Does dietary β-carotene influence ontogenetic colour change in the southern corroboree frog?

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Summary Statement

This study examined the effects of long-term dietary β-carotene supplementation and ontogenetic colour change in an aposematic frog. We found evidence for consistent ontogenetic colour change, but no effect of dietary β-carotene.

Abstract

Ontogenetic colour change occurs in a diversity of vertebrate taxa, and may be closely linked to dietary changes throughout development. In various species, red, orange, and yellow colouration can be enhanced by the consumption of carotenoids. However, a paucity of long-term dietary manipulation studies means that little is known of the role of individual carotenoid compounds in ontogenetic colour change. We know even less about the influence of individual compounds at different doses (dose effects). The present study aimed to use a large dietary manipulation experiment to investigate the effect of dietary β-carotene supplementation on colouration in southern corroboree frogs (*Pseudophryne corroboree*) during early post-metamorphic development. Frogs were reared on four dietary treatments with four β-carotene concentrations (0, 1, 2 and 3 mg g^{-1}), with frog colour measured every 8 weeks for 32 weeks. β-carotene was not found to influence colouration at any dose. However,

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colouration was found to become more conspicuous over time, including in the control treatment. Moreover, all frogs expressed colour maximally at a similar point in development. These results imply that for our study species: (i) β-carotene may contribute little or nothing to colouration, (ii) frogs can manufacture their own colour, (iii) colour development is a continual process, and (iv) there may have been selection for synchronised development of colour expression. We discuss the potential adaptive benefit of ontogenetic colour change in *P. corroboree*. More broadly, we draw attention to the potential for adaptive developmental synchrony in the expression of colouration in aposematic species.

Introduction

Conspicuous colouration is found in most animal taxa and is known to play a significant role in male-male competition, female mate choice and anti-predator defence (Casas-Cardona et al. 2018; Gomez et al. 2009; Sztatecsny et al. 2012). Such varied function has resulted in considerable phenotypic variation in colouration, both within and between species (Hofreiter & Schöneberg 2010). Adding to this variation, colour expression in vertebrates is rarely static (Booth 1990), with a diversity of species known to change colour (Nilsson Sköld et al. 2013; Strickland & Doucet 2021; Zimova et al. 2018). This change can be rapid and reversible, occurring within a matter of minutes (termed 'dynamic' colour change (Kindermann et al. 2014)), or take place over the entire course of an individual's life and can be irreversible (termed 'ontogenetic' colour change (Booth 1990; Bulbert et al. 2018)).

Among vertebrates, colour production often relies on pigment-based systems (Bagnara 1983; Ligon & McCartney 2016), whereby pigment granules are stored in specialised cells called 'chromatophores'. Vertebrate chromatophores include melanophores, xanthophores, erythrophores, leucophores and iridophores, which contain pigment granules responsible for producing brown, yellow, orange and red, white, and structural effects, respectively (Hofreiter & Schöneberg 2010; Mills & Patterson 2009; Suga & Munesada 1988). In the context of sensory ecology, the production of red, orange, and yellow colouration is of particular interest as these colours are known to play an important role in sexual signalling (Baeta et al. 2008; Davis & Grayson 2008) and aposematism (warning colouration) (Blount et al. 2012; Dreher et al. 2015). Xanthophores produce red, orange, and yellow colours through a combination of long-wave shifted reflecting pigments, including pteridines and carotenoids

(Steffen & McGraw 2009). Pteridines are synthesised by vertebrates during purine production (Ziegler 2003), and are thus expected to be present in chromatophores from early development. Carotenoids are a group of more than 1000 different hydrocarbon molecules produced by all photosynthetic organisms (Fernandes et al. 2018). Unlike pteridines, they cannot be produced de novo by vertebrates and thus must be obtained through the diet (Bendich & Olson 1989; Fernandes et al. 2018).

In species where dietary carotenoids contribute to colouration, we can expect colour characteristics (such as hue, chroma and luminance) to change during ontogeny for a number of reasons. First, if carotenoids are used to produce ornaments and sexual signals, colouration may become more pronounced as individuals approach sexual maturity (Booth 1990). Second, as individuals age, they will typically undergo changes in foraging behaviour, diel activities, habitat use and body size. These changes can influence conspicuousness to visually-oriented predators, pressure for the development of defence mechanisms, and expression of warning colouration (Booth 1990; Higginson & Ruxton 2010). As such, a common manifestation of ontogenetic colour change is a switch in defensive strategies, most often seen where drab juveniles become more colourful as they mature (Higginson & Ruxton 2010; for notable exceptions, see Bulbert et al. 2018; Wilson et al. 2007). Third, because carotenoids must be acquired via the diet and can be limited, trade-offs can occur between investment of carotenoids in colouration versus the maintenance of essential bodily functions. Specifically, when individuals experience periods of high metabolic activity during growth, it may be critical for carotenoids to function as antioxidants and remove reactive oxygen species (ROS) that can damage cells and DNA (Alonso- Alvarez et al. 2008; Lozano 1994). As growth slows, carotenoids may become increasingly available for investment in colour. Considering these life history relationships, it is logical to predict that investment in carotenoid-based colouration will increase during ontogeny.

The extent to which dietary carotenoids contribute to ontogenetic colour change may critically depend on carotenoid class and concentration. Carotenoids are a diverse group of compounds split into two broad classes (carotenes and xanthophylls) based on their structural characteristics (Miller et al. 1996; Pérez- Rodríguez 2009). Carotenoid-based colouration ranges from pale to bright yellow, through to orange, red, and occasionally purple (Maoka 2020). The chemical structure of any given carotenoid compound has been found to influence colour expression (Meléndez-Martínez et al. 2007). As the number of conjugated double bonds (CDB) in a carotenoid increases, so does its wavelength absorption range. Thus,

carotenoids such as β-carotene and lycopene (11 CDB) are perceivably more red than carotenoids such as violaxanthin and neoxanthin (9 CDB), which are more yellow (Meléndez-Martínez et al. 2007). Carotenoids which share the same number of conjugated bonds can vary in colour due to differences in the location of CDB within their chemical structure (Meléndez-Martínez et al. 2007). For example, the absorption maxima of β-carotene (11 CDB) are located at shorter wavelengths than that of lycopene (11 CDB) as two of the CDB of β-carotene are located in its rings, making it noncoplanar (Meléndez-Martínez et al. 2007). Due to the unique influence that carotenoid structure exerts on colouration, careful examination of single carotenoid compounds is crucial. Moreover, it is important to examine a carotenoid compound at multiple concentrations to test for dose effects. Under stable physiological conditions, the positive effects of carotenoids on pigment-based colouration are expected to increase up to a threshold (Cothran et al. 2015; McInerney et al. 2019). When concentrations are below this threshold, carotenoids may be limited and preferentially invested in other functions (Baeta et al. 2008). When the threshold dose is exceeded, carotenoids begin acting as pro-oxidants and have toxic effects that inhibit colour expression (Palozza 1998; Young & Lowe 2001). Identifying the concentrations at which individual carotenoids begin to influence colouration is thus crucial for understanding their effects on signalling. Despite the influence that carotenoids may exert on colour development, few studies have examined the role of individual carotenoid compounds (Ho et al. 2012; Prado-Cabrero et al. 2020; Toomey & McGraw 2011; Weaver et al. 2018; Weaver et al. 2020; Yasir & Qin 2010; Yi et al. 2014), or tested effects across a range of dosages (Koch et al. 2016).

Amphibians are frequently used as models to investigate the proximate and ultimate causes of colour variation as their colour can depend on carotenoids. Amphibian colour is controlled by a specialised dermal grouping of chromatophores called the dermal chromatophore unit (DCU). The DCU is composed of three chromatophores (melanophores, iridophores and xanthophores (Bagnara et al. 1968; Kindermann & Hero 2016)), each containing unique pigment granules responsible for brown-black, blue-green, and red-yellow colour respectively (Suga & Munesada 1988). The DCU is thought to be responsible for producing both dynamic and ontogenetic colour change in amphibians (Bagnara et al. 1968), and dietary carotenoids are predicted to play an important role in ontogenetic colour change. In support of this notion, carotenoids have been identified in the skin of various amphibian species (Bonansea et al. 2017; Brenes-Soto et al. 2017; Matsui et al. 2002), and positive relationships have been found between carotenoid presence and skin reflectance and hue

(Brenes-Soto et al. 2017). Despite these advances, we still have a limited understanding of the influence of carotenoid compounds at different doses on amphibian colour change. To date, only three studies have manipulated dietary carotenoid availability to test for effects on colouration in amphibians. These studies have provided evidence that dietary carotenoids can make frogs more yellow, red and orange (Brenes-Soto & Dierenfeld 2014; Ogilvy et al. 2012; Umbers et al. 2016). Unfortunately, however, these studies have either used mixtures of multiple carotenoid types (Ogilvy et al. 2012; Umbers et al. 2016), or failed to mention the doses of specific carotenoids tested (Brenes-Soto & Dierenfeld 2014).

