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Dietary carotenoids change the colour of Southern corroboree frogs

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Animal coloration can be the result of many interconnected elements, including the production of colour-producing molecules *de novo*, as well as the acquisition of pigments from the diet. When acquired through the diet, carotenoids (a common class of pigments) can influence yellow, orange, and red coloration and enhanced levels of carotenoids can result in brighter coloration and/or changes in hue or saturation. We tested the hypothesis that dietary carotenoid supplementation changes the striking black and yellow coloration of the southern corroboree frog (*Pseudophryne corroboree*, Amphibia: Anura). Our dietary treatment showed no measurable difference in colour or brightness for black patches in frogs. However, the reflectance of yellow patches of frogs raised on a diet rich in carotenoids was more saturated (higher chroma) and long-wave shifted in hue (more orange) compared to that of frogs raised without carotenoids. Interestingly, frogs with carotenoid-poor diets still developed their characteristic yellow and black coloration, suggesting that their yellow colour patches are a product of pteridines manufactured *de novo.* © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **00**, 000– 000.

KEYWORDS: amphibian – anuran – carotenoid – coloration – diet – morphological colour change – nutrition – ontogenetic colour change – pigment.

INTRODUCTION

The mechanisms by which animals produce and change colour over their lifetimes are truly diverse (Bagnara, Taylor & Prota, 1973; Suga & Munesada, 1988). In vertebrates, coloration of the integument is often (but not exclusively) caused by the presence of chromatophores, cells that contain molecules (e.g. pigments) and/or structures with optical properties (Bagnara & Hadley, 1969). Chromatophores commonly found in vertebrates include xanthophores, erythrophores, leucophores, melanophores, and iridophores, which can give rise to yellows, reds, whites, browns, and structural optical effects, respectively (Mills & Patterson, 2009). Although optically active molecules in some chromatophores are produced *de novo* (e.g. melanins and pteridines), other pigments such as carotenoids must be acquired from the diet. Observable variation in animal coloration can thus reflect one or more of several intrinsic or extrinsic contributing factors. Influences on the mechanisms of coloration include intrinsic factors such as ontogeny, hormones, metabolism and genetics, and extrinsic factors such as diet, presence of conspecifics, predators, temperature, and habitat background, as well as their interactions (Booth, 1990; Stuart-Fox & Moussalli, 2009).

In vertebrates, yellow coloration is generally a product of the presence of pteridines and/or carotenoids (Steffen & McGraw, 2009; Weiss *et al.* 2012). Pteridines are synthesized during purine production and are produced *de novo* by the animal (Zeigler, 2003).

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Given that guanine production is a fundamental physiological process, pteridines are readily available and consistently replenished. Pteridine-based yellow coloration is therefore less likely to be variable because it is not greatly influenced by extrinsic factors. By contrast, the availability of other pigments can be variable over time and space.

Manufactured by plants and fungi, carotenoids cannot be made by animals *de novo* but, instead, must be acquired through their diet (Goodwin, 1984; Feltl *et al.*, 2005). Where carotenoids deposited into skin and feathers enhance the fitness of the consumer via signalling, the limitation of dietary acquisition is considered to keep signals honest (Hill & McGraw, 2006). The classic example is the house finch (*Carpodacus mexicanus*), where males laboriously acquire carotenoids through the diet and females prefer males with the reddest plumage as mates (Hill, 1991). Beyond coloration, carotenoids have a remarkable breadth of benefits for the animals that sequester them (Goodwin, 1984; von Lintig, 2010; Svensson & Wong, 2011). Carotenoids have a variety of functions in immune function, reproduction, exercise performance, and coloration, and can directly influence fitness. For example, carotenoid-rich diets have been shown to improve the escape performance of zebra finches (*Taeniopygia guttata*; Blount & Matheson, 2006) and southern corroboree frogs (Silla, McInerney & Byrne, 2016), and the addition of carotenoid supplements to the diet of strawberry poison frogs (*Oophaga pumilio*) leads to an increase in the number of offspring that successfully metamorphose (Dugas, Yeager & Richards-Zawacki, 2013). Recently, research has focused on understanding the co-occurrence of pteridines and carotenoids in animal coloration. For example, in striped plateau lizards (*Sceloporus virgatus*) Weiss *et al.* (2012) found that females trade off by utilizing pteridines for ornamental coloration, leaving carotenoids free for use in egg production. In anoles (*Norops sagrei* and *Norops humilis*), pteridines and carotenoids occupy different parts of the dewlap and this varies between the sexes and species (Steffen and McGraw, 2007, 2009).

