Pteridine pigments compensate for environmental availability of carotenoids

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Abstract

Carotenoid-based colours are a textbook example of honest signalling because carotenoids must be acquired from the environment. However, many species produce similar colours using self-synthesised pteridine pigments. A compelling but untested hypothesis is that pteridines compensate for low environmental availability of carotenoids because it is metabolically cheaper to synthesise pteridines than to acquire and sequester carotenoids. Based on a phylogenetic comparative analysis of 11 pigment concentrations in skin tissue of agamid lizards, we show that pteridine concentrations are higher and carotenoid concentrations lower in less productive environments. Both carotenoid and pteridine pigments were present in all species, but only pteridine concentrations explained colour variation among species. Furthermore, pigment concentrations were uncorrelated with indices of sexual selection. These results suggest that variation among species in pteridine synthesis compensates for environmental availability of carotenoids and challenge the paradigm of honest carotenoid signalling in vertebrates with complex colour production mechanisms.

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Data accessibility: The datasets and code used during the current study are available from Dryad**.** *Data will be archived upon acceptance of the manuscript.*

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Abstract

Carotenoid-based colours are a textbook example of honest signalling because carotenoids must be acquired from the environment. However, many species produce similar colours using self-synthesised pteridine pigments. A compelling but untested hypothesis is that pteridines compensate for low environmental availability of carotenoids because it is metabolically cheaper to synthesise pteridines than to acquire and sequester carotenoids. Based on a phylogenetic comparative analysis of 11 pigment concentrations in skin tissue of agamid lizards, we show that pteridine concentrations are higher and carotenoid concentrations lower in less productive environments. Both carotenoid and pteridine pigments were present in all species, but only pteridine concentrations explained colour variation among species. Furthermore, pigment concentrations were uncorrelated with indices of sexual selection. These results suggest that variation among species in pteridine synthesis compensates for environmental availability of carotenoids and challenge the paradigm of honest carotenoid signalling in vertebrates with complex colour production mechanisms.

INTRODUCTION

Colour is one of the most striking and varied components of visual signals throughout the natural world. The cost of producing vivid colours can be critical for maintaining signal honesty. Colours produced by carotenoid pigments are a text-book example of honest signals of individual quality because carotenoids cannot be synthesized by animals (i.e. must be ingested), and have a range of essential physiological functions (Olson & Owens 1998; Svensson & Wong 2011). Consequently, supply may limit carotenoid intake and there may be trade-offs in allocation of pigments to ornamentation versus other physiological roles (Koch & Hill 2018). The literature on carotenoid-based coloration is dominated by studies of birds; however, the majority of vertebrates (ectothermic vertebrates – fish, reptiles, amphibians) can produce yellow to red skin colours using carotenoids and/or a biochemically distinct class of pigments called pteridines (Bagnara & Matsumoto 2006). Pteridines can be used instead of, or together with carotenoids to produce yellow-red colours and the two pigment classes can frequently be found together in coloured integument (Bagnara & Hadley 1973; Bagnara & Matsumoto 2006). Pteridines are synthesised *de novo*within pigment cells from abundant purine molecules (Bracher *et al.* 1998; Ziegler 2003; Braasch *et al.* 2007) and have limited antioxidant function (McGraw 2005). This suggests that, in contrast with carotenoids, production costs of pteridine-based colours may be minimal and it is unlikely that supply is limited or that there are trade-offs in allocation. However, few studies have examined pteridine pigments in the context of colour signalling and the physiological costs and evolutionary drivers of variation in pteridine pigments remain largely unknown.

What explains the use of carotenoid or pteridine pigments when both can produce spectrally similar colours? One possibility is that pteridines compensate directly or indirectly for variation in the environmental availability of carotenoids (Grether *et al.* 1999). Under direct compensation, pteridines directly replace carotenoids with a similar hue (carotenoid-mimicry hypothesis). This is expected to result in a negative correlation between the concentrations of similarly coloured carotenoid and pteridine pigments. Under indirect compensation, pteridines compensate for geographic variation in carotenoids, irrespective of hue. Different species or populations may have different colours, depending on local selective pressures, but use a higher proportion of pteridines when carotenoid availability is low. In this case, we expect a positive association between combined carotenoid concentration and environmental carotenoid availability, and a corresponding negative association with combined pteridine concentration, but we do not necessarily expect correlations between specific carotenoid and pteridine pigments. Whether direct or indirect compensation for environmental carotenoid availability explains the prevalence of pteridine pigments among species has not been tested in any taxonomic group, to our knowledge, due to insufficient data on pigment concentrations.

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, or low dietary availability for terrestrial animals, ketocarotenoids are more strongly associated with measures of individual quality and sexual selection than dietary yellow carotenoids, particularly in birds (Weaver *et al.*2018). Pteridines similarly vary in colour from yellow (e.g. sepiapterin, xanthopterin) to red (e.g. drosopterin, erythropterin, riboflavin) but other pteridines (e.g. pterin, biopterin, isoxanthopterin) found in skin pigment cells (chromatophores) are assumed to be colourless. Colourless pteridines can be found in large quantities within chromatophores, though it is unclear whether or how they may contribute to integument coloration (Bagnara & Matsumoto 2006). The different costs and roles in colour production for different types of carotenoids and pteridines influence expected associations with environmental factors and sexual selection. To understand evolutionary drivers of pigment variation, it is therefore essential to quantify specific metabolites in integument tissue; however, this has only been attempted for carotenoids, and only in some groups of birds (Prum*et al.* 2012; Friedman *et al.* 2014b, a; Ligon *et al.*2016).

Here, using an extensive dataset of concentrations of 11 pigments, we test whether pteridine pigments compensate, directly or indirectly, for environmental availability of carotenoids among 27 species of Australian agamid lizards (186 skin samples, 79 individuals, 28 populations with distinct coloration). Specifically, 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 pigment concentrations are associated with environmental gradients indicative of carotenoid availability. Since this relationship 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). To distinguish direct*versus* indirect compensation, we examined correlations between carotenoid and pteridine pigments with similar hue. Lastly, we evaluated how carotenoid or pteridine concentrations covary with skin colour (hue, saturation, luminance).

MATERIALS AND METHODS

Study Species and Sample Collection

We captured three to five sexually mature males (with adult coloration) 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 S4). 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). Skin samples for liquid chromatography-mass spectrometry analysis were biopsied and stored in methanol at -20°C and tubes were wrapped with foil to exclude light. Immediately post-mortem, we took standardized 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).

Pigment concentrations

To identify the specific metabolites responsible for yellow-red coloration, 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. The pigment extraction protocol was based on McLean et al. (2017). In brief, we sequentially extracted carotenoids then pteridines: Samples were weighed and then homogenized in methanol:ethylacetate (6:4 v/v + 0.01% butylated hydroxy toluene) using a TissueLyser II system (with two 3mm tungsten-carbide beads; Qiagen, Hilden, Germany). The resulting carotenoid extract was collected following centrifugation and the extraction repeated once on the remaining tissue pellet. Pteridines were subsequently extracted from the remaining tissue pellet using 2% ammonium hydroxide in two serial extractions. Respective fractions were combined. Internal standards were added directly to respective extraction solvents. 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) in two separate liquid chromatography-mass spectrometry analyses on an Agilent 6490 triple quadrupole MS system with a Jet Stream electrospray ionization source coupled to an Agilent 1290 series LC system (Agilent Technologies Inc, Santa Clara, CA). 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 analyzed further.

Data were analysed using Agilent MassHunter Workstation Software (version B.07.00). All peak assignments were matched against commercial or purified standards, confirmed with a qualifier ion and quantified against the linear range from six-point calibration curves (all $R^2 > 0.95$). Final concentrations were normalized against tissue weight: i.e. 'pigment per gram of tissue' referred to as "concentration" throughout for brevity. Commercial standards were used for all metabolites except drosopterin, which we extracted and purified from fruit flies, *Drosophila melanogaster*(per Wilson & Jacobson 1977). Peaks for lutein and zeaxanthin could not be reliably distinguished chromatographically so they were combined into a single category). For drosopterin we use the relative response because we lacked a commercial standard, thus, we refer to this as "level". For subsequent analyses, we calculated the total concentration of carotenoids and pteridines, as well as the concentration of 5 subcategories: dietary yellow-orange carotenoids (lutein/zeaxanthin, 3'-dehydrolutein, β-carotene), red ketocarotenoids (astaxanthin, canthaxanthin), yellow pteridines (xanthopterin), red pteridines (drosopterin) and colourless pteridines (pterin, 6-biopterin, isoxanthopterin, pterine-6-carboxylic acid).

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 focussed 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 S5). 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, broadleafed) plants, aridity, radiation and temperature of the warmest quarter (Figure S5; Table S5). 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 S5). 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 S6, Figure S6). We used a subset of 1300 post-burnin trees (subsampled using logcombiner (Bouckaert *et al.*2019) and pruned of 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) colourless pteridines (pterin, 6-biopterin, isoxanthopterin, pterine-6-carboxylic acid).

Lastly, we tested whether the concentration of pigments present in skin tissue was associated with its colour. The response variables were luminance (the brightness of the colour), saturation (the intensity of the colour)

or hue. For luminance and saturation, we ran two models with the following predictors: 1) total carotenoids; and 2) total pteridines. For hue we ran four models with concentrations of pigment subcategories as predictors: 1) dietary carotenoids (yellow-orange); 2) xanthopterin (yellow); 3) ketocarotenoids (red); and 4) drosopterin (red). 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

Skin tissues from all 27 species contained both carotenoid and pteridine pigments (Figure 1). Carotenoids with the highest concentrations among our samples were lutein/zeaxanthin (yellow) and β-carotene (orange), while the pteridines with the highest concentrations were isoxanthopterin and pterin-6-carboxilic acid (colourless) but there were also substantial concentrations of yellow xanthopterin and red drosopterin (Figure S1).

Total carotenoid concentration was significantly associated with environmental gradients. Individuals living in environments with higher productivity (and thus higher carotenoid availability; environmental PC1) had a higher concentration of total carotenoids (Figure 2, Table 1, r^2=0.16). Furthermore, individuals in more productive environments had a lower concentration of total pteridines (Figure 2, Table 1, r^2=0.14), and therefore there was a significantly higher ratio of carotenoids to pteridines in more productive environments (Figure 2, Table 1, r^2=0.17). There was no significant association between sexual selection indices and pigment concentration in the whole dataset analysis (Table 1) or at the species-level (Table S1), although there was a trend for higher total carotenoid concentration in species with higher sexual size dimorphism (Figure 2D).

To test for direct compensation (carotenoid mimicry), we examined the relationships between carotenoids and pteridines of a similar hue. There was no association between either red ketocarotenoids and drosopterin, or yellow-orange dietary carotenoids and xanthopterin (Figure 3). Interestingly, there was a significant positive correlation between the concentration of dietary carotenoids and ketocarotenoids, and between colourless pteridines and xanthopterin, and a negative correlation between xanthopterin and ketocarotenoids (Figure 3).

Variation in skin colours was associated with the concentration of pteridines but not carotenoids (Figure

4). Specifically, tissues with higher concentrations of drosopterin had redder hues (lower hue values), and tissues with higher xanthopterin, colourless pteridines and total pteridines had more saturated colours (Table 2). Tissues with higher concentrations of colourless pteridines also had lower luminance (darker). Yellow-red (including browns, N=150) tissues had higher concentrations of drosopterin (Figure 4C), colourless 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 S2, Figure S2). Additionally, the luminance of skin colours was associated with habitat productivity (PC1 95% CIs 2.56 – 10.18), with darker colours in more productive environments (Figure S3), but environmental PCs did not predict hue or saturation (Table S3).

DISCUSSION

We tested whether pteridine pigments compensate for environmental carotenoid availability among agamid lizards, using a large interspecific dataset of pigment concentrations in coloured skin tissue. We found that the total concentration of carotenoids was positively associated with habitat productivity, and therefore presumably environmental carotenoid availability. Individuals in more productive environments not only had higher concentrations of total carotenoids, but they also had a lower concentration of total pteridines and consequently, a higher 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, although there was a weak negative association between red ketocarotenoid and yellow xanthopterin concentrations. This indicates that variation in carotenoid availability is not compensated directly by replacing carotenoids with pteridine pigments of the same hue (carotenoid mimicry). Instead, compensation appears to be indirect. In environments where carotenoids are scarce, pteridines are used in relatively higher concentrations but different combinations of pteridine and carotenoid pigments are used to produce different hues. Pigment concentrations correspond to variation in yellow-red skin colours among species, and this was primarily driven by pteridines: redder hues were associated with higher concentrations of drosopterin, and more saturated colours were associated with higher concentration of pteridines (xanthopterin, colourless and total). We found no relationship between carotenoid or pteridine concentrations and indices of sexual selection (sexual dichromatism and sexual size dimorphism). This is consistent with the lack of association between carotenoid concentration and skin colour and the hypothesised low metabolic cost of pteridine synthesis (Bagnara & Matsumoto 2006). Taken together, these results suggest that costs associated with the production and allocation of pigments to skin colour are unlikely to maintain colour signal honesty in agamid lizards.

In reptiles, yellow-red skin coloration can be produced through diverse mechanisms and pigment compositions. Yellow, orange and red hues can all be produced exclusively by carotenoids (Fitze *et al.* 2009) or pteridines (snakes; Kikuchi & Pfennig 2012; Kikuchi *et al.*2014). Furthermore, red hues can be produced by a higher proportion of red ketocarotenoids relative to both dietary yellow carotenoids and to pteridines (McLean *et al.* 2019), or exclusively by drosopterin (Merkling *et al.* 2018). In the majority of lizards, however, both carotenoids and pteridines contribute to colour variation within and between species, with yellow produced by relatively higher concentrations of dietary carotenoids and orange-red 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). We found similar patterns 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. Thus, yellow-red ornamentation in agamid lizards is not an indicator of carotenoid content.

Carotenoids vary in their dietary availability within and between species; however, this 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 metabolism (e.g. 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). Accordingly, there is limited and inconsistent evidence for an association between carotenoid concentrations in the integument and diet, at least for birds (Mahler *et al.* 2003; McGraw *et al.* 2003; Olson & Owens 2005). By contrast, we found that in agamid lizards, total carotenoid concentrations in coloured skin tissue were associated with habitat productivity. All species of agamid lizard in this study are insectivorous, though some occasionally eat plant material including yellow flowers (Cogger 2018; Melville 2019). Most species occupy semi-arid to arid environments, often with little vegetation, suggesting that environmental carotenoid availability may well be limiting in this clade.

Concentrations of ketocarotenoids were generally low (particularly astaxanthin) relative to other carotenoids. Although ketocarotenoids are common in red integument tissue in vertebrates and may be obtained from dietary sources, they are rare in the diets of most terrestrial vertebrates. Despite being rare in the diet, in some species, ketocarotenoids 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, 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 mechanisms for ketocarotenoid conversion may explain the prevalence of drosopterin to produce orange and red hues in lizards and some other groups of poikilothermic vertebrates.

We found that the ratio of carotenoids to pteridines was higher in more productive environments and concentrations of total pteridines were lower in habitats with higher productivity. Variation in pteridine synthesis is likely to have a genetic basis. This is the case among populations of guppies in which genetic differences in pteridine synthesis among populations compensate for environmental carotenoid availability (Grether *et al.* 2005). Furthermore, the positive correlation of carotenoid and drosopterin concentrations in guppies is driven by female preference for a specific orange hue (Deere *et al.* 2012). We found that higher concentrations of red ketocarotenoids were associated with lower concentrations of yellow xanthopterin in agamid lizards, which further indicates compensation, though not carotenoid mimicry. This may be due to selection for specific hues, particularly in species in which ketocarotenoids contribute to integument coloration.

Variation in the ratio of carotenoids to pteridines in association with habitat productivity among agamid lizards may reflect variation in sexual or natural selection in different environments. Sexual selection can vary in relation to environmental gradients via, for example, environmental effects on population density (Littleford-Colquhoun*et al.* 2019). However, we found no relationship between pigment concentrations and indices of sexual selection, apart from a weak trend for increased total carotenoids with higher sexual dimorphism. By contrast, we found that species with darker colours (lower luminance) were more likely to be found in more productive or vegetated environments. This pattern has been documented in birds and butterflies and may enhance camouflage (Dalrymple *et al.* 2018). More saturated and darker colours were

associated with a higher concentration of colourless pteridines. Thus, one possibility is that variation in pteridine synthesis among species partly reflects variation in selection to minimise predation risk in different environments, leading to lower pteridine concentrations in productive habitats.

Our comparative analysis uncovered broad patterns in pigment concentration; 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, or by structural colour alone, but never by pigments alone (Kemp *et al.* 2012). For example, within a mimicry complex of poison frogs (Dendrobatidae), model species and different morphs of the mimic species use different combinations of dietary and metabolically converted carotenoids (Twomey*et al.* 2020b). In these species, drosopterin contributes to orange coloration but variation in hue across the group is predominantly associated with the thickness of platelets within iridophores (i.e. structural; Twomey *et al.* 2020b). Furthermore, skin tissue commonly contains high concentrations of colourless pteridines such as isoxanthopterin, pterin and biopterin (Bagnara & Matsumoto 2006; McLean*et al.* 2017; McLean *et al.* 2019; Twomey *et al.*2020b). We found an association between the concentration of colourless pteridines and skin colour saturation and luminance. However, it is not clear whether or how colourless pteridines contribute to skin coloration (e.g. light scattering). For example, isoxanthopterin (a pteridine analog of guanine that forms the crystalline platelets in iridophores) forms crystalline structures that act as reflectors in the eyes of crustaceans (Palmer *et al.* 2018), but whether it contributes to structural coloration in vertebrates is unknown. The role and contribution of such colourless pteridines to integument coloration is a fascinating area for future research.

Overall, our results support a scenario where limited carotenoid availability in low productivity environments is compensated by higher concentrations of pteridines. This has important implications for honest colour signalling. A dominant paradigm is that costs associated with carotenoid signalling maintain the honesty of yellow, orange and particularly red colour signals; however, this paradigm derives largely from literature on birds. Our results suggest that expression of yellow-red signalling colours in agamid lizards is unlikely to convey information on individual quality related to carotenoid acquisition or allocation. Instead, the honesty of these colour signals may be maintained by other costs such as predation risk associated with conspicuous coloration. Our study suggests that the paradigm of honest carotenoid signalling may not apply broadly to other major vertebrate groups, such as reptiles, that use a combination of pteridine and carotenoid pigments to generate yellow-red hues and have complex colour generation mechanisms.

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Competing Interests

The authors declare that they have no competing interests

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Figures and Tables

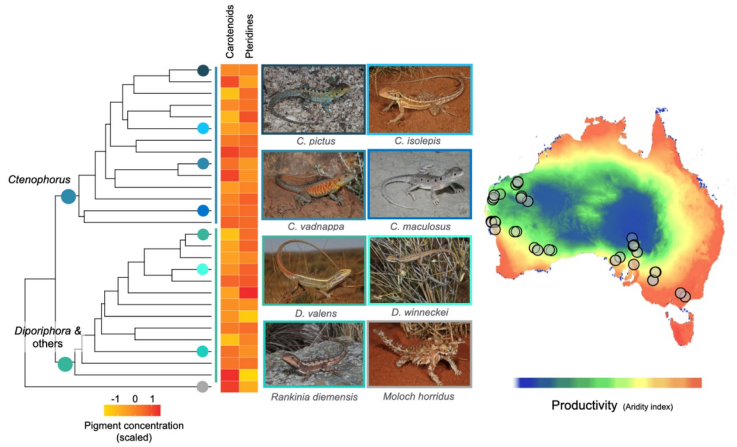

Figure 1. Phylogenetic relationships between the 28 clades (27 species) of Australian agamid lizards included in this study, with images of representative species from the *Ctenophorus* and*Diporiphora* & others (genera *Amphibolurus* ,*Gowidon* , *Pogona* , *Rankinia* , *Tympanocryptis*) clades as well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

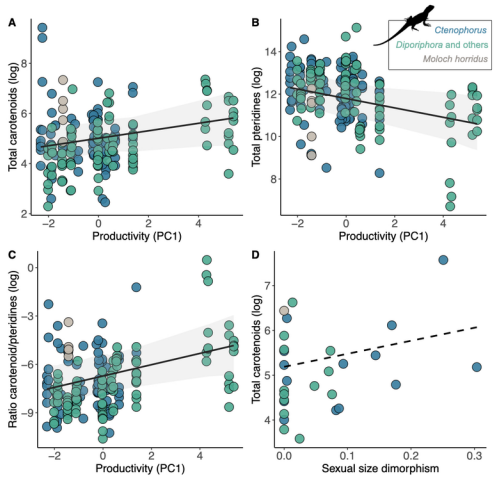

Figure 2. Associations between (A) concentration of total carotenoids, (B) concentration of total pteridines, and (C) ratio of total carotenoids to total pteridine and a habitat productivity (PC1*-1), as well as (D) concentration of total carotenoids and sexual size dimorphism. Solid lines indicate a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

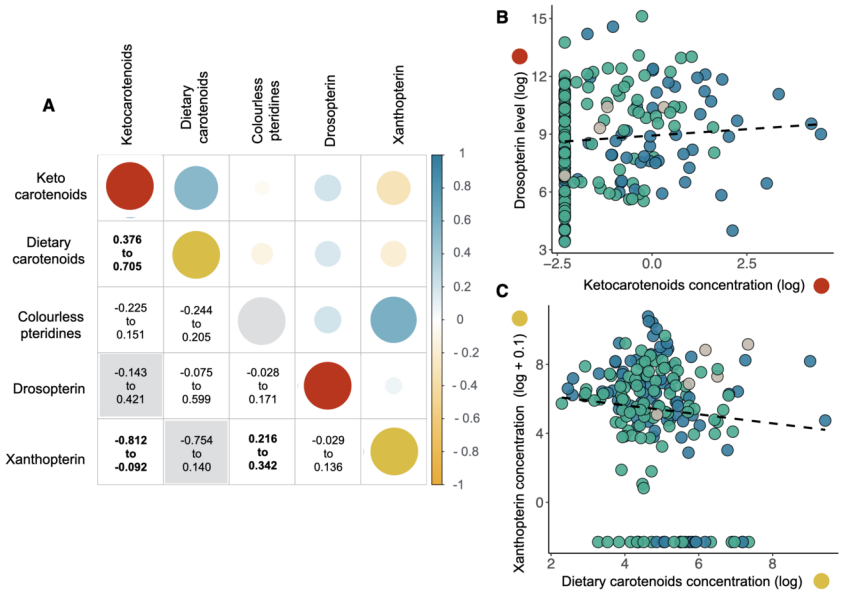

Figure 3. Correlations between carotenoid and pteridine subcategories. **(A)** Correlation matrix showing HPD intervals of estimates from GLMMs. Size and color of upper diagonal circles indicate the strength and direction of the relationship. Central circles indicate the color of pigment subcategories.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

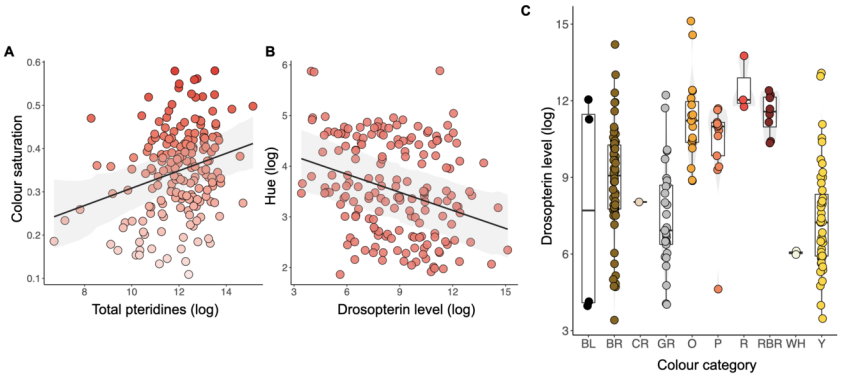

Figure 4. Associations between (A) saturation and the total concentration of pteridines; (B) hue and the concentration of drosopterin. Orange-red colored skin had higher concentrations of drosopterin than other skin colors (C). BL = black; BR = brown, CR = cream; GR = grey; O =orange, P = pink, R = red,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

Table 1. Association 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 carotenoids)**	**log(Total pteridines)**	**log(Total pteridir**
Predictors	**Lower**	**Upper**	**Lower**	**Upper**
PC1	**-0.354**	**-0.006**	**0.021**	**0.423**
PC2	-0.089	0.261	-0.228	0.182
Size dimorphism	-1.071	7.057	-4.216	5.889
Dichromatism	0.039	0.089	-0.093	0.068

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. Association between pigment concentration and color traits (N=180).

	Luminance	**Luminance**	**Saturation**	**Saturation**	**Hue**	**Hue**
Predictors	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
Dietary carotenoids	-2.389	7.536	-0.008	0.020	-0.066	0.192
Ketocarotenoids	-1.677	7.062	-0.006	0.017	-0.047	0.148
Xanthopterin	-2.604	0.632	**0.001**	**0.010**	-0.067	0.017
Drosopterin	-3.867	0.525	-0.003	0.008	**-0.173**	**-0.07**
Colourless pteridines	**-6.96**	**-0.565**	**0.009**	**0.026**	-0.129	0.021
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.