The Southern Corroboree Frog (Pseudophryne corroboree, Moore, 1953) is an excellent model species to investigate ontogenetic changes in colouration, and how colour change may be influenced by the availability of dietary carotenoids. P. corroboree displays striking yellow and black colouration thought to function as an aposematic signal (Umbers et al. 2020), and this species is known to feed on carotenoid-rich algae as tadpoles, and insects as adults (Osborne 1991; Umbers et al. 2016). Moreover, there is some evidence that consumption of carotenoids can influence P. corroboree skin colour. A recent dietary manipulation study reported that the colour of frogs fed a mixture of carotenoids for 50 weeks became increasingly orange-shifted (Umbers et al. 2016). Analysis of the skin of several experimental frogs using High-Performance Liquid Chromatography (HPLC) showed that the main carotenoid compounds present were lutein and β-carotene (Byrne and Silla, unpublished data). However, the extent to which each compound influenced colouration remains unknown. Dietary β-carotene has been shown to make the skin of false tomato frogs (Dyscophus guineti) more yellow (Brenes-Soto & Dierenfeld 2014), and this species sits in the same suborder (Neobatrachidae) as P. corroboree. Therefore, the aim of the present study was to test the effect of multiple doses of β-carotene on colour expression in P. corroboree. The specific aims were to investigate: 1) the influence of different concentrations of β-carotene on colouration, 2) whether colouration changes during post-metamorphic development, and 3) whether the effect of treatment dose on colouration changes over time. If the availability of carotenoids increases during individual development, we expect more pronounced dose effects over time. Specifically, later in development, significant investment in colour may only be possible under high dose treatments where carotenoid availability is not limited.

Methods

Ethics Statement

All procedures described in this study were evaluated and approved by the University of Wollongong's Animal Ethics Committee (AE18/15), and were conducted under the scientific license number #SL102197. All relevant institutional and national guidelines for the care and use of animals were followed.

Study Animals

On February 5th, 2019, 136 P. corroboree metamorphs from eight clutches were transported to the Environmental Research Centre (ERC) at the University of Wollongong (UOW) from a captive colony maintained at Taronga Zoo Sydney. At the time of collection, individuals ranged in age from 4-8 weeks post-metamorphosis.

Husbandry and Diet Treatments

Upon arrival at UOW, metamorphs were separated into individual enclosures (21 cm x 12 cm x 12 cm) and randomly assigned to one of four experimental diet treatments: 0 mg g^{-1} β-carotene (T0); 1 mg g^{-1} β-carotene (T1); 2 mg g^{-1} β-carotene (T2); 3 mg g^{-1} β-carotene (T3). Of note, the values represent milligrams of carotenoid per gram of feed. Individuals from each clutch were evenly distributed amongst treatment groups to control for genetic differences influencing colour expression. Enclosures contained a 2 cm layer of aquarium-grade pebbles, 1 cup (approx. 220 mL) of loosely packed sphagnum moss (*Sphagnum cristatum*) and a singular, small PVC pipe (inner diameter = 4.4 cm; length = 5.5 cm) to provide refuge. Enclosures were flushed thoroughly with Reverse Osmosis (R.O.) water twice weekly. Sphagnum moss was removed and replaced with fresh moss fortnightly to remove carotenoid residue and excrement, and to avoid the accumulation of ammonia. Experimental enclosures were positioned in rows in two-deep pairs along five shelves, and containers remained on their allocated shelf for the duration of the treatment period. Enclosures were kept in an artificially illuminated constant-temperature room maintained at 20°C for the duration of the experiment. UV+Visible lights (Reptisun 10.0 T5 High Output 36" bulb; Pet Pacific, Australia) were suspended approx. 20 cm above enclosures and were maintained on a 9:15-hour light:dark cycle. Husbandry procedures were modelled on the P.

corroboree husbandry protocols employed at Zoos Victoria and Taronga Conservation Society Australia.

Following arrival, all metamorphs were fed a basal diet consisting of commercially-available crickets (Acheta domesticus) which are known to contain negligible levels of carotenoids (McInerney et al. 2020) for five weeks until experimental diets commenced on March 15th, 2019. Experimental diets were administered twice weekly and were prepared by dusting a standardised weight of crickets (approx. 15g per treatment) with one of four treatment powders, corresponding to each experimental diet: 0 mg g^{-1} β-carotene (T0); 1 mg g^{-1} β-carotene (T1); 2 mg g^{-1} β-carotene (T2); 3 mg g^{-1} β-carotene (T3) (Table 1). Cellulose microcrystalline powder (435 236; Sigma-Aldrich, Castle Hill, NSW) was added to each dietary supplement to ensure that feed quantity was balanced across experimental diets (Table 1). Cellulose was used as a dietary bulking agent as it has no nutritional value, and is commonly used to balance feed quantity in amphibian dietary manipulation studies (Keogh et al. 2018; McInerney et al. 2019). To prevent developmental disorders, all treatment diets were supplemented with a standard amount of calcium powder (0.25 g; Repti-Cal, Aristopet, Melbourne, Australia) per feed (equivalent to 16.7 mg g^{-1}). Carotenoid doses were based on recent carotenoid supplementation studies on Litoria booroolongensis and P. corroboree (Keogh et al. 2018; McInerney et al. 2019). In these studies, β-carotene doses tested ranged from 0.1-10mg g^{-1} and 0.1-1 mg g^{-1} respectively, and the optimal doses identified in both studies was 1 mg g^{-1}. All doses were found to be sublethal. Crickets were coated with diet treatments in a humidity and temperature-controlled room, and fed to frogs immediately after dusting to prevent the loss of carotenoids caused by crickets grooming off powder (Li et al. 2009). At each feed, individual frogs were provided with 10-15 crickets (total mass = 0.38-0.47 g) to be eaten ad libitum. The age of crickets ranged from 7-10 days old at the beginning of the experimental period, and was increased to 9-12 days old as the size of the frogs increased.

Colour Quantification

To quantify temporal changes in colour, individuals were photographed over a 32 week period from March 15th, 2019 until October 21st, 2019. The dorsal surface of each frog was photographed immediately prior to the commencement of experimental diets, and every eight weeks thereafter (weeks 0, 8, 16, 24, 32).

Photographs were taken using a Canon EOS70D DSLR camera (Settings: IOS 400; shutter speed 1/250; f-stop 11; lens 55 mm) attached to a copy stand positioned at a height of 43 cm above the photo staging area. The photo staging area was enclosed within two opaque cylindrical containers stacked on top of each other to reduce ambient light. The container lid included a hole for the camera lens to project into the chamber, with black foam around to prevent stray light from entering. To create a standardised lighting environment, white LED lights were positioned in a ring around the inside of the container. A ColorChecker Passport Classic Target (XRite Inc.) pad consisting of 24 coloured squares was included in the photo staging area to enable colour standardisation during data quantification (Fig. 1). The ColorChecker and ID tags were designed to be minimally reflective (matte) to reduce reflectance altering colour in the photos. Photographs were taken and saved in RAW (CR2) format to prevent colour alterations associated with file formatting (Frey & Haworth 2014). At each sampling time (weeks 0, 8, 16, 24, 32), the body mass of each individual was recorded to the nearest 0.001 g using a Kern PCB-350-3 balance immediately after they were photographed.

Frog colour was quantified using specially written software written in MatLab (R2019a). Prior to colour data collection, images were white balanced in the software with reference to the white square of the XRite ColorChecker (Fig. 1). Colour averages were collected for all areas of yellow colouration over the dorsal surface of each frog. Colour values were recorded as red, green, and blue (RGB) values using image analysis techniques within the MatLab software (as in Cadena et al. 2018). RGB data was then transformed into hue, chroma and luminance (HCL) values using another MatLab programme (for MatLab code used for the analyses see Appendix S1). Hue is commonly represented as degrees on a circular scale, in which red = 0° (or 360°), green = 120° and blue = 240°. To translate our data to this scale, hue (H) was transformed according to the formula: $H = H_0 + 120$ (where H_0 represents the untransformed hue value from MatLab).