The present study aimed to determine whether the striking, putatively aposematic (Wells, 2007), yellow and black coloration of the critically endangered Southern corroboree frog (*Pseudophryne corroboree*) is influenced by, or independent of, dietary carotenoids. We manipulated dietary carotenoid availability to test the hypothesis that different levels of dietary carotenoids affect corroboree frog colour and pattern. For colour, we made three broad predictions: (1) if corroboree frog yellow is primarily carotenoid-based, frogs that received no carotenoids will not develop yellow stripes; (2) if corroboree frog yellow is primarily pteridine-based, frogs that receive no carotenoids will still develop yellow stripes; and (3) if corroboree frog yellow is purely pteridine-based, frogs that receive carotenoids will be indistinguishable in colour to frogs that did not. For colour pattern, we predicted that there would be a difference between frogs on carotenoid-rich and carotenoid-poor diets where frogs on carotenoid-rich diets would develop a pattern with a greater proportion of yellow to black as a result of an abundance of available carotenoids (Price, 2006).

MATERIAL AND METHODS

Rearing and husbandry

The Southern corroboree frog (*P. corroboree*) is endemic to the sub-alpine regions of Australia. These relatively small frogs (snout–vent length of approximately 25 mm) harbour toxic alkaloids and have a striking black and yellow colour pattern that may function to deter predators (Daly *et al.*, 1990; Osborne, 1991). Corroboree frogs are currently listed as critically endangered and are the subject of an extensive captive breeding programme hosted at multiple institutions across Australia.

Throughout our experiment, frogs developed through three stages: egg, tadpole, and metamorph. Eggs ($N = 64$) were obtained from a captive colony maintained at Melbourne Zoo. Eggs were stimulated to hatch via flooding with reverse-osmosis water. All tadpoles ($N = 64$) hatched within 11 days, and were immediately transferred to individual plastic containers (diameter 10 cm, height 10.5 cm) filled with 600 mL of reverse-osmosis water. Tadpoles were kept in a constant temperature room held at approximately 12 °C (range 11.4–12.9 °C). To prevent developmental disorders associated with ultraviolet deficiencies (Lannoo, 2008), a single ultraviolet-B light bulb (36″ fluorescent strip bulb) was suspended approximately 20 cm above tadpoles, providing 1 h of ultraviolet-B light per day between 11.30 h and 12.30 h. The room's fluorescent lighting was under an 11.5 : 12.5 h light/dark cycle (including twilight for 15 min at both dawn and dusk). These conditions approximate the conditions in the Australian alps and those of Standard Operating Procedures used in captive breeding programmes at Taronga Zoo.

Immediately after hatching, tadpoles were fed an *ad libitum* basal diet three times per week. This basal diet consisted of ground fish flakes (75 : 25 mixture of Sera Flora/Sera Sans; SERA) suspended in 10 mL of reverse-osmosis water. To prevent water fouling, excess food and tadpole excrement was siphoned from each container once a week and half the water was replenished three times per week using an automated irrigation system (Aqua

Systems) integrated with a reverse-osmosis water system (Sartorius Stedim Biotech). Water quality was tested in three haphazardly selected containers per treatment every 4 weeks (Aqua One). Throughout the experiment, ammonia concentrations remained low (0.5–1.0 mg L^{-1}).

