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Dietary carotenoids change the colour of Southern corroboree frogs

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Abstract
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.

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Dietary carotenoids change the colour of southern corroboree frogs

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Running Title: Colouration of corroboree frogs

ABSTRACT

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

Keywords: carotenoid, pigment, colouration, coloration, diet, nutrition, ontogenetic colour change, morphological colour change, anuran, amphibian

Introduction

The mechanisms by which animals produce and change colour over their lifetimes are truly diverse (Bagnara *et al*., 1973; Suga & Munesada, 1988). In vertebrates, colouration 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 (the last being non-pigmentary) which can give rise to yellows, reds, whites, browns and structural optical effects, respectively (Mills & Patterson, 2009). While 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 colouration can thus reflect one or more of several intrinsic or extrinsic contributing factors. Influences on the mechanisms of colouration include intrinsic factors such as ontogeny, hormones, metabolism and genetics, and extrinsic factors such as diet, presence of conspecifics, predators, temperature and habitat background; and their interactions (Booth, 1990, Stuart-Fox & Moussalli, 2009).

In vertebrates, yellow colouration is generally a product of the presence of pteridines and/or carotenoids (Weiss *et al.* 2012, Stephan & McGraw, 2009). Pteridines are synthesised during purine production and are produced *de novo* by the animal (e.g. Ziegler, 2003). Given that guanine production is a fundamental physiological process, pteridines are readily available and consistently replenished. Pteridine-based yellow colouration is therefore less likely to be variable as it is not greatly influenced by extrinsic factors. In contrast, the availability of other pigments can be patchy 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 thought to keep signals honest (Hill and McGraw, 2006). The classic example is the house finch (*Carpodacus mexicanus*), the male of which laboriously acquires carotenoids through his diet and the females of which prefer the males with the reddest plumage as mates (Hill, 1991). Beyond colouration,

carotenoids have a remarkable breadth of benefits for the animals that sequester them (von Lintig, 2010, Goodwin, 1984, Svensson and Wong, 2011). Carotenoids have a variety of functions in, immune function, reproduction, exercise performance, and colouration and can directly influence fitness. For example, carotenoid rich diets have been shown to improve the escape performance of zebra finches (*Taeniopygia guttata*; Blount and Matheson 2006) and southern corroboree frogs (Silla et al 2016). While 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 *et al*., 2013). Recently, research has focused on understanding the co-occurrence of pteridines and carotenoids in animal colouration. For example, in striped plateau lizards (*Sceloporus virgatus*), Weiss *et al.* (2012) found that females trade off by utilising pteridines for ornamental colouration leaving carotenoids free for use in egg production. In anoles (*Norops sagrei* and *N. humilis*) pteridines and carotenoids occupy different parts of the dewlap and this varies between the sexes and species (Steffen and McGraw, 2009, Steffen and McGraw, 2007).

The purpose of our study was to determine whether the striking, putatively aposematic (Umbers et al unpublished data), yellow and black colouration 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: (a) if corroboree frog yellow is primarily carotenoid-based, frogs that received no carotenoids will not develop yellow stripes, (b) if corroboree frog yellow is primarily pteridine-based, frogs that receive no carotenoids will still develop yellow stripes and (c) 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 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 due to an abundance of available carotenoids.

Results

The spectral reflectance of a subset of corroboree frogs in this study showed that there was no ultraviolet component to their yellow colouration shown by a lack of reflectance below 450 nm (Fig. 1). From swatches cut from photos of frog dorsal surfaces we plotted standardised RGB values in 2D colour space to visualise the spread of colouration 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, but no differences between hue and saturation between treatments for the black patches (Table 1, Figs 3 and 4). Values of saturation and values of hue in the yellow skin were higher in frogs from carotenoid-rich treatments than 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, there was substantial variation in colour diversity, pattern complexity and proportion of yellow:black (see Fig. 3 for examples).

Discussion

Our data show that at the end of the experimental period the yellow skin of corroboree frogs whose diet was supplemented with carotenoids was significantly different in colour compared to individuals that were not supplemented with carotenoids. 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 colouration).