Statistical Analysis

In order to examine the relationship between diet treatment, time and the interaction between diet treatment and time on colouration, Linear Mixed-effects Models (LMM) were used. Three separate models were conducted with hue, saturation, and luminance as the dependent response variables in each model, respectively. In each model, diet treatment (0 mg g^{-1}, 1 mg g^{-1}, 2 mg g^{-1}, 3 mg g^{-1}), test week (0, 8, 16, 24, 32), and the interaction between

diet treatment and test week were treated as fixed effects. Frog ID and clutch were included as random effects to account for repeated sampling of the same individuals across the experimental period. Frog body weight (measured at each sampling point) was included as a covariate in all models to account for size-dependent effects. Prior to analysis, a Shapiro-Wilks W Test was conducted on hue, chroma and luminance variables to test for distribution normality. Chroma data were transformed using an arcsine square root transformation ($\sin^{-1}(\sqrt{x})$; $x = \text{chroma}$) to improve normality and homogeneity. In models where significant effects were detected, post-hoc comparisons were made using Tukey's HSD tests. During the study, 18 individuals (3-8 frogs per treatment) died from unexpected adverse events or failure to thrive and were excluded from all analyses. The final number of replicates in each experimental treatment were as follows: T0: n = 30; T1: n = 31; T2: n = 29; T3: n = 26; total n = 116.

All statistical analysis was performed using JMP® 14 (SAS Institute Inc., North Carolina, USA). Results were considered significant at $p < 0.05$.

Results

Hue

Overall, there was no effect of diet treatment on hue (Linear mixed-effects model: $F_{3, 122.6}$=1.5708, P=0.1999; ***Error! Reference source not found.***Fig. 2), and no significant interaction between time and diet treatment (Linear mixed-effects model: $F_{12, 470.5}$=1.4277, P=0.1494; Fig. 2). There was a significant effect of time on hue (Linear mixed-effects model: $F_{4, 485.6}$ =8.1314, P<0.0001; Fig. 2), with hue becoming slightly more yellow-shifted over the 32-week experimental period (W0 mean range = 68.36°–69.10°, W32 mean range = 69.14 ° - 70.46 °; Fig. 2). Hue was not significantly associated with body weight (Linear mixed-effects model: $F_{1, 538.8}$=1.8606, P=0.1731).

Chroma

Overall, there was no effect of diet treatment on chroma over the 32-week experimental period (Linear mixed-effects model: $F_{3, 104.4}$=1.6820, P=0.1751; Fig. 3). There was a significant effect of time on chroma (Linear mixed-effects model: $F_{4, 489.5}$=18.6314; P<0.0001; Fig. 3) and a significant treatment-by-time interaction (Linear mixed-effects model: $F_{12, 463.9}$=2.2824, P=0.0080; Fig. 3). Chroma values were variable at the

commencement of the experiment (week 0), with mean values ranging between 45.11% and 48.82% (Fig. 3). From week 0 to week 8, chroma values for all diet treatments rose and converged on a similar value (range=50.79-51.39%; Fig. 3) before exhibiting a plateau for the remainder of the experimental period (Fig. 3). Chroma was not significantly associated with body weight (Linear mixed-effects model: $F_{1, 219}$= 2.3576, P = 0.1260).

Luminance

Overall, there was no significant effect of diet treatment on luminance (Linear mixed-effects model: $F_{3, 123.1}$=0.7974, P=0.4976; Fig. 4), and no significant interaction between time and treatment (Linear mixed-effects model: $F_{12, 472.6}$=1.7251, P=0.0586; Fig. 4). There was a significant effect of time on luminance (Linear mixed-effects model: $F_{4, 500.1}$=4.2999, P=0.0020; Fig. 4). Luminance increased linearly between week 0 and week 32 across all treatment groups (Fig. 4). At week 0, luminance ranged on average between 64.59% and 67.35%, and by the end of the experiment (week 32), luminance ranged on average between 72.09% and 73.31% (Fig. 4). Body weight had a significant influence on the model (Linear mixed-effects model: $F_{1, 585.4}$=6.6655, P=0.0101), whereby luminance was positively correlated with body weight.

Discussion

The present study showed that dietary β-carotene supplementation did not cause significant changes in corroboree frog colouration (measured as hue, chroma and luminance) at any of the doses tested. Therefore, our study provides no evidence that dietary β-carotene influences the degree of corroboree frog colouration. However, across all diet treatments, the colour of frogs became more yellow-shifted and more saturated over time, evidenced by significant changes in hue and increases in chroma and luminance over the 32 week experimental period. These results indicate that southern corroboree frogs experience colour changes during post-metamorphic development, providing evidence for intrinsic ontogenetic colour change.

Our finding that β-carotene supplementation failed to change colouration was unexpected because a previous study by Umbers et al (2016) reported that corroboree frogs fed a diet supplement consisting of multiple carotenoid types (including β-carotene) exhibited significantly different colour (more saturated chroma and orange-shifted hue) compared to the control group. It is possible that β-carotene consumption has no, or very limited, influence

on corroboree frog colouration, and that the colour change reported by Umbers et al (2016) was caused by carotenoids other than β-carotene (e.g. lutein). Alternatively, it is possible that certain aspects of our methodology restricted our capacity to detect effects of β-carotene on colouration. Because β-carotene is a light sensitive molecule, the lighting conditions we imposed may have altered our findings. In the present study, frogs were exposed to UV (ultraviolet) radiation for nine hours per day, which was much higher than the exposure level provided by Umbers et al (2016) (one hour of UV radiation per day). This approach was taken to simulate UV radiation in the wild and to prevent frogs from developing metabolic bone disease, but it may have obfuscated potential benefits of β-carotene supplementation. Specifically, elevated UV levels may have either degraded β-carotene prior to consumption, making the supplement ineffective, or caused the destruction of pigment granules in the frogs' skin, restricting colour expression (McNett & Marchetti 2005; Surmacki 2008). At temperatures similar to those used in our experiment, complete UV degradation of β-carotene is expected to take several hours to several days (Scita 1992). As such, we consider it unlikely that UV damage completely nullified any positive effects of β-carotene on colouration because the supplement was only exposed to UV light for a few minutes prior to ingestion. Furthermore, as the colour of frogs did not decline over the experimental period, we expect that UV exposure did not degrade pigmentation within the skin.

An alternative reason why we did not observe an effect of β-carotene dose on colouration might be that the doses administered were incorrect. One possibility is that the tested doses were too high. Even the negligible concentrations of carotenoids provided in the control treatment (0.005 mg g^{-1}), may have been sufficient to saturate the corroboree frog system and enable colour production. In support of this argument, a large number of studies for other vertebrate species have failed to find significant relationships between colouration and carotenoid dose in dietary supplements (ranging from approximately 0.00007-0.25 mg g^{-1}, though as high as 291 mg g^{-1}), or circulating levels of carotenoids in the blood (Cantarero, Andrade, et al. 2020; Cantarero, Mateo, et al. 2020; Koch & Hill 2018; Koch et al. 2018; Koch et al. 2019; Mahler et al. 2003; McCoy et al. 2021; McGraw et al. 2003; Olson & Owens 2005; Powers & Hill 2021; Steffen et al. 2010; Weiss et al. 2012). As for many other vertebrate taxa, amphibians may require surprisingly low carotenoid amounts to develop colouration. An alternative explanation is that the doses administered were all too low. Two lines of evidence support this supposition. First, compared to our previous study where frogs received a mixed carotenoid at 20 mg g^{-1}, the colour of experimental frogs in the present

study (given β-carotene at1-3 mg g^{-1}) was less saturated (previous study = 0.78, present study = 0.50 - 0.51). Second, for a subset of frogs (n=32) that were recaptured approximately three and a half months post release, the colour of individuals spanning all treatment groups became significantly brighter and more orange shifted (unpublished data). This change may have resulted from the natural diet supplying either a higher concentration of β-carotene, or a different combination of carotenoids (for example lutein and others rather than beta-carotene). Moreover, colouration may have changed in response to an increased consumption of macronutrients. It is well established that more fat in the diet can aid in carotenoid absorption, while, more protein can influence light absorbing properties of carotenoids, and modify hue (Shawkey & D'Alba 2017). Clearly, further manipulative dietary studies spanning a broader range of carotenoid types, doses and dietary macronutrients will be needed to elucidate the extent to which variation in individual compounds influences corroboree frog colouration. However, before this work proceeds, a critical step will be to assay concentrations of carotenoids and macronutrients in the natural diet to ensure that the quantities of different carotenoids tested are ecologically relevant. Until this work has been done, any explanations for our lack of dose effect will remain speculative.