Tadpole development was assessed every 2 days using Gosner staging tables (Gosner, 1960). Just prior to metamorphosis (stage 43–46; Gosner, 1960), individuals were rehoused in new containers of the same size, which incorporated a half-submerged sponge to provide a substrate on which tadpoles could crawl from the water to undergo metamorphosis. During the transition from forelimb emergence (Gosner stage 42) to full tail absorption (Gosner stage 46), individuals satisfy their nutritional needs through the absorption of the tail tissue (Wassersug and Wilbur, 1974); consequently, food was not provided during this period (duration = 25.24 ± 0.65 days). Once individuals had completed metamorphosis (tails reabsorbed), they were moved into experimental containers (of the same size as above). The experimental containers contained a thick layer of moist sphagnum moss (*Sphagnum cristatum*, 5 cm deep) on a pebble base (2 cm deep). Experimental containers were cleaned once per week by flushing with 500 mL of reverse-osmosis water to drain out excess food and faecal matter.

For 2 weeks prior to the second period of dietary manipulation, metamorphs received first-instar crickets (*Acheta domesticus*) fed twice a week *ad libitum*. Crickets were reared on apples, containing negligible carotenoids; prey were not fed carotenoid rich diets (e.g. carrots). Once each week, crickets were dusted with approximately 0.2 g of a calcium supplement (Repti-Cal; Aristopet) to prevent developmental disorders associated with calcium deficiencies (Lannoo, 2008).

Experimental treatments

On day 1 of the first experimental period, tadpoles were randomly assigned to one of two diet treatments: (1) carotenoid-poor diet consisting of 1.0 g of basal diet (see above) ($N = 32$) or (2) a carotenoid-rich diet consisting of 1.0 g of basal diet plus 20 mg of carotenoid mixture (Superpig; Repashy) ($N = 32$), providing approximately 20 mg g^{-1} carotenoids. Tadpoles were fed two drops of food (0.059–0.069 g wet mass, 0.015–0.018 g dry mass), three times a week until aged 8 weeks old. From 9 weeks old until the tadpoles metamorphosed, individuals were fed four drops of food (range = 0.117–0.137 g wet mass, 0.030–0.036 g dry mass), three times per week.

Two weeks after metamorphosis, survivors were weighed, and continued on their dietary manipulations: carotenoid-rich ($N = 32$) or carotenoid-poor ($N = 32$). For metamorphs, the carotenoid-rich diet consisted of first-instar crickets twice per week. Crickets were carotenoid enriched via gut-loading for 48 h with carrot, as well as being dusted with approximately 1.0 g of carotenoid mixture (Superpig; Repashy). The carotenoid-poor diet consisted of the same quantity of prey but without carotenoid supplementation. Metamorphs were maintained on their respective diets until the end of the experimental period, which lasted 50 weeks.

Quantifying colour and pattern

At the end of the experimental period, all individuals were photographed using a 600D camera with a 60 mm macro lens (Canon Inc.). Photographs were taken in camera raw format with the settings: ISO = 400, f-stop = 6.3, shutter speed = $\frac{1}{5}$ s and all included the ColorChecker Passport Classic Target (X-Rite Inc.), which consists of 24 coloured squares against which photo colours can be standardized. Prior to analysis, images were white balanced and colour corrected by applying the custom image profile in LIGHTROOM (Adobe Systems Inc.) using the X-rite ColorChecker Passport plugin (X-Rite Inc.). Once the images were colour corrected, square 'swatches' of the entire dorsum of each frog were excised from each photograph by cutting out the largest possible square within its four legs (see Appendix, Fig. A1) and saved as separate files for both colour and pattern analysis.

For colour analysis, mean RGB values were gathered from each image (the dorsal swatches) employing a method adapted from Endler's adjacency analysis (Endler, 2012) and using the image processing and statistical toolboxes in MATLAB (MathWorks Inc.). We used the same images (dorsal swatches) to generate our estimates of pattern complexity. Because frogs were wet and knobbly, the images contained shiny spots, which we accounted for using a custom smoothing function (see Appendix, Fig. A2). Pixels for each image were then k-means clustered into two classes (representing yellow and black) and the mean RGB values of the two colour classes for each frog were calculated. The number of pixels averaged varied with the size of the frogs (large frogs had relatively larger swatches). *Sensu* Stevens *et al.* (2007) we inspected the raw RGB values for linearity and RGB equality and found them to be satisfactory (see Appendix, Fig. A3). The standardized differences between mean RGB values for both colour classes were calculated *sensu* Endler (1990) [$x = (R - G)/(R + G)$; $y = (G - B)/(G + B)$] and plotted in two-dimensional (2D) colour space. We estimated luminance (L) saturation (S), and hue (H) with normalized RGB values

[dividing each by their maximum (255); R/255, G/255, and B/255]. We derived luminance (L) by summing the minimum normalized value among R, G, and B with the maximum normalized value among R, G, and B, and dividing it by 2. The value for luminance determined the equation for calculating S. Luminance (L) values for black patches were < 0.5, and so $S_{black} = (max - min)/(max + min)$. For the yellow patches, L was > 0.5, and so $S_{yellow} = (max - min)/(2 - max - min)$. Hue ($H$) was calculated by $H = (G - B)/(max - min) \times 60$. R was excluded because it was the maximum value among the normalized R, G, and B, and the ratio was multiplied by 60 to convert to circular degrees. S and H were then compared between treatments using Wilcoxon rank sum tests in R, version 2.8.1 (R Foundation for Statistical Computing).

For small animals with small colour patches, data on coloration and colour pattern are more efficiently and reliably collected via photography than by spectrophotometry. Accordingly, although we recognize that taking spectrometric measurements of individual colour patches may require fewer assumptions than photography, almost all of the frogs and their patterns in the present study were too small (snout–vent length of approximately 2 cm) to obtain reliable readings of individual colour patches with our available spectrometry equipment. The yellow and black stripes were too thin and any small edges of black contaminating readings of yellow would reduce the overall brightness, although this would not be easily detectable during measurements. Nevertheless, to check whether the frogs' yellow reflected in the ultraviolet, we managed to take reliable spectral readings of the largest yellow patch on eight of the largest frogs in the colony. Spectra were collected using a portable spectrophotometer (Jaz, Ocean Optics Inc.) with a fibre-optic cable and light source (model PX-2; Ocean Optics Inc.) and SPECTRASUITE (Ocean Optics Inc.; integration time: 40, spectra averaged: 3, boxcar width: 10). The spectrometer was calibrated using a white standard (WS-1 Diffuse Reflectance Standard, Ocean Optics Inc., > 98% reflectance across 250–1500 nm) and black velvet for the black standard. The light probe was fitted with a custom probe holder to maintain a consistent distance (5 mm) and angle (45°) of light to colour patch. When measuring, we ensured that light did not leak from the edges of the probe and recalibrated the spectrophotometer with the white and black standards between every frog. We took the mean of three measurements of each yellow patch to maximize the accuracy of spectral data. Spectra were analyzed using *pavo* package for R, version 2.8.1 (Maia *et al.* 2013, White *et al.*, 2015).

Pattern analysis was performed on the smoothed swatches (above) (see Appendix, Fig. A2) using a method adopted from Endler (2012) to estimate three pattern metrics: 'colour diversity', 'pattern complexity', and the proportion of each colour present. After each smoothed swatch was k-means clustered into two classes (above), the fraction of pixels in each colour class was calculated as the first pattern metric. Next, transects were taken across each image every two pixels (both vertically and horizontally). Neighbouring pixels along the transect were recorded as being either in the same colour class (a transition within a colour class) or of different colour classes (a transition between different colour classes). We calculated the 'colour diversity' metric, which measures how equally the two colour classes are represented. Also, for each image, we calculated the overall pattern 'complexity', defined as the proportion of transitions between colour classes to the total number of transitions possible. A complexity score close to 1 indicates a more complex colour pattern (e.g. a checkerboard pattern). These analyses were performed using MATLAB (MathWorks Inc.).

Statistical analysis

To test the effect of treatment on frog colour, statistical analyses where carried out on two colour values: (1) estimates of saturation for black and yellow and (2) hue for black and yellow from RGB values, as well as two pattern parameters: (1) pattern complexity and (2) proportion of yellow to black coloration. Wilcoxon rank sum tests and Student's t-tests were conducted in R to compare colour and pattern parameters between the treatment groups.

RESULTS

The spectral reflectance of a subset of corroboree frogs in the present study showed that there was no ultraviolet component to their yellow coloration, as shown by a lack of reflectance below 450 nm (Fig. 1). From swatches cut from photographs of frog dorsal surfaces, we plotted standardized RGB values in 2D colour space to visualize the spread of coloration across the two treatments (Fig. 2). Employing Wilcoxon rank sum tests, we found a significant difference between treatments for saturation and hue for the yellow patches, whereas there were no differences between hue and saturation between treatments for the black patches (Figs 3, 4; Table 1). Values of saturation and values of hue in the yellow skin were higher in frogs from carotenoid-rich treatments compared to those from carotenoid-poor treatments. There was no significant effect of treatment on the metrics of colour diversity, pattern complexity or proportion of yellow to black (Table 2). Overall,

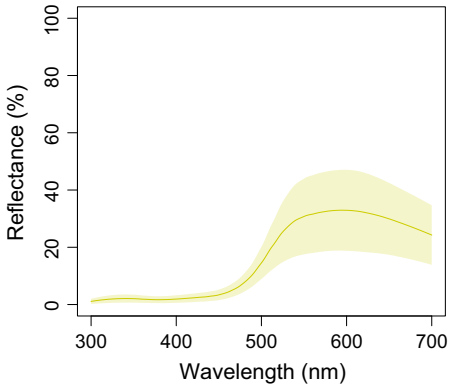

Figure 1. Mean ± SE spectral reflectance of yellow patches of eight corroboree frogs showing no ultraviolet component present.

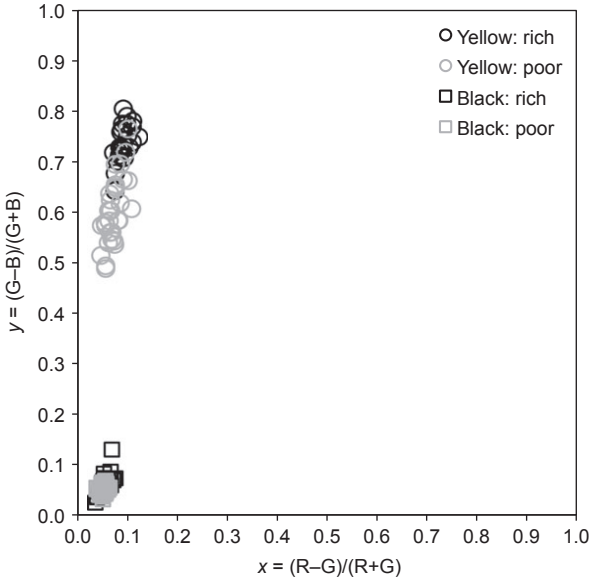

Figure 2. Standardized RGB values plotted in two-dimensional colour space for yellow patches and black patches. Data from black and yellow patches are split into treatment groups.

there was substantial variation in colour diversity, pattern complexity and proportion of yellow:black (Fig. 3).

DISCUSSION

The data obtained in the present study show that at the end of the experimental period the yellow skin of corroboree frogs receiving a diet supplemented with carotenoids was significantly different in colour compared to individuals not receiving a supplemented diet. The yellow patches on frogs fed carotenoid-rich diets were more orange than yellow, compared to those fed carotenoid-poor diets. However, frogs that received carotenoid-poor diets still developed the characteristic yellow and black corroboree frog markings. We found no effect of dietary carotenoids on the colour of black patches or any measures of pattern (i.e. colour diversity, pattern complexity, and proportion of yellow to black coloration).

Corroboree frogs raised on a supplement-free diet appear to have developed their natural yellow and black coloration. This suggests that natural coloration in this species is not reliant on dietary carotenoids and perhaps that colours are produced *de novo*. Thus, as in other vertebrates, black patches are likely to be produced by concentrations of melanophores (melanin-containing cells), whereas the yellow patches are likely produced by pteridine-containing xanthophores (yellow pigment-containing cells). Xanthophores are common in vertebrates, especially fish and amphibians, and these cells can also accommodate carotenoids, should they be available (Bagnara & Hadley, 1969). Our data suggest that when carotenoids are available, corroboree frogs possess the mechanisms to sequester and incorporate them into their integument, altering their coloration. This result is consistent with other studies on the effect of carotenoid sequestration on animal coloration (Svensson & Wong, 2011), including anuran amphibians. For example, in false tomato frogs (*Dyscophus guineti*), β-carotenoids turned frogs yellow over a 9-week period, and a mix of four types of carotenoids,including leutins, canthaxantins, and xanthophylls, turned frogs red (Brenes-Soto & Dierenfeld, 2014).

Ideally, we would compare the results of the present study with baseline coloration in wild corroboree frog populations to gauge the level of carotenoids that frogs access under natural conditions. However, corroboree frogs are critically endangered and the small number of individuals that remain in the wild are susceptible to chyridiomycosis (amongst other threats) and thus are highly protected. Consequently, the natural population is not available for comparison. However, from past dietary studies, corroboree frogs are known to eat ants (e.g. *Iridomyrmex prociduus*) and other invertebrates, many of which are sources of dietary carotenoids (Andersen, 1991; Green & Osborne, 1994; Slattery, 1998). Therefore, with a broad diet, corroboree frogs probably have the opportunity to sequester carotenoids in the wild. It would be of interest to analyze the diet of re-introduced animals (from breeding programmes) to

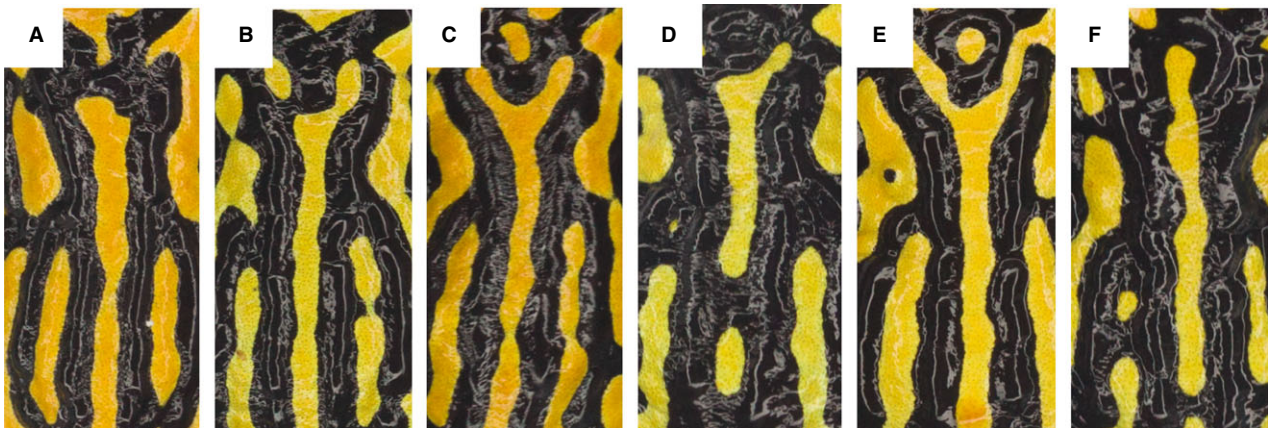

Figure 3. Examples of swatches of corroboree frog colour and pattern. A, high yellow saturation score. B, low yellow saturation score. C, high complexity value. D, low complexity value. E, high ratio of yellow : black. F, low ratio of yellow : black.

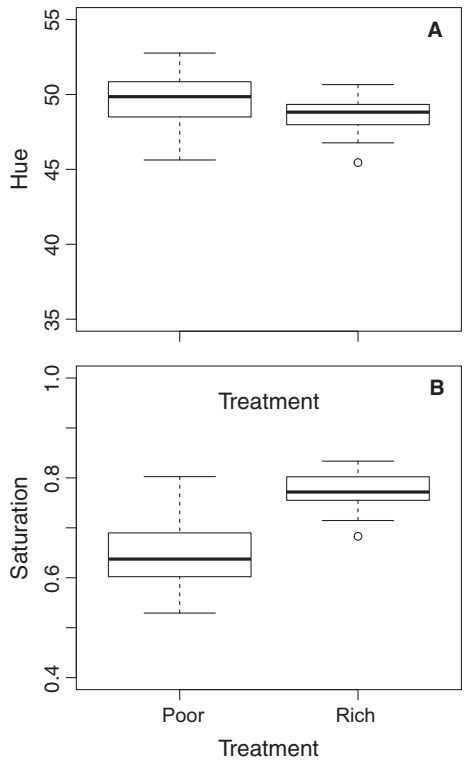

Figure 4. Boxplots showing hue (A) and saturation (B) of yellow patches of frogs from carotenoid diet manipulations. The box encompasses the first and third quartile. Whiskers indicate the first quartile −1.5 × the interquartile range and the third quartile +1.5 × the interquartile range. Open circles indicate outliers.

Table 1. Results of Wilcoxon rank sum tests for differences between treatments with respect to measures of saturation and hue for black and yellow colour patches

Patch colour	Colour metric	Hodges–Lehmann estimator	*W*	*P*
Yellow	Saturation	0.14	686	< 0.01
Yellow	Hue	−1.24	192	< 0.01
Black	Saturation	< 0.01	446	0.14
Black	Hue	0.58	403	0.46

Table 2. Results of *t*-tests for measures of pattern between treatments

Pattern parameter	*r*	d.f.	*t*	*P*
Colour diversity	0.02	49.08	−0.16	0.87
Pattern complexity	0.12	51.79	0.87	0.39
Ratio yellow : black	0.03	52.44	−0.23	0.82

determine the propensity of corroboree frogs to sequester carotenoids in nature.

Although the results of the present study demonstrate that carotenoid availability affects corroboree frog coloration, there was no effect on colour pattern (i.e. size, shape, and regularity of colour patches). We found no variation in pattern with our dietary manipulation despite the highly variable patterns across individuals. Although colour patterns in frogs vary with development, season, temperature, and background (Davison, 1963; Hoffman & Blouin, 2000; Wente & Phillips, 2005), this is perhaps not surprising given that the pattern in at least 26 species of anuran is genetically determined and highly heritable (Hoffman & Blouin, 2000). In the present study, individuals across all treatments were maintained under a constant laboratory environment, with no environmental variation other than diet. Therefore,

it is likely that the high level of variation reported in the colour patterns of corroboree frog metamorphs in the present study is not the product of variation in environmental conditions but, instead, reflects an intrinsic factor such as genetic variation. To validate this idea, future studies could focus on quantitative genetic breeding designs that aim to determine the pattern of heritability (Evans, 2010).

The present study advances our understanding of corroboree frog coloration by showing that they possess the physiological architecture for a diet-induced colour change, as well as the pathways for synthesizing yellow and black coloration with minimal (or no) dietary pigments. Further opportunities for understanding the evolution of corroboree frog coloration are limited by their critically endangered status, although some exciting avenues are still available. For example, understanding the function of their coloration in an antipredator context (e.g. aposematism: Wells, 2007; pattern variability: Price, 2006), could be investigated in plasticine model experiments that determine their natural predators and the protective value of their coloration.

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CONFLICT OF INTERESTS

The authors declare that there are no competing financial interests.

AUTHOR CONTRIBUTIONS

PB and AJS conceived the study. AJS, PB, KU and AKS developed the methods. JAB, AJS and PB were responsible for frog husbandry, generation of the experimental groups, and data collection. KU, AKS and JAB were responsible for data analysis. KU wrote the paper with input from all of the other authors.

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APPENDIX

Figure A1. Photograph of a corroboree frog showing a representative swatch selection for pattern and colour analysis.

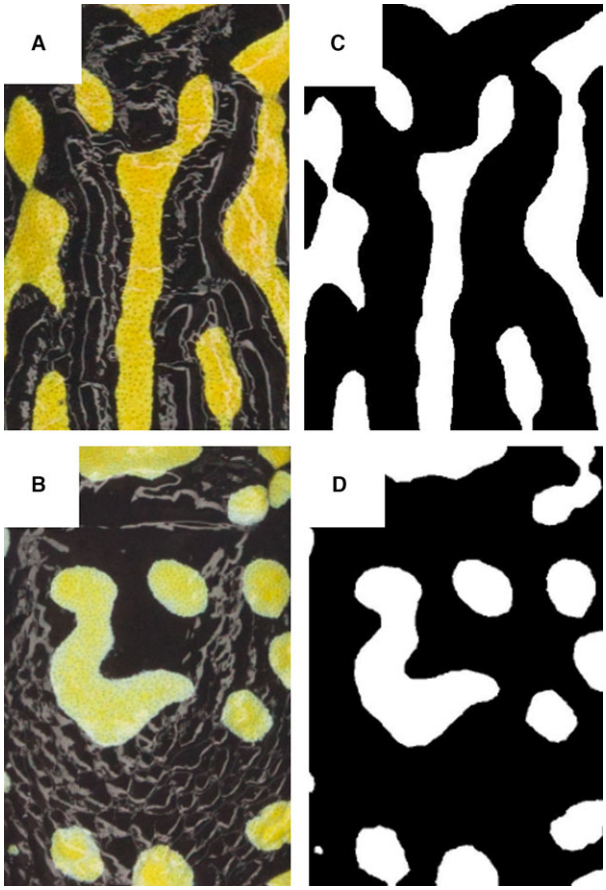

Figure A2. Cropped photographs of the corroboree frog pattern and black and white representations of the same photographs after application of the smoothing function. The black and white pixels represent those that were clustered into each of the two colour classes during the analysis.

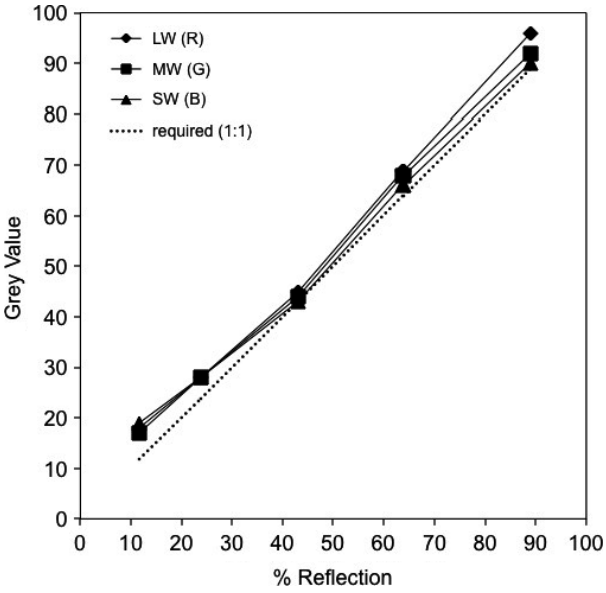

Figure A3. Linearity and equality of RGB values used in the presrent study (*sensu* Stevens *et al.*, 2007) showing that each channel conforms very closely to a 1 : 1 relationship compared to the reflectance of grey standards.