Corroboree frogs raised on a diet not supplemented with carotenoids seem to have developed their natural yellow and black colouration. This suggests that natural colouration 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) while 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 colouration. This result is consistent with other studies on the effect of carotenoid sequestration on animal colouration (Svensson & Wong, 2011), including anuran amphibians. For example, in false tomato frogs (*Dyscophus guineti*), β-carotenoids turned frogs yellow over a nine-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 our study to baseline colouration in wild corroboree frog populations to gauge the level of carotenoids that frogs access under natural conditions. However, corroboree frogs are critically endangered and any individuals that remain in the wild are susceptible to chyridiomycosis and thus highly protected. As a consequence, 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 are likely to have the opportunity to sequester carotenoids in the wild. It would be of interest to analyse the diet of re-introduced animals from breeding programs to determine the propensity of corroboree frogs to sequester carotenoids in nature. Although opportunities for behavioural ecology studies are limited due to the critically endangered status of this species, understanding the function of their colouration in an antipredator context, and how carotenoid loading influences their development and immune function, are exciting avenues for further investigation.

While the results of this study show that carotenoid availability affects corroboree frog colouration, there was no effect on colour pattern (size, shape and regularity of colour patches). Although colour patterns in frogs vary with development, season, temperature and background (Hoffman & Blouin, 2000, Davison, 1964, Wente & Phillips, 2005), this is perhaps not surprising given that pattern in at least 26 species of anuran is genetically determined and highly heritable (Hoffman & Blouin, 2000). We found no

variation in pattern with our dietary manipulation despite the highly variable patterns across individuals. In this study individuals across all treatments were maintained in 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 this 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 using quantitative genetic breeding designs to determine the pattern of heritability (Andrea *et al*., 2009; Evans, 2010).

In conclusion, the aim of our study was to investigate the effect of dietary carotenoid supplementation on the colour and patterning in corroboree frogs. Our results show that carotenoid supplementation made the corroboree frogs' distinctive yellow patches more orange and imply that their coloration is labile with respect to diet. Our results also show that characteristic yellow and black corroboree frog colouration can be developed on a basic laboratory diet without carotenoids, and thus, is likely to be produced *de novo*. This study advances our understanding of corroboree frog colouration by showing that they possess the existing physiological architecture for diet-induced colour change.

Materials and Methods

Rearing and husbandry

The Southern corroboree frog (*Pseudophryne corroboree*) is endemic to the sub-alpine regions of Australia. These relatively small frogs (~25 mm snout-vent length) harbor toxic alkaloids and have a striking black and yellow colour pattern which 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 program 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 (10 cm diameter and 10.5 cm high) filled with 600 mL of R.O. water. Tadpoles were kept in a constant temperature room held at approx. 12˚C (range = 11.4˚C – 12.9˚C). To prevent developmental disorders associated with ultraviolet deficiencies (Lannoo, 2008), a single UV-B light bulb (36” fluorescent strip bulb) was suspended approx. 20 cm above tadpoles, providing 1 hour of UV-B light per day between 11:30 am - 12:30 pm. The room’s fluorescent lighting was on an 11.5 h / 12.5 h day/night 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 programs 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®, Germany) suspended in 10 mL of R.O. 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, Melbourne, VIC Australia) integrated with a reverse osmosis water system (Sartorius Stedim Biotech, Germany). Water quality was tested in three haphazardly selected containers per treatment every four weeks (Aqua One ®,

Australia). Throughout the experiment ammonia concentrations remained low (0.5 – 1.0 mg L^{-1}).

Tadpole development was assessed every two 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 nutritional needs through the absorption of the tail tissue (Wassersug, 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). Experimental containers contained a thick layer of moist sphagnum moss (*S. 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 two 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, Australia) in order to prevent developmental disorders associated with calcium deficiencies (Lannoo and Ebrary, 2008).

Experimental treatments

On day one of the first experimental period, tadpoles were randomly assigned to one of two diet treatments: i) carotenoid-poor diet consisting of 1.0 g of basal diet (see above) (N = 32), or ii) a carotenoid-rich diet consisting of 1.0 g of basal diet plus 20 mg of carotenoid mixture (Superpig, Repashy ®, USA) (N = 32), providing approximately 20 mg g^{-1} carotenoids. Tadpoles were fed two drops of food (0.059 g – 0.069 g wet mass, 0.015 g – 0.018 g dry mass), three times a week until eight weeks old. From nine weeks

old until tadpoles metamorphosed, individuals were fed four drops of food (range = 0.117 g – 0.137 g wet mass, 0.030 – 0.036 g dry mass), three times per week.