To better understand mechanisms underlying skin colour in corroboree frogs it would also be prudent to consider the possibility that colouration is the outcome of complex interactions between multiple pigment types. In many ectothermic vertebrates (including amphibians) colour is produced by a combination of carotenoids and pteridines; a distinct class of pigments synthesised de novo from carbohydrates and proteins (Braasch et al. 2007). Pteridines may be critically important for maintaining colour across different environments where carotenoid availability varies. For example, in guppies it has been demonstrated that geographic variation in carotenoid availability influences carotenoid content in skin ornaments, but that ratios of carotenoids to the primary pteridine (drosopterin) are conserved, yielding low variation in hue. This indicates that interactions between pigments are needed to maintain a specific hue (i.e. that pteridine synthesis balances carotenoid availability), and that these ratios have been targets of selection (Grether et al. 2005). Given that corroboree frogs are restricted to alpine habitats where the availability of invertebrate prey (and thus dietary carotenoids) fluctuates seasonally, the species might have experienced strong selection for colour production mechanisms involving both carotenoids and pteridines. Moving forward, to clarify the relative importance of pteridines versus carotenoids in the control of corroboree frog colouration, it will be necessary to biochemically characterise skin pigments, and

quantify the relationship between skin pigment composition and skin colour expression (Bonansea et al. 2017). However, given the critically endangered status of corroboree frogs, such research will need to occur opportunistically using animals in conservation breeding programs.

Another important finding from our study was that hue became more yellow shifted, and chroma and luminance significantly increased, producing a stronger colour signal over the experimental period. These results suggest that colour change is related to ontogenetic development in corroboree frogs. Mechanisms of ontogenetic colour development in amphibians have not been well documented (Beukema 2011), and few studies have examined the structure of the amphibian DCU throughout development (However, see Ide 1986; Yasutomi & Yamada 1998). Nevertheless, based on available information, it appears that the DCU is not fully formed until after metamorphosis (Mahalwar et al. 2014; Thibaudeau & Altig 2012), and that changes in colour during development are caused by the progressive production and accumulation of pigment within chromatophores (Matsui et al. 2002; Nilsson Sköld et al. 2013). As such, ontogenetic colour change in southern corroboree frogs most probably reflects the gradual differentiation and maturation of chromatophore cells, and possibly the production and accumulation of pteridine pigment granules. This progressive change would explain our finding that colour variation between treatment groups was greatest at the start of the experiment (week 0), and why at this time some treatments had higher mean chroma values. By chance, in certain treatment groups (such as T1), a subset of metamorphs may have had a slightly more developed DCU, elevating mean colour values. This may be explained, at least in part, by our metamorphs differing in age by four weeks. Despite these initial differences, frogs in all treatment groups converged on similar mean values within a period of two months (which manifested as a significant treatment by time interaction for chroma). Critically, this synchronised colour change indicates that corroboree frogs have experienced selection pressure to maximally express colour soon after metamorphosis.

Ontogenetic colour change in corroboree frogs may be indicative of a developmental switch in defensive strategies between life stages (Bulbert et al. 2018). Tadpoles develop in dark pools within peat bogs and are uniformly jet black in colour, which presumably conceals individuals from visually-hunting predators (for an example, see Davis et al. (2020)). The onset of yellow colouration begins in the days immediately prior to metamorphic climax, and based on our findings, colour is maximally expressed during early post-metamorphic development. This pattern is consistent with the development of colour in a diversity of

aposematic amphibian and insect species (Booth 1990; Grant 2007; Rudh & Qvarnström 2013), adding support to the notion that the yellow colouration of corroboree frogs functions as a warning signal. Theoretically, an evolutionary switch in defensive strategy from crypsis to aposematism can occur when prey species start to incur opportunity costs (such as lost foraging opportunities) by remaining cryptic (Speed & Ruxton 2005). Increasing costs are predicted to drive prey to invest in anti-predatory defences, which in turn allows them to exploit more rewarding habitats. In the process, prey become more behaviourally conspicuous, which is predicted to selectively favour the expression of aposematic signals (Grant 2007; Speed & Ruxton 2005). Our current knowledge of corroboree frog habitat use and movement behaviour fits this pattern. Frogs metamorphose in summer and after leaving natal pools they move into more open terrestrial habitats, where they forage intensively for several months before hibernating over winter. Frogs are known to forage diurnally (presumably because warmer daytime temperatures enable heightened activity and more efficient foraging in alpine habitats) and they rapidly increase in body size (which likely reflects a need to reach a threshold body size before hibernation to ensure survival). Such behavioural and morphological changes are expected to make frogs increasingly conspicuous to visually-hunting predators.

More broadly, our finding that experimental frogs showed maximal expression of yellow colouration at a similar point in development draws attention to the potential for developmental synchrony in the expression of colouration in aposematic species. Theoretically, aposematic colouration should be under strong stabilising selection, with conspecifics converging on similar colouration and patterning to strengthen avoidance learning by predators (Endler 1988; Wang & Shaffer 2008). Based on this reasoning, we might also expect limited variation in the onset of colouration, with colour expressed maximally at a similar point in development. Selection for synchronised expression of colour may be particularly strong in seasonal breeders where cohorts of offspring develop together, and large numbers of individuals become exposed to predators at a similar time. This possibility seems particularly likely if frogs have evolved to utilise very low doses of carotenoids to optimise colour expression. We propose that anurans offer excellent opportunities to test this idea because ontogenetic colour change has been reported in a diversity of species characterised by varying degrees of seasonality and colour change (Hoffman & Blouin 2000; Rudh & Qvarnström 2013; Stangel et al. 2015). In species with compressed breeding seasons, metamorphic climax is usually highly synchronised, with mass

emergence of metamorphs from breeding ponds triggered by abiotic cues, such as pond drying (Wells 2010). Synchronised expression of colouration could strengthen the intensity of the aposematic signal and perceived unprofitability, facilitating predator learning and the effectiveness of aposematism.

In conclusion, the aim of this study was to investigate the influence of dietary β-carotene on the putative aposematic colouration of corroboree frogs. Varying β-carotene supplementation had no detectable effect on hue, chroma or luminance over the course of frog maturation. This indicates that β-carotene did not influence colouration at the doses tested. Another significant finding was that frogs acquired colour progressively during development, providing evidence for ontogenetic colour change. Maximal expression of colour was synchronised in the months following metamorphosis, and we argue that this reflects changes in conspicuousness to predators linked to a rapid increase in body size and heightened foraging activity in terrestrial habitats post metamorphosis. Our study highlights the potential for anurans to be used as model systems to investigate the adaptive significance of ontogenetic colour change, and the selective drivers underpinning the evolution of colour based anti-predatory strategies.

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COMPETING INTERESTS

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

P.B. and A.S. conceived and designed the study. S.W., P.B. and A.S. collected the data. J.E., S.W. and P.B. processed, analysed and interpreted the data. The manuscript was written by S.W. and P.B. with input and revision from A.S. and J.E.

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Data

DATA AVAILABILITY STATEMENT

Data including MatLab code deposited on dryad: https://doi.org/10.5061/dryad.2jm63xsqc

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Figures

Figure 1. Arrangement of the internal photo staging area. Shown is the placement of the XRite ColorChecker, identification tag, and a southern Corroboree frog, Pseudophryne corroboree.

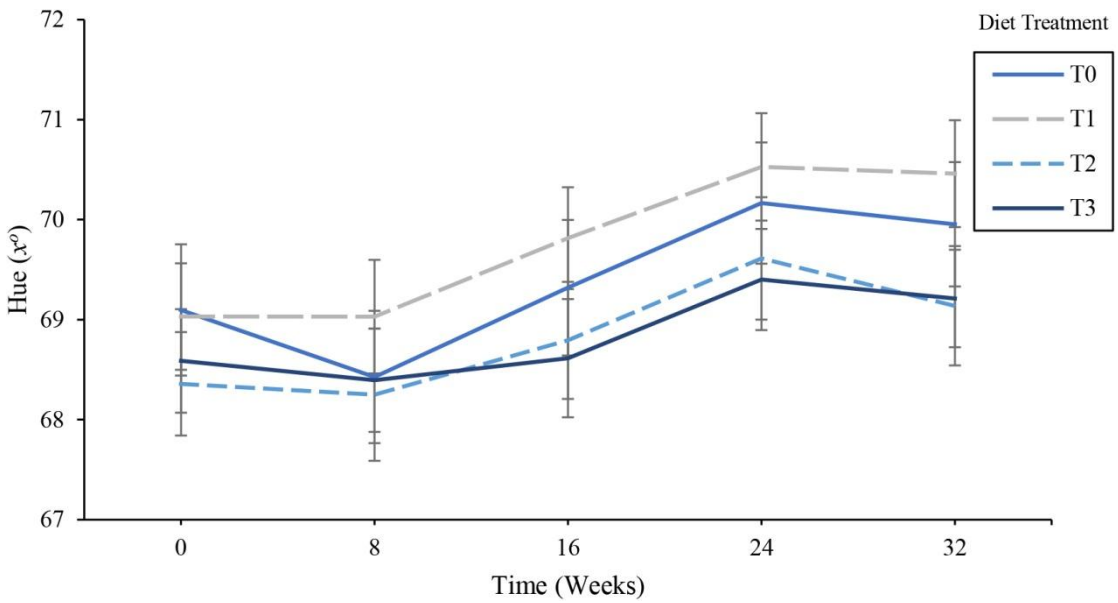

Figure 2. Effect of dietary β-carotene supplementation on hue over the 32 week experimental period. Data shown are untransformed mean ± S.E. Sample sizes for each diet treatment were as follows: T0 (0 mg g^{-1}), n = 30; T1 (1 mg g^{-1}), n = 31; T2 (2 mg g^{-1}), n = 29; T3 (3 mg g^{-1}), n = 26.

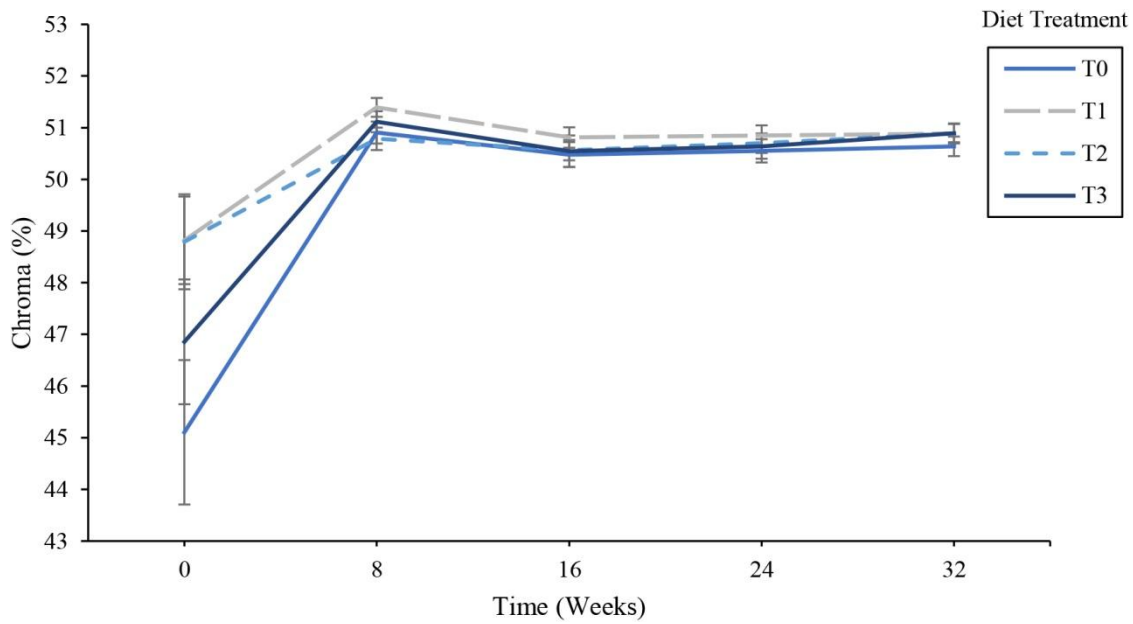

Figure 3. Effect of dietary β-carotene supplementation on chroma over the 32 week experimental period. Data shown are transformed ($\sin^{-1}(\sqrt{x})$; x = chroma) mean ± S.E. Sample sizes for each diet treatment were as follows: T0 (0 mg g^{-1}), n = 30; T1 (1 mg g^{-1}), n = 31; T2 (2 mg g^{-1}), n = 29; T3 (3 mg g^{-1}), n = 26.

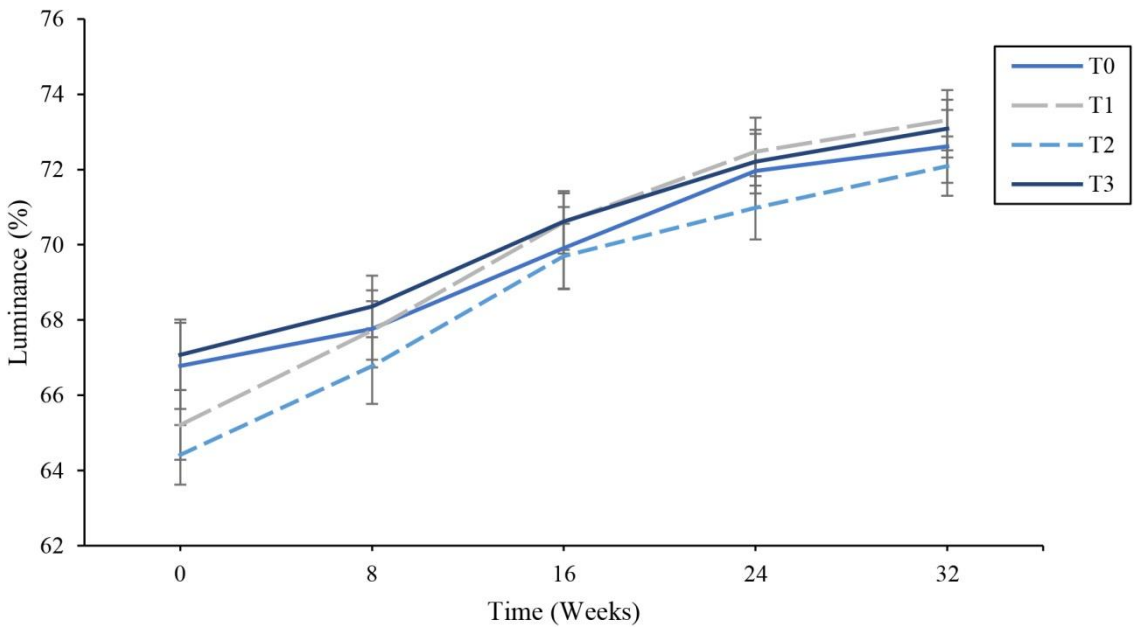

Figure 4. Effect of dietary β-carotene supplementation on luminance over the 32 week experimental period. Data shown are untransformed mean ± S.E. Sample sizes for each diet treatment were as follows: T0 (0 mg g^{-1}), n = 30; T1 (1 mg g^{-1}), n = 31; T2 (2 mg g^{-1}), n = 29; T3 (3 mg g^{-1}), n = 26.

Table 1. Composition of experimental diets fed to *Pseudophryne corroboree* metamorphs (n = 34 for each treatment group). Diet supplements were dusted onto approximately 15g of crickets per treatment, per feed.

Diet Treatment	β-carotene mass (g)	β-carotene concentration per g of feed (mg g^{-1})	Cellulose mass (g)	Calcium mass (g)	Total supplement mass (g)
T0	0.000	0	0.045	0.250	0.295
T1	0.015	1	0.030	0.250	0.295
T2	0.030	2	0.015	0.250	0.295
T3	0.045	3	0.000	0.250	0.295

Appendix S1. MATLAB script used for the conversion of RBG into HSL colour values. Note that this requires actual reflectance values for the six grayscale squares in the Xrite color checker; you will need to replace the rfl values with those of your own Xrite. This function reads a csv file with columns: directory, photo, patch, R, G, B, total reflectance. It creates a new output file with the results in the same number of rows as the input file.

This file contains several functions, where CalibrateRGBandSave calls the others, so everything in this document should be placed in an .m file, call it CalibrateRGBandSave.m When run it will ask for the input .csv file name.

```
function CalibrateRGBandSave;
%get fitting functions for each RGB value in each standard photograph
%assumes RGBs for grayscale standards in each standard photo in
RGBstandards.txt
rfl=zeros(6,1);
rfl(1)=0.0310; rfl(2)=0.0910; rfl(3)=0.1950;
rfl(4)=0.3720; rfl(5)=0.6090; rfl(6)=0.9480; %actual values for Xrite
grayscale (from Jair)

% rfl=log(rfl); %

%VARIABLES
*********************************************************************
% rfl(6)      Actual log reflectances of each grayscale square in
ColorChecker
%*********************************************************************
*******

[fname,path]=uigetfile('*.csv','Select a csv photo data file');
fn=[path fname];
fid=fopen(fn);
rd=textscan(fid,'%s %s %s %s %s %s %s %s',1,'delimiter',',');
heads=[rd{1:end}];
rd=textscan(fid,'%s %s %s %f %f %f %f %s','delimiter',',');
fclose(fid);
heads=[heads 'Chroma' 'HueAngle'];

pdir=rd{1}; photo=rd{2}; patch=rd{3}; Red=rd{4}; Grn=rd{5}; Blu=rd{6};
Brt=rd{7};
Area=rd{8};
n=length(Red); clear fid rd;
%set up output file
k=strfind(fn,'.csv'); if isempty(k) k=0; end; k=k-1;
oname=[fn(1:k) 'Calibrated.csv'];
ofid=fopen(oname,'wt');
k=length(heads); fprintf(ofid,'%s',heads{1});
for i=2:k fprintf(ofid,',%s',heads{i}); end;
fprintf(ofid,'\n');

%identify each photo in order to extact standards and calibrate RGB
phlist=unique(photo); np=length(phlist);
phid=zeros(np,1);
```

```
for i=1:n
  str=photo{i};
  phid(i)=find(strcmp(phlist,str));
end; clear i str;
%do each photo
for p=1:np  %photo number p
  pdr=pdir(phid==p); pho=photo(phid==p); pch=patch(phid==p);
ars=Area(phid==p);
  R=Red(phid==p); G=Grn(phid==p); B=Blu(phid==p); BT=Brt(phid==p);
nd=length(BT);
  %1-24 are the standards  25:nd are the bird samples
  %1 is black 6 is white, corresponding to refl(1:6);
  rbd=R(25:nd); gbd=G(25:nd); bbd=B(25:nd); btbd=BT(25:nd); %bird
measurements
  pdbd=pdr(25:nd); phbd=pho(25:nd); ptchbd=pch(25:nd); arbd=ars(25:nd);
%bird metadata
  rst=R(1:6); gst=G(1:6); bst=B(1:6); btst=BT(1:6);         %gray
standards
%  %plot raw data actual on x axis
%  figure;
%  plot(rfl,rst,'r',rfl,rst,'or'); hold on;
%  plot(rfl,gst,'g',rfl,gst,'og');
%  plot(rfl,bst,'b',rfl,bst,'ob');
%  plot(rfl,btst,'k--',rfl,btst,'ok'); hold off; %log shape, log(rfl) not
quite linear
%  xlabel('actual reflectance'); ylabel('R,G,B,BT'); hold on;

 %get function to correct RGB to white (R=G=B), first fit lines to all
points
  x=(0.03:0.005:1)';
  [xData,yData]=prepareCurveData(rfl,rst); ft=fittype('exp2');
  opts=fitoptions('Method','NonlinearLeastSquares');
  opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
  fitted=fit(xData,yData,ft,opts); yr=feval(fitted,x);
  [xData,yData]=prepareCurveData(rfl,gst); ft=fittype('exp2');
  opts=fitoptions('Method','NonlinearLeastSquares');
  opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
  fitted=fit(xData,yData,ft,opts); yg=feval(fitted,x);
  [xData,yData]=prepareCurveData(rfl,bst); ft=fittype('exp2');
  opts=fitoptions('Method','NonlinearLeastSquares');
  opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
  fitted=fit(xData,yData,ft,opts); yb=feval(fitted,x);
  [xData,yData]=prepareCurveData(rfl,btst); ft=fittype('exp2');
  opts=fitoptions('Method','NonlinearLeastSquares');
  opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
  fitted=fit(xData,yData,ft,opts); ybt=feval(fitted,x);
  clear xData Ydata opts fitted ft;
  % yr,yg,yb,ybt are the continuous best fits to the grayscale data
%     figure;
%     plot(rfl,rst,'or',rfl,gst,'og',rfl,bst,'ob',rfl,btst,'ok'); hold
on;
%     plot(x,yr,'r',x,yg,'g',x,yb,'b',x,ybt,'k'); xlabel('actual total
reflectance');
```

```
%     ylabel('R,G,B,Brt'); title('fitted data for irradiance
correction');
  %excange x y and get fits from RGB to actual reflectance
  [xData,yData]=prepareCurveData(yr,x); ft=fittype('exp2');
  opts=fitoptions('Method','NonlinearLeastSquares');
  opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
  eqR=fit(xData,yData,ft,opts);
  [xData,yData]=prepareCurveData(yg,x); ft=fittype('exp2');
  opts=fitoptions('Method','NonlinearLeastSquares');
  opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
  eqG=fit(xData,yData,ft,opts);
  [xData,yData]=prepareCurveData(yb,x); ft=fittype('exp2');
  opts=fitoptions('Method','NonlinearLeastSquares');
  opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
  eqB=fit(xData,yData,ft,opts);
  [xData,yData]=prepareCurveData(ybt,x); ft=fittype('exp2');
  opts=fitoptions('Method','NonlinearLeastSquares');
  opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
  eqBt=fit(xData,yData,ft,opts);
%     figure; plot(yr,x,'r',yg,x,'g',yb,x,'b',ybt,x,'k:'); hold on;
%     xlabel('R,G,B,Bt'); ylabel('reflectance+light');
%     title('Fitted data to convert to light+reflectance');
%     yR=feval(eqR,rst); plot(rst,yR,'or'); yG=feval(eqG,rst);
plot(rst,yG,'og');
%     yB=feval(eqB,rst); plot(rst,yB,'ob'); yBt=feval(eqBt,rst);
plot(rst,yBt,'ok');
  %convert rgb for birds to reflectance+light
  % rbd gbd bbd btbd bird measurements,  heads has original headings--use
again
  % pdbd directory, phbd photo, ptchbd patch name, arbd area on bird
%bird metadata
  r=feval(eqR,rbd); g=feval(eqG,gbd); b=feval(eqB,bbd);
bt=feval(eqBt,btbd);
  nb=length(r); %number of bird samples
  %use rst,gst,bst,btst(4) to get white balance coefficients
  wh=[rst(4) gst(4) bst(4) btst(4)]; whc=wh/wh(4); %like the von Kries!
  cal=zeros(nb,4); %corrected for both reflectance and white balance
(illumination)
  for s=1:nb
    bd=[r(s) g(s) b(s) bt(s)]./whc;
    cal(s,:)=bd;
  end;
  for s=1:nb
    fprintf(ofid,'%s,%s,%s',pdbd{s},phbd{s},ptchbd{s});
    for k=1:4 fprintf(ofid,',%6.4f',cal(s,k)); end;
    fprintf(ofid,',%s',arbd{s});
    rc=cal(s,1); gc=cal(s,2); bc=cal(s,3); t=rc+gc+bc;
    rc=rc/t; gc=gc/t; bc=bc/t;
    [hue,chr]=TriToHSV(rc,gc,bc);
    fprintf(ofid,',%6.4f,%6.2f\n',chr,hue);
  end;
  fprintf(1,'Finished photo %s\n',phbd{1});
end; %photo number p
fclose(ofid);
```

```
fprintf(1,'Finished file %s \n   in directory %s\n',fname,path);
end

function [hue,chr]=TriToHSV(R,G,B)
% [hue,chr]=TriToHSV(R,G,B);
%get HSV from calibrated RGB in Triangle
%INPUT   R,G,B  calibrated R G and B values as column vectors
%OUTPUT  hue    hue angle R at -120, G at 0, B at +120 degrees
%        chr    chroma (maximum possible 1.0)
%
[xa,ya]=ToTriangle([1 0 0]); %vertex for maximum chroma 0.5774
[xw,yw]=ToTriangle([1/3 1/3 1/3]); %  0.5000    0.2887
[xc,yc]=ToTriangle([R G B]);
xa=xa-xw; ya=ya-yw;
xc=xc-xw; yc=yc-yw; %make gray pont at 0,0
[h,c]=cart2pol(ya,xa); %max chroma value 0.5774
[hue,chr]=cart2pol(yc,xc);
hue=(180/pi)*hue;  chr=chr/c; %red at -120, green at 0, blue at +120
degrees
end

function [tx,ty]=ToTriangle(xyz)
% [tx,ty]=ToTriangle(xyz)
%    converts data matrix xyz=[x y z] of x,y,z coordinates
%    to triangular coordinates tx,ty (both column vectors)
%    with sides=1, height sqrt(3)/2
% Will temporarily make xyz rows sum to 1
% Coordinates of triangle are trix=[0 1 0.5 0]; triy=[0 0 sqrt(3)/2 0];
% Same as plottri but only saves triangular coordinates
[N,C]=size(xyz); ht=sqrt(3)/2;
su=sum(xyz')'; for i=1:N xyz(i,:)=xyz(i,:)/su(i); end; %row sums=1
sx=(sqrt(3)/2)*xyz; % sx=xyz to make height=1 instead of sides
for i=1:N tx(i)=(sx(i,2)+2*sx(i,3))/sqrt(3); ty(i)=sx(i,2); end;
tx=tx'; ty=ty'; %convert to column vectors
end


function DigitizeFrogsIntactXriteCR2files
%  DigitizeFrogsIntactXriteCR2files;
%digitize frog pictures, intact XRITE standard
%this version for CR2 files
% epm509@uowmail.edu.au   srk649@uowmail.edu.au
clear;

head='fmeanR,fmeanG,fmeanR,fmeanTot,fsdR,fsdG,fsdB,fsdTot';
head=[head ',hmeanR,hmeanG,hmeanR,hmeanTot,hsdR,hsdG,hsdB,hsdTot'];
head=[head ',tmeanR,tmeanG,tmeanR,tmeanTot,tsdR,tsdG,tsdB,tsdTot'];
head=[head ',vmeanR,vmeanG,vmeanR,vmeanTot,vsdR,vsdG,vsdB,vsdTot,Photo'];

[dirs,nds]=GetDIRList('./');
[s,v]=listdlg('ListString',dirs,'SelectionMode','single',...
    'PromptString','Select directory with photo files','Name','Select
Directory',...
    'ListSize',[200 (20*nds)]);
 dname=dirs{s}; dr=['./' dname '/'];

 oname=['YellowPatchData' dname '.csv'];
 fid=fopen(oname);
```

```
 if fid<0 new=1; else new=0; fclose(fid); end;
 if new==1
   fid=fopen(oname,'wt');  %create new file for output data
   fprintf(fid,'Calibrated RGB and total reflectance estimates for frog,
head(h), torso(t), vent(v) in directory "%s"\n',dr);
   fprintf(fid,'%s\n',head);
 else
   fid=fopen(oname,'at');  %append to existing file if present
 end;

 [files,nf]=GetFileList(dr,'.CR2');
 if nf <1
   fprintf(1,'No .CR2 files found in directory %s\n',dname);
   fclose('all');
   stop;
 end;

 v=1;
 while v>0
  [s,v]=listdlg('ListString',files,'SelectionMode','single',...
    'PromptString',{'Select photo file to use','Cancel to end pgm'},...
    'Name','Select Photo');
  close all;
  if v>0
    fname=files{s}; fprintf(1,'doing %s\n',fname);
    fn=[dr fname];
    img=imread(fn);
    [rows,cols,d]=size(img); if rows>cols img=imrotate(img,270); end;
    [rows,cols,d]=size(img);
    img=flipud(img); %CR2 files flipped up and down
    imshow(img,'initialmagnification','fit');

    ch=questdlg('Is grayscale on right edge of Xrite','Image
mirrored?','Yes','No','Yes'); %last one default
    switch ch  %some cr2 files dark, rescale for outlining only
     case 'Yes'
        dark=0;
     case 'No'
        img=fliplr(img);
    end;
    imshow(img,'initialmagnification','fit');

    ch=questdlg('Is image upside-down','Image
mirrored?','Yes','No','No'); %last one default
    switch ch  %some cr2 files dark, rescale for outlining only
     case 'Yes'
        img=flipud(img);
     case 'No'
        dark=0;
    end;
    imshow(img,'initialmagnification','fit');

    dark=0;
    ch=questdlg('Is this image dark?','Image Dark?','Yes','No','No');
%last one default
    switch ch  %some cr2 files dark, rescale for outlining only
     case 'Yes'
        simg=double(img);
```

```
        simg(img>128)=128; simg=2.2*simg; simg=uint8(simg);
        dark=1;
     case 'No'
        simg=img;
        dark=0;
    end;

    figure(1); set(gcf,'Position',[816 270 1102 860]);
    imshow(simg,'initialmagnification','fit');
    drawnow;

    msg='CLICK carefully ON 4 WHITE CORNER MARKS, adjust, then double-
click on corner';
    hold on;  drawnow;
    title(msg,'FontSize',16,'FontWeight','bold');
    drawnow;
    [mask,mx,my]=roipoly(simg); hold on; plot(mx,my,'w');
    x1=mx(1); x2=mx(2); y1=my(1); y2=my(2);
    dst=sqrt((x1-x2)^2 + (y1-y2)^2);
    scale=dst/60;  %pixels/mm, 60mm between corners at narrower dimension
    clear x1 x2 y1 y2 dst;

    msg='CLICK and drag to CROP around frog, then double-click on last
corner';
    title(msg,'FontSize',16,'FontWeight','bold');
    drawnow;
    frogimg=imcrop(img);
  %   figure; imshow(frogimg);
  %   msg='CLICK around edge of frog''s body, then double-click on first
point';
  %   title(msg,'FontSize',16,'FontWeight','bold');
  %   [frmask,xx,yy]=roipoly(frogimg); hold on; plot(xx,yy,'w'); clear xx
yy;
  %   title('Body pattern outlined','FontSize',16,'FontWeight','bold');
drawnow;
  %   figure(1);
    title('Getting standard RGBs & frog patches, takes a few seconds',...
        'FontSize',16,'FontWeight','bold'); drawnow;
    %find angle to make mx,my aligned 0 and 90 degrees
    x=mx(2)-mx(1); y=my(2)-my(1); [rot,R] = cart2pol(x,y);
    %temporary rotation of outline
    [pcx,pcy]=polycenter(mx,my); tmx=mx-pcx; tmy=my-pcy; %center on 0,0
    [ang,dst]=cart2pol(tmx,tmy); [tx,ty]=pol2cart((ang-rot),dst);
    [X,Y]=meshgrid(1:4,1:6);
    xrange=abs(max(tx)-min(tx)); yrange=abs(max(ty)-min(ty));
    xin=xrange/4; yin=yrange/6; xs=min(tx)+xrange/8;
ys=min(ty)+yrange/12;
    x=(xs-xin)+X*xin; y=(ys-yin)+Y*yin;
    %rotate back
    [ang,dst]=cart2pol(x,y); [px,py]=pol2cart((ang+rot),dst);
    px=px+pcx; py=py+pcy;
    clear x y rot R pcx pcy tmx tmy ang dst tx ty X Y xrange yrange xs
ys;
    cx=px; cy=py; % plot(cx,cy,'ok'); %cx,cy contain square centres
    xr=xin/3.4; yr=yin/3.4; px=zeros(5,1); py=px;
    masks=cell(24,1);
    for c=1:24
      x=cx(c); y=cy(c);
```

```
      px(1)=x-xr; py(1)=y-yr; px(2)=x+xr; py(2)=y-yr;
      px(3)=x+xr; py(3)=y+yr; px(4)=x-xr; py(4)=y+yr; px(5)=px(1);
py(5)=py(1);
      plot(px,py,'g'); text(x,y,num2str(c));
      %save each mask and extract r,g,b,gry from each
      msk=roipoly(img,px,py); masks{c}=msk;
    end;
    clear x y px py msk  ;
    r=zeros(rows,cols); g=r; b=r;
    r=img(:,:,1); g=img(:,:,2); b=img(:,:,3); gry=rgb2gray(img);
    rgbg=zeros(24,4);
    for c=1:24
      msk=masks{c}; stn=num2str(c);
      mr=mean(r(msk==1)); mg=mean(g(msk==1)); mb=mean(b(msk==1));
      mgy=mean(gry(msk==1));
      rgbg(c,:)=[mr mg mb mgy];
    end;
    clear r g b c* d masks msk stn mr mg mb mgy mx my cols rows x* y* msg
gry mask simg;
    title(['doing frog in '
fname],'FontSize',16,'FontWeight','bold','interpreter','none');
    drawnow;
    %VARIABLES
*********************************************************************
    %  fn      file name
    %  scale   %pixels/mm, derived from 50mm in scale
    %  img     original colour image
    %  rgbg(24,4) mean RGB and Gray values for the 24 colour & gray
standards
    %  frogimg frog colour image, cropped for greater efficiency

%*********************************************************************
*********
    %in order to threshold frogimage, need to rescale
    test=double(frogimg);
    if dark>0
       test(test>125)=125; test=uint8(4*test);  %dark images
    else
       test=uint8(2*test);                      %light images
    end;
    % imshow(test);  %this is only for thresholding, not for RGB values


%*********************************************************************
*******
    figure; imshow(test); hold on;
    r=test(:,:,1); g=test(:,:,2); b=test(:,:,3); [rw,cl]=size(r);
    h=text(50,50,'<CR> then draw around head'); drawnow; pause;
    delete(h); [maskhead,pc1,pr1]=roipoly(test); plot(pc1,pr1,'b--');
    testhead=uint8(zeros(rw,cl,3));
    rm=r; rm(maskhead==0)=255; gm=g; gm(maskhead==0)=255; bm=b;
bm(maskhead==0)=255;
    testhead(:,:,1)=rm; testhead(:,:,2)=gm; testhead(:,:,3)=bm;

    h2=text(50,50,'<CR> then draw around torso'); drawnow; pause
    delete(h2); [masktorso,pc2,pr2]=roipoly(test); plot(pc2,pr2,'b--');
    testtorso=uint8(zeros(rw,cl,3));
```

```
    rm=r; rm(masktorso==0)=255; gm=g; gm(masktorso==0)=255; bm=b;
bm(masktorso==0)=255;
    testtorso(:,:,1)=rm; testtorso(:,:,2)=gm; testtorso(:,:,3)=bm;
    delete(h);

    h3=text(50,50,'<CR> then draw around vent'); drawnow; pause
    delete(h3); [maskvent,pc,pr]=roipoly(test);
    plot(pc1,pr1,'r--',pc2,pr2,'g--',pc,pr,'b--');
    testvent=uint8(zeros(rw,cl,3));
    rm=r; rm(maskvent==0)=255; gm=g; gm(maskvent==0)=255; bm=b;
bm(maskvent==0)=255;
    testvent(:,:,1)=rm; testvent(:,:,2)=gm; testvent(:,:,3)=bm;
    clear mask* rm gm bm; delete(h);
    % testhead,testtorso,testvent contain 3 parts, test contains all
    h4=text(50,50,'<CR> to continue'); drawnow; pause;
    delete(h4);


%*************************************************************************
********
    %do entire frog image
    r=test(:,:,1); g=test(:,:,2); b=test(:,:,3);
    df=imlincomb(0.5,r,0.5,g,-1,b); bw=im2bw(df,0.2); % figure(2);
imshow(bw);
    cc=bwconncomp(bw);
    if dark>0
      crts=40;
    else
      crts=20;
    end;
    stats=regionprops(cc,'Area'); idx=find([stats.Area] > crts);
    bw2=ismember(labelmatrix(cc),idx);
    %  bdy=bw; bdy(frmask==0)=0;  %restrict to outlined parts
    if dark>0
      se=strel('disk',6);
    else
      se=strel('disk',2);     %erode to avoid boundaries
    end;
    bw2=imerode(bw2,se,12); % figure; imshow(bw2);
    yellow=bw2; %contains pixels to be measured (yellow to orange spots)
    % figure; imshow(yellow)
    fr=test(:,:,1); fg=test(:,:,2); fb=test(:,:,3);
    fr(yellow==1)=0; fg(yellow==1)=0; fb(yellow==1)=150;
    shw=test; shw(:,:,1)=fr; shw(:,:,2)=fg; shw(:,:,3)=fb;
   figure(2); set(gcf,'Position',[842 292 1068 834]);
subplot(1,2,1); imshow(test); subplot(1,2,2); imshow(shw);
    title([fname ', blue=measured'],'interpreter','none',...
        'FontSize',16,'FontWeight','bold');
    clear x1 x2 y1 y2 slp ic r g b df bw bw2 stats fr fg fb tle tls idx
cc shw;
    %VARIABLES
*********************************************************************
    %  fn      file name
    %  scale   %pixels/mm, derived from 50mm in scale
    %  img     original colour image
    %  rgbg(24,4) mean RGB and Gray values for the 24 colour & gray
standards
```

```
    %  frogimg(rows,cols,3) frog colour image, cropped for greater
efficiency
    %  yellow(rows,cols)    mask: white for yellow blotches away from
edges

%*********************************************************************
********

    % testhead,testtorso,testvent contain 3 parts, test contains all

%*********************************************************************
********
    %do head image
    r=testhead(:,:,1); g=testhead(:,:,2); b=testhead(:,:,3);
    df=imlincomb(0.5,r,0.5,g,-1,b); bw=im2bw(df,0.2); % figure(2);
imshow(bw);
    cc=bwconncomp(bw);
    if dark>0
      crts=40;
    else
      crts=20;
    end;
    stats=regionprops(cc,'Area'); idx=find([stats.Area] > crts);
    bw2=ismember(labelmatrix(cc),idx);
    %  bdy=bw; bdy(frmask==0)=0;  %restrict to outlined parts
    if dark>0
      se=strel('disk',6);
    else
      se=strel('disk',2);     %erode to avoid boundaries
    end;
    bw2=imerode(bw2,se,12); % figure; imshow(bw2);
    yellowhead=bw2; %contains pixels to be measured (yellow to orange
spots)
    % figure; imshow(yellowhead)
    clear x1 x2 y1 y2 slp ic r g b df bw bw2 stats fr fg fb tle tls idx
cc shw;
    %VARIABLES
*******************************************************************
    %  fn      file name
    %  scale   %pixels/mm, derived from 50mm in scale
    %  img     original colour image
    %  rgbg(24,4) mean RGB and Gray values for the 24 colour & gray
standards
    %  frogimg(rows,cols,3) frog colour image, cropped for greater
efficiency
    %  yellow(rows,cols)     mask: white for yellow blotches away from
edges
    %  yellowhead(rows,cols) same for head region

%*********************************************************************
********

    % testhead,testtorso,testvent contain 3 parts, test contains all

%*********************************************************************
********
    %do torso image
    r=testtorso(:,:,1); g=testtorso(:,:,2); b=testtorso(:,:,3);
```

```
    df=imlincomb(0.5,r,0.5,g,-1,b); bw=im2bw(df,0.2); % figure(2);
imshow(bw);
    cc=bwconncomp(bw);
    if dark>0
      crts=40;
    else
      crts=20;
    end;
    stats=regionprops(cc,'Area'); idx=find([stats.Area] > crts);
    bw2=ismember(labelmatrix(cc),idx);
    %  bdy=bw; bdy(frmask==0)=0;  %restrict to outlined parts
    if dark>0
      se=strel('disk',6);
    else
      se=strel('disk',2);     %erode to avoid boundaries
    end;
    bw2=imerode(bw2,se,12); % figure; imshow(bw2);
    yellowtorso=bw2; %contains pixels to be measured (yellow to orange
spots)
    % figure; imshow(yellowtorso)
    clear x1 x2 y1 y2 slp ic r g b df bw bw2 stats fr fg fb tle tls idx
cc shw;
    %VARIABLES
*********************************************************************
    %  fn      file name
    %  scale   %pixels/mm, derived from 50mm in scale
    %  img     original colour image
    %  rgbg(24,4) mean RGB and Gray values for the 24 colour & gray
standards
    %  frogimg(rows,cols,3) frog colour image, cropped for greater
efficiency
    %  yellow(rows,cols)      mask: white for yellow blotches away from
edges
    %  yellowhead(rows,cols)  same for head region
    %  yellowtorso(rows,cols) same for torso region

%*********************************************************************
********

    % testhead,testtorso,testvent contain 3 parts, test contains all

%*********************************************************************
********
    %do vent image
    r=testvent(:,:,1); g=testvent(:,:,2); b=testvent(:,:,3);
    df=imlincomb(0.5,r,0.5,g,-1,b); bw=im2bw(df,0.2); % figure(2);
imshow(bw);
    cc=bwconncomp(bw);
    if dark>0
      crts=40;
    else
      crts=20;
    end;
    stats=regionprops(cc,'Area'); idx=find([stats.Area] > crts);
    bw2=ismember(labelmatrix(cc),idx);
    %  bdy=bw; bdy(frmask==0)=0;  %restrict to outlined parts
    if dark>0
      se=strel('disk',6);
```

```
    else
      se=strel('disk',2);     %erode to avoid boundaries
    end;
    bw2=imerode(bw2,se,12); % figure; imshow(bw2);
    yellowvent=bw2; %contains pixels to be measured (yellow to orange
spots)
    % figure; imshow(yellowtorso)
    clear x1 x2 y1 y2 slp ic r g b df bw bw2 stats fr fg fb tle tls idx
cc shw;
    %VARIABLES
*********************************************************************
    %  fn      file name
    %  scale   %pixels/mm, derived from 50mm in scale
    %  img     original colour image
    %  rgbg(24,4) mean RGB and Gray values for the 24 colour & gray
standards
    %  frogimg(rows,cols,3) frog colour image, cropped for greater
efficiency
    %  yellow(rows,cols)      mask: white for yellow blotches away from
edges
    %  yellowhead(rows,cols)  same for head region
    %  yellowtorso(rows,cols) same for torso region
    %  yellowvent(rows,cols) same for torso region

%*********************************************************************
********
%
%     figure;
%     subplot(2,2,1); imshow(yellow);
%     subplot(2,2,2); imshow(yellowhead);
%     subplot(2,2,3); imshow(yellowtorso);
%     subplot(2,2,4); imshow(yellowvent);
%

    % figure; imshow(yellