiles and other animals is produced by the interaction of pigments and structural components (Kemp *et al.* 2012). For example, within a mimicry complex of poison frogs (Dendrobatidae), drosopterin contributes to orange coloration but variation in hue across the group is predominantly associated with the thickness of crystalline platelets within iridophores (i.e. structural; Twomey *et al.* 2020b). Furthermore, skin tissue commonly contains high concentrations of crystalline pteridines such as isoxanthopterin and pterin (Bagnara & Matsumoto 2006; McLean *et al.* 2017; McLean *et al.* 2019; Twomey *et al.* 2020b). We found an association between the concentration of these pteridines and skin colour saturation and luminance, as expected if these pigments contribute to colour saturation and luminance through the reflection and scattering of light (Oliphant & Hudon 1993; Palmer *et al.* 2018).

Overall, our results support a scenario where environmental carotenoid availability influences the relative concentrations of carotenoid and pteridine pigments used to generate yellow to red skin colours in lizards. Environmental gradients may shape the ecology and evolution of animal coloration by altering the relative costs of environmentally acquired and self-synthesised pigments.

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Competing Interests

The authors declare that they have no competing interests

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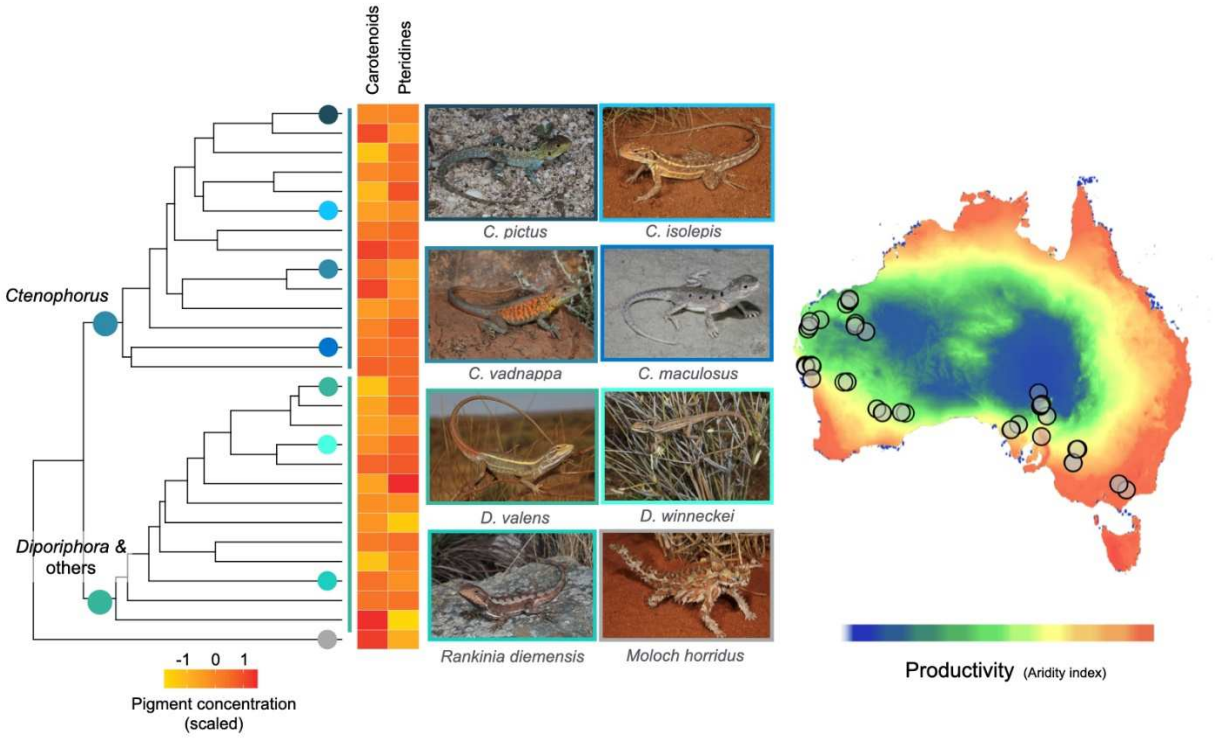
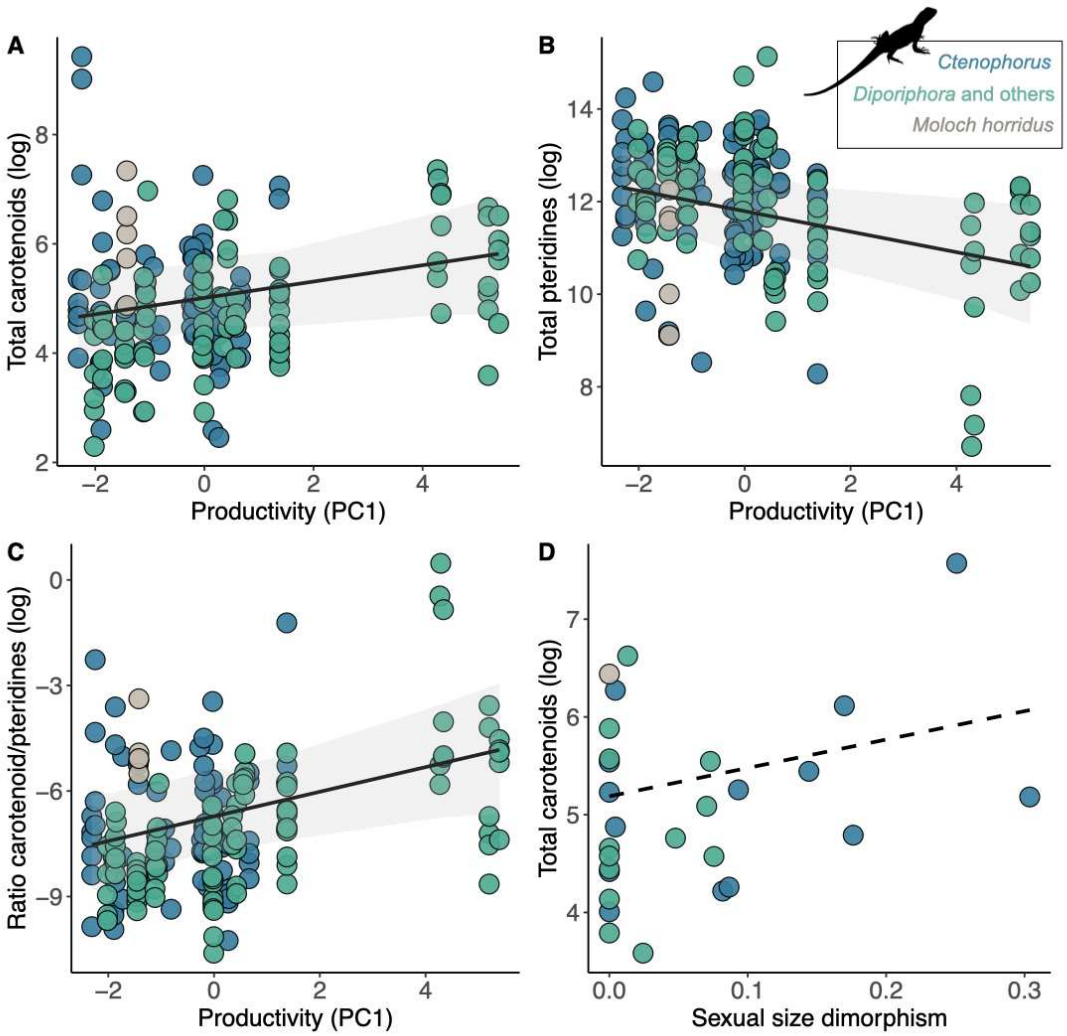
690 **Figures and Tables**



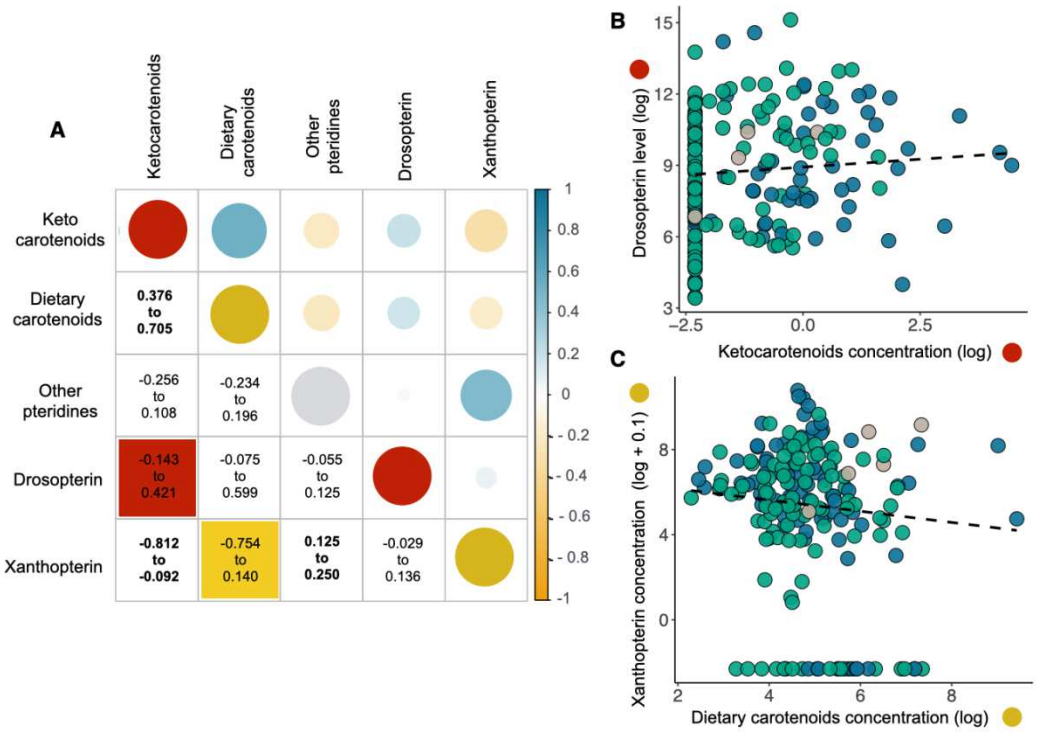
691

692 **Figure 1.** Phylogenetic relationships between the 28 clades (27 species) of Australian agamid
693 lizards included in this study, with images of representative species from the *Ctenophorus* and
694 *Diporiphora* & others (genera *Amphibolurus*, *Gowidon*, *Pogona*, *Rankinia*, *Tympanocryptis*)
695 clades as well as basal *Moloch horridus*. Heatmap shows variation in the concentrations of total
696 carotenoids and total pteridines among species. Map shows extensive geographic sampling (186
697 skin samples from 79 individuals across 30 populations) against a measure of habitat
698 productivity (annual mean aridity index: monthly ratio of precipitation to potential evaporation).



699

700 **Figure 2.** Associations between (A) concentration of total carotenoids, (B) concentration of total
701 pteridines, and (C) ratio of total carotenoids to total pteridine and habitat productivity (PC1*-1),
702 as well as (D) concentration of total carotenoids and sexual size dimorphism. Solid lines indicate
703 a significant relationship with 95% confidence bounds (grey shading), broken lines indicate a
704 non-significant trend between variables (N = 186).



705

706 **Figure 3.** Correlations between carotenoid and pteridine subcategories. **(A)** Correlation matrix
707 showing HPD intervals of estimates from GLMMs. Size and color of upper diagonal circles
708 indicate the strength and direction of the relationship. Central circles indicate the color of
709 pigment subcategories. Significant correlations are in bold. There was no relationship between
710 similarly hued carotenoids and pteridines: red ketocarotenoids and drosopterin (B), and yellow
711 dietary carotenoids and xanthopterin (C). Broken lines indicate a non-significant trend between
712 variables (N = 186).

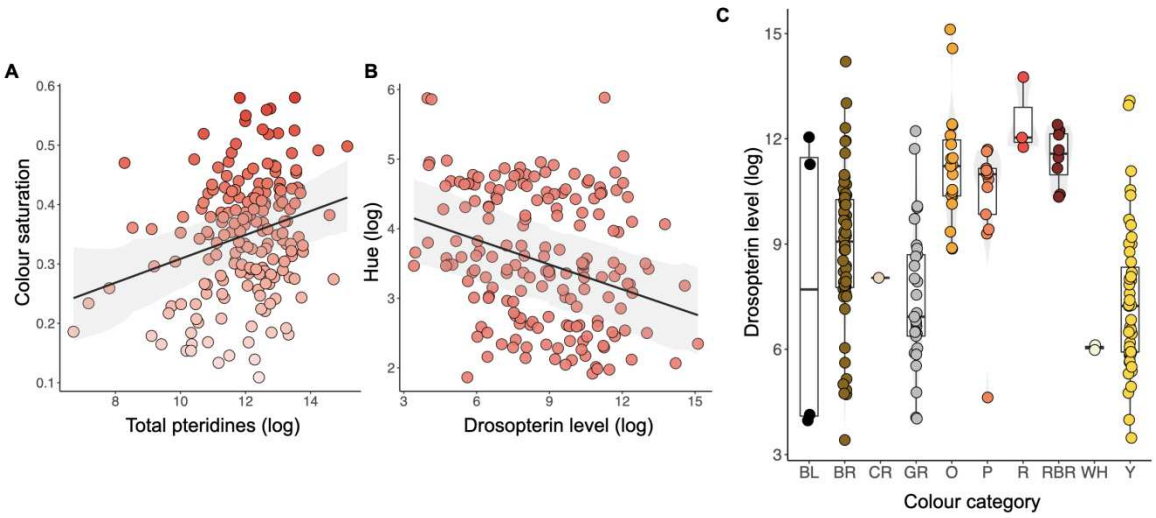


Figure 4. Associations between (A) saturation and the total concentration of pteridines; (B) hue and the concentration of drosopterin. Orange-red colored skin had higher concentrations of drosopterin than other skin colors (C). BL = black; BR = brown, CR = cream; GR = grey; O =orange, P = pink, R = red, RBR = red-brown, Y = yellow skin samples (N = 180).

Table 1. Associations between total carotenoid, total pteridine and the ratio of carotenoid to pteridine pigment concentration and environmental and sexual selection variables (N = 186).

	log(Total carotenoids)		**log(Total pteridines)**		**log(Ratio)**	
Predictors	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
PC1	**-0.354**	**-0.006**	**0.021**	**0.423**	**-0.703**	**-0.087**
PC2	-0.089	0.261	-0.228	0.182	-0.133	0.439
Size dimorphism	-1.071	7.057	-4.216	5.889	-6.428	9.697
Dichromatism	0.039	0.089	-0.093	0.068	-0.053	0.172

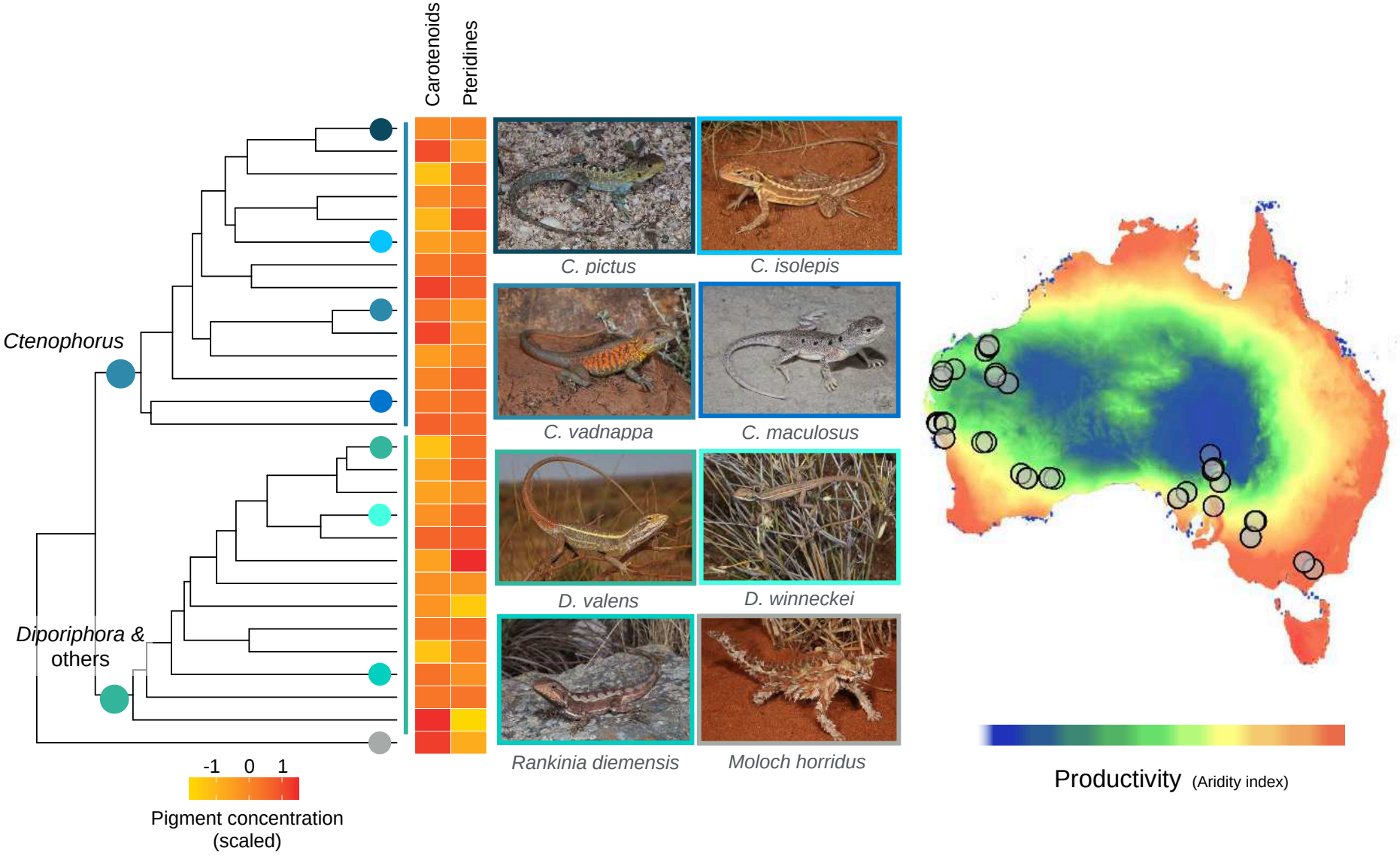
Lower and upper represent the 95% confidence bounds from the posterior distribution of the estimate based on phylogenetic mixed models run on 1300 phylogenies. Values in bold represent cases where the upper and lower confidence bounds not overlap zero and thus there is evidence of a significant effect.

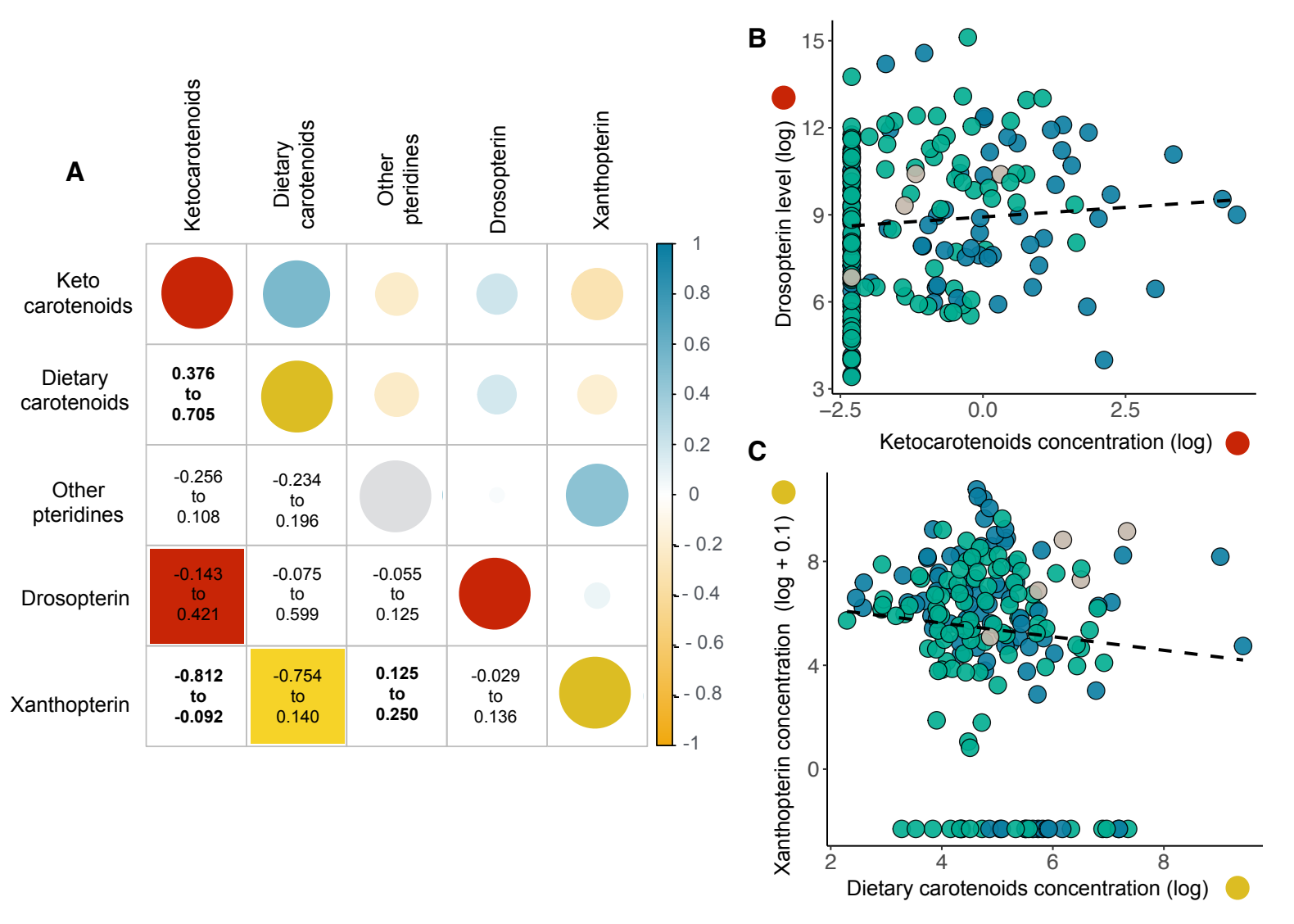
Table 2. Associations between pigment concentrations and color traits (N = 180).

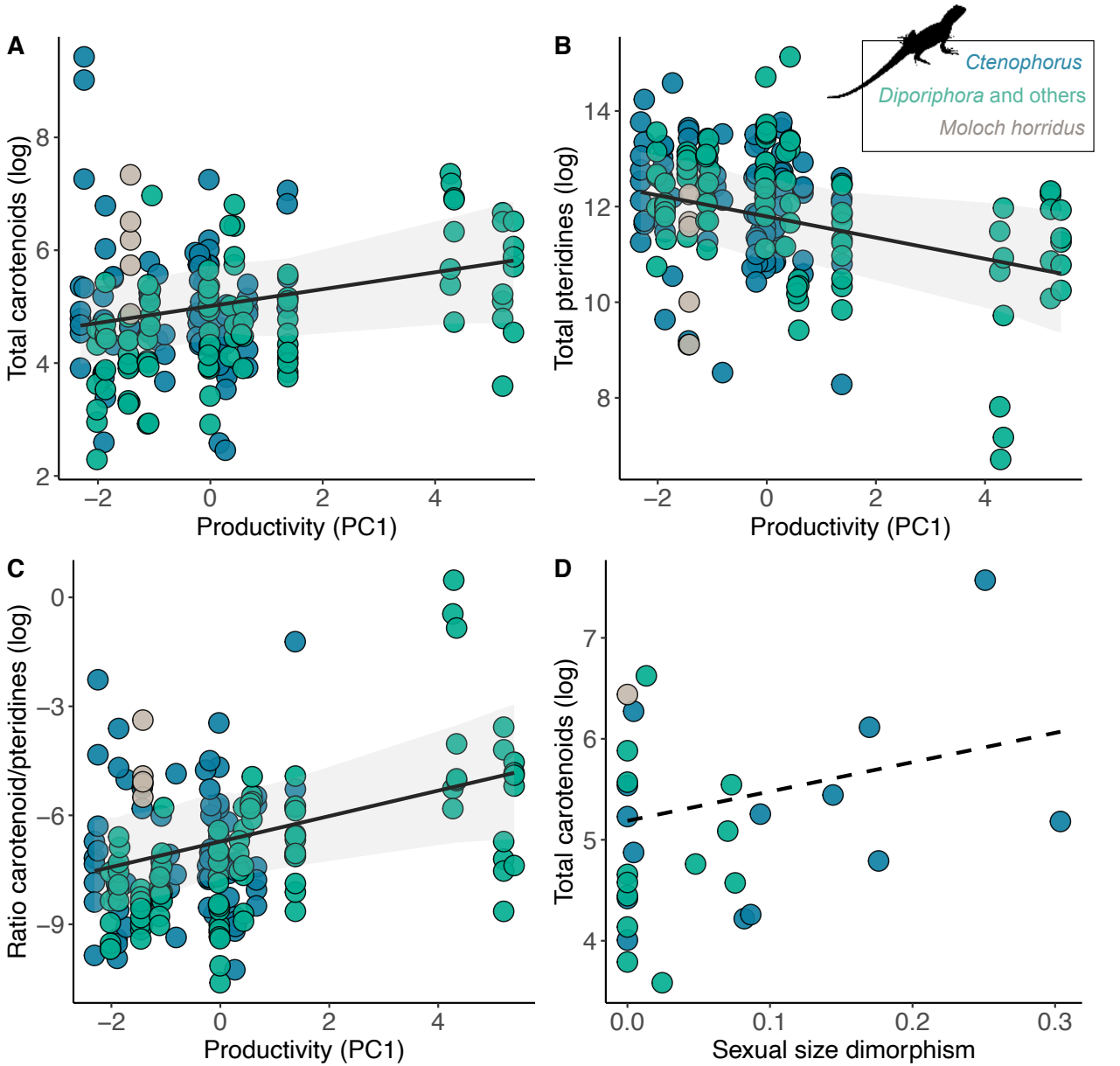
	Luminance		**Saturation**		**Hue**	
Predictors	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
Dietary carotenoids	-2.476	7.596	-0.006	0.022	-0.006	0.207
Ketocarotenoids	-2.045	6.061	-0.004	0.019	-0.056	0.149

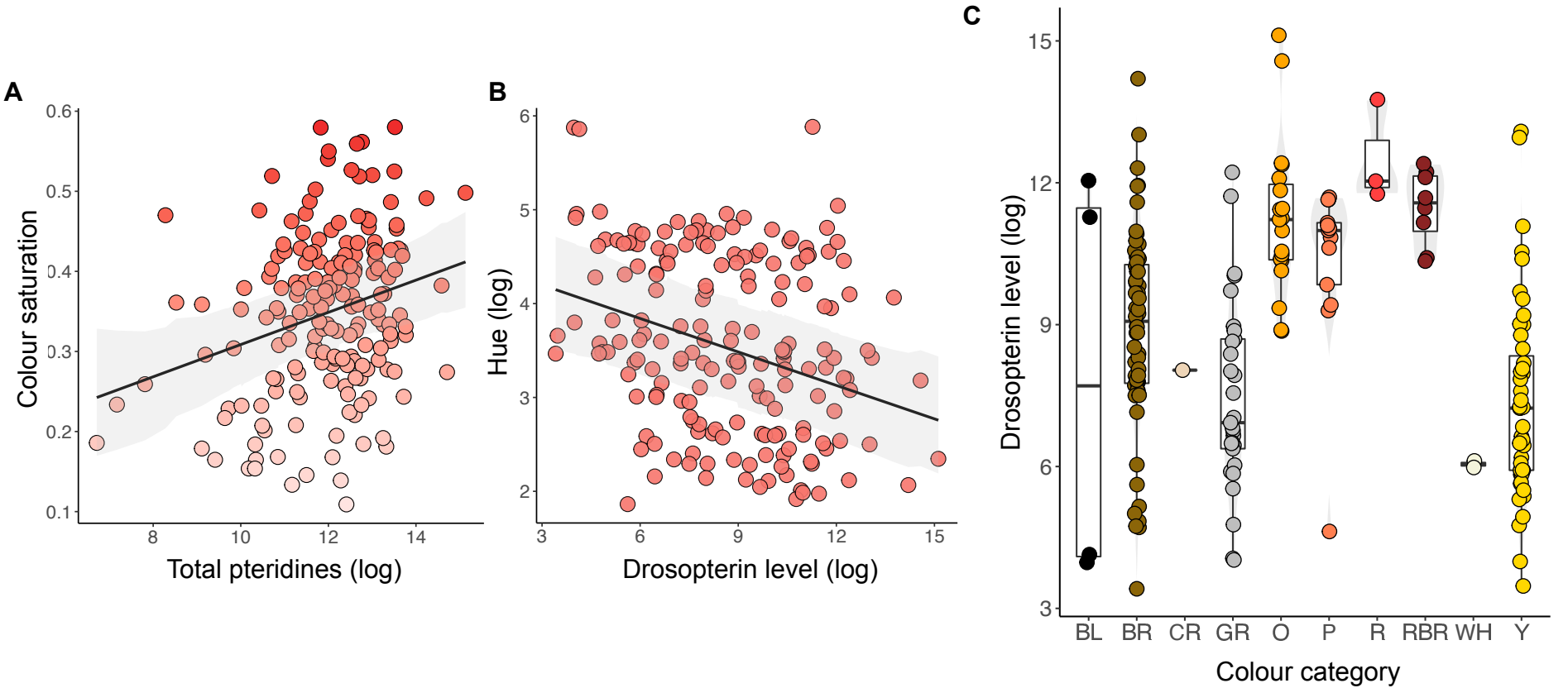
Xanthopterin	-2.533	0.649	**0.001**	**0.011**	-0.061	0.019
Drosopterin	-4.170	0.161	-0.002	0.01	**-0.170**	**-0.069**
Other pteridines	**-7.431**	**-0.254**	**0.002**	**0.02**	-0.110	0.054
Total carotenoids	-1.644	7.789	-0.007	0.0212	**–**	**–**
Total pteridines	-7.576	1.256	**0.007**	**0.0317**	**–**	**–**

Lower and upper represent the 95% confidence bounds from the posterior distribution of the estimate based on phylogenetic mixed models run on 1300 phylogenies. Values in bold represent cases where the upper and lower confidence bounds not overlap zero and thus there is evidence of a significant effect.


Carotenoids
Pteridines
Ctenophorus
Diporiphora & others
-1
0
1
Pigment concentration (scaled)
C. pictus
C. isolepis
C. vadnappa
C. maculosus
D. valens
D. winneckei
Rankinia diemensis
Moloch horridus
Productivity (Aridity index)


A
Ketocarotenoids
Dietary carotenoids
Other pteridines
Drosopterin
Xanthopterin
Keto carotenoids
Dietary carotenoids
Other pteridines
Drosopterin
Xanthopterin
0.376 to 0.705
-0.256 to 0.108
-0.234 to 0.196
-0.143 to 0.421
-0.075 to 0.599
-0.055 to 0.125
-0.812 to -0.092
-0.754 to 0.140
0.125 to 0.250
-0.029 to 0.136
1
0.8
0.6
0.4
0.2
0
- 0.2
- 0.4
- 0.6
- 0.8
-1
B
Drosopterin level (log)
Ketocarotenoids concentration (log)
C
Xanthopterin concentration (log + 0.1)
Dietary carotenoids concentration (log)


A
Total carotenoids (log)
Productivity (PC1)
B
Total pteridines (log)
Productivity (PC1)
Ctenophorus
Diporiphora and others
Moloch horridus
C
Ratio carotenoid/pteridines (log)
Productivity (PC1)
D
Total carotenoids (log)
Sexual size dimorphism


A
Colour saturation
0.6
0.5
0.4
0.3
0.2
0.1
8
10
12
14
Total pteridines (log)
B
Hue (log)
6
5
4
3
2
3
6
9
12
15
Drosopterin level (log)
C
Drosopterin level (log)
15
12
9
6
3
BL
BR
CR
GR
O
P
R
RBR
WH
Y
Colour category