Two weeks after metamorphosis survivors were weighed, split evenly into treatments 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-hours with carrot, as well as being dusted with approximately 1.0 g of carotenoid mixture (Superpig; Repashy®, USA). 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 Canon 600D camera with a 60 mm Canon macro lens (Canon Inc., Japan). Photographs were taken in camera raw format with the following settings: ISO = 400, f-stop = 6.3, shutter speed = ⅕ s and all included the X-rite ColorChecker Passport Classic Target which consists of 24 coloured squares against which photo colours can be standardised. Prior to analysis, images were white balanced and colour corrected by applying the custom image profile in Adobe Lightroom using the X-rite ColorChecker Passport plugin (X-rite Inc. USA). Once the images were colour corrected, square 'swatches' of the entire dorsum of each frog were excised from each photo by cutting out the largest possible square within its four legs (Fig. A1) and saved as separate files for both colour and pattern analysis.

For colour analysis, average RGB values were gathered from each image (the dorsal swatches) using a method adapted from Endler's adjacency analysis (Endler, 2012), using the image processing and statistical toolboxes in MATLAB (The MathWorks, USA). 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 (Fig. A2). Pixels for each

image were then k-means clustered into two classes (representing yellow and black) and the average RGB values of the two colour classes for each frog calculated. The number of pixels averaged varied with the size of the frogs (large frogs had relatively larger swatches). Following Stevens *et al*. (2007) we inspected the raw RGB values for linearity and RGB equality and found them to be satisfactory (Fig. A3). The standardised differences between average RGB values for both colour classes were calculated following Endler (1990) (x = (R-G)/(R+G); y = (G-B)/(G+B)) and plotted in 2D colour space. From our RGB values we estimated saturation (S) by first normalising them (R/255, G/255, B/255), finding the minimum and maximum normalised value among R, G and B, finding the luminance by dividing the sum of the minimum and maximum by 2. Then, for black the luminance was < 0.5 so we calculated S = (max-min/max+min). For the yellow patches luminance was >0.5 so, S = (max-min/(2-max-min). Hue was calculated by (G-B)/max-min) (because red was the max value) and multiplied by 60 to convert to circular degrees. Saturation and hue were then compared between treatments using Wilcoxon Rank Sum tests in R (v. 2.8.1, R Foundation).

For small animals with small colour patches, data on colouration and colour pattern are more efficiently and reliably collected via photography than with spectrophotometry. So, while we recognise that taking spectrometric measurements of individual colour patches may require fewer assumptions than photography, almost all the frogs and their patterns in this study were too small (snout-vent length: ~ 2 cm) to get 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, but 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., Dunedin, USA) with a fibre-optic cable and light source (model PX-2, Ocean Optics Inc., Dunedin, USA) and SpectraSuite software (Ocean Optics Inc., Dunedin, USA, 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., Dunedin, USA, >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 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 average of three measurements of each yellow patch to maximise accuracy in spectral data. Spectra were analysed using the PAVO package for R (v. 2.8.1, R Foundation, Maia *et al*. 2013, White *et al*. 2015).

Pattern analysis was performed on the smoothed swatches (above) (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 2 pixels (both vertically and horizontally). Neighboring 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 total transitions that were between different colour classes. A complexity score close to 1 indicates a more complex colour pattern (e.g. a checkerboard pattern). These analyses were performed using MATLAB software (The MathWorks, USA).

Statistical analyses

In order 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, and two pattern parameters: (1) pattern complexity and (2) proportion of yellow to black colouration. Wilcoxon Rank Sum tests and Student's t-tests were conducted in R to compare colour and pattern parameters between the treatment groups.

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Competing Interests

The authors declare no competing financial interests.

Author contributions

P.B. and A.J.S. conceived the study A.J.S., P.B., K.U. and A.K.S. developed the methods, J.B., A.J.S. and P.B. were responsible for frog husbandry, generation of the experimental groups and data collection. K.U., A.K.S. and J.B. were responsible for data analysis, K.U. wrote the paper with input from all other authors.

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Figure legends

Figure 1. Average Spectral reflectance of yellow patches of eight corroboree frogs (±SE) showing no ultraviolet component present.

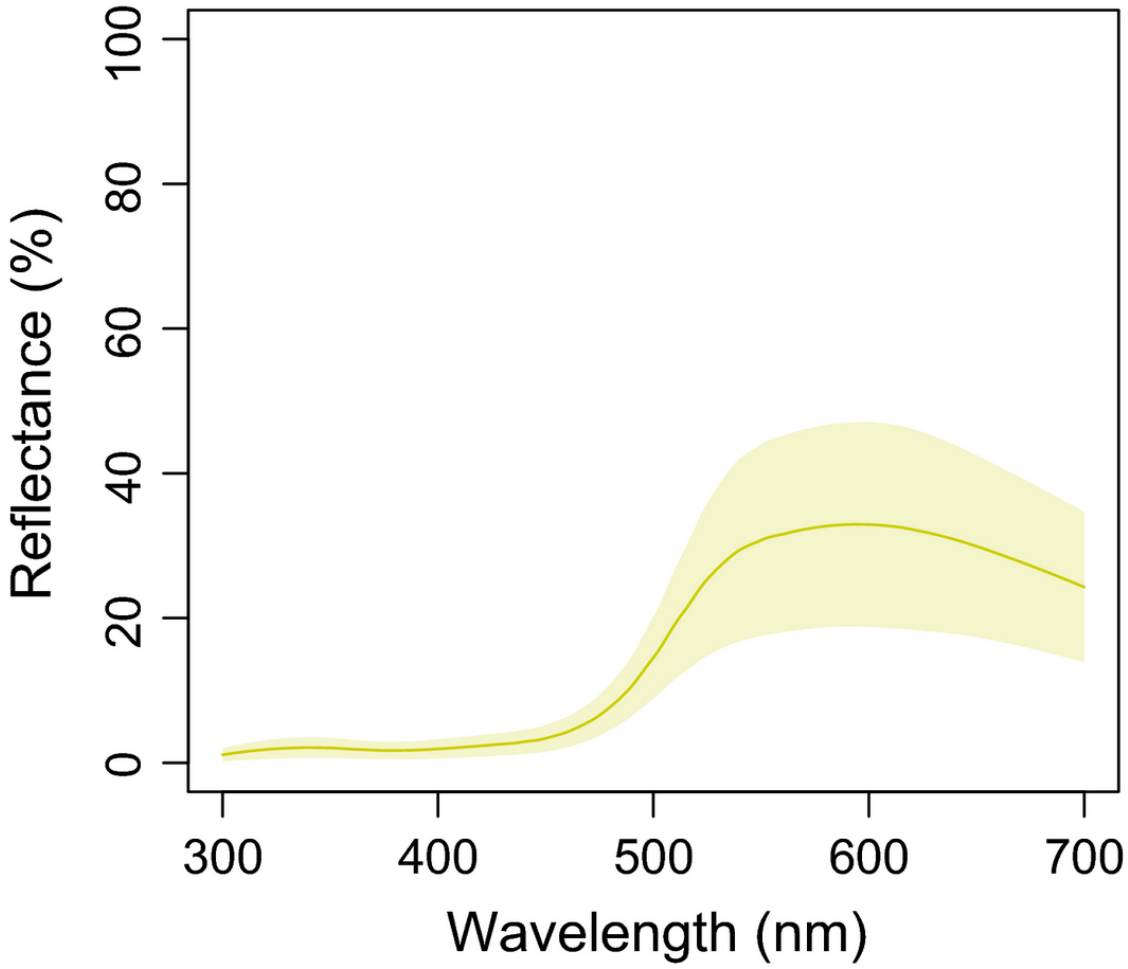


Figure 2. Standardised RGB values plotted in 2D colour space for yellow patches and black patches. Data from yellow patches is split into treatment groups, data from black patches are presented in a single icon and not split into treatments.

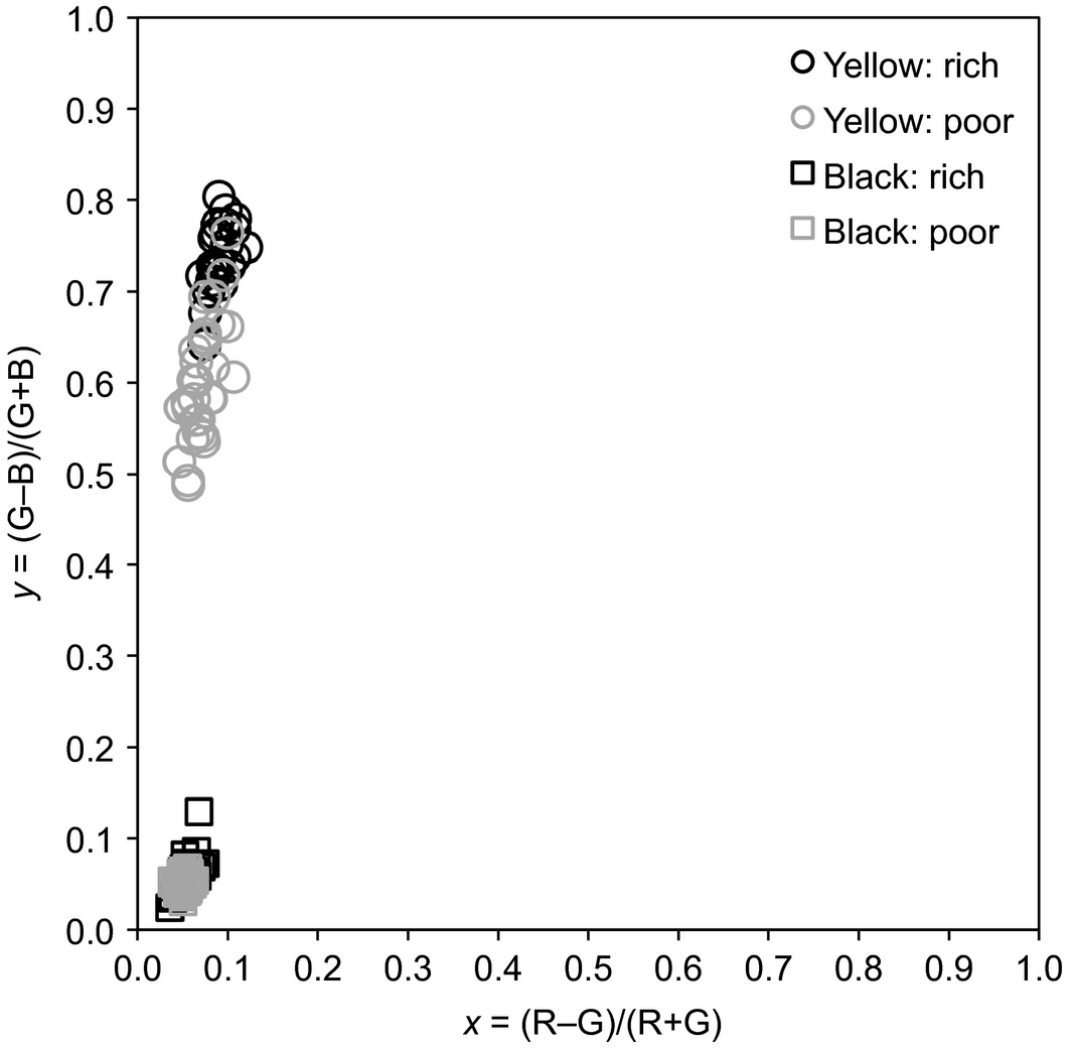


Figure 3. Examples of swatches of corroboree frog colour and pattern: (A) high yellow saturation score, (B) low yellow saturation score, (C) high complexity value, (D) low complexity value, (E) high ratio of yellow:black, (F) low ratio of yellow:black.

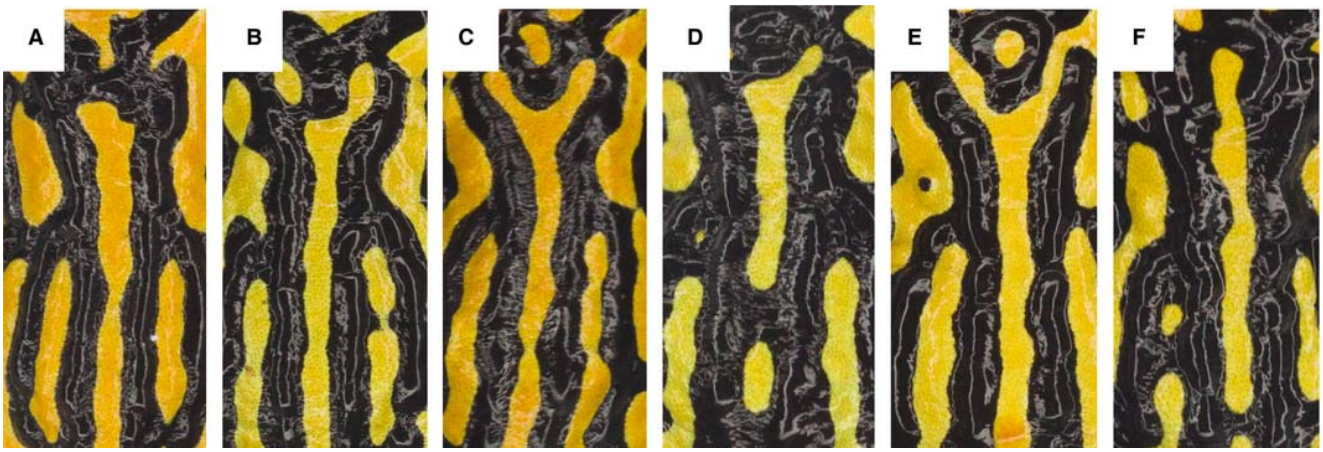
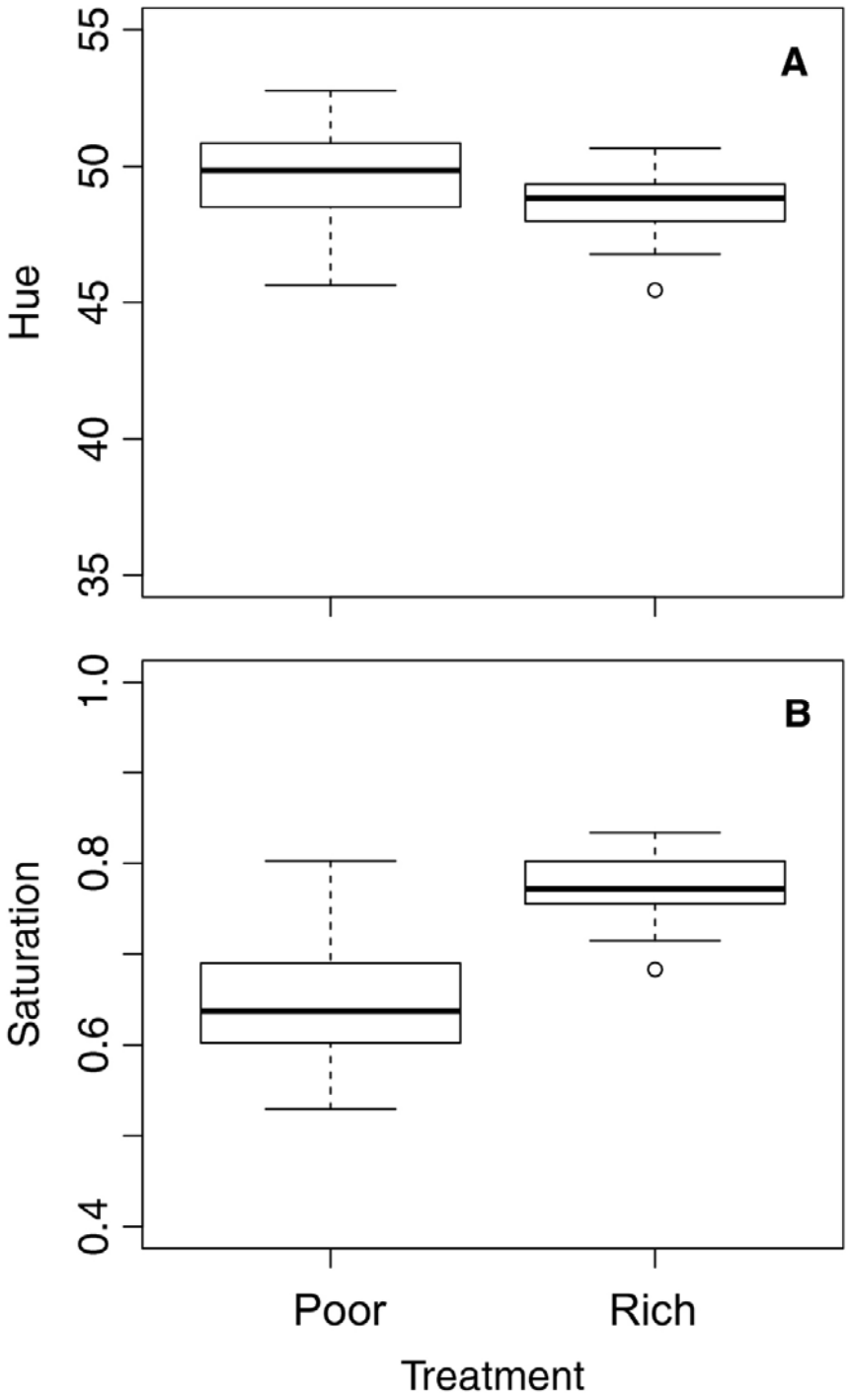


Figure 4. Boxplots showing (A) hue and (B) saturation of yellow patches of frogs from carotenoid diet manipulations. Box encompasses first and third quartile, whiskers indicate the first quartile minus 1.5 times the inter-quartile range and the third quartile plus 1.5 times the inter-quartile range, and open circles indicate outliers.



Tables

Table 1. Results of Wilcoxon rank sum tests for differences between treatments for 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	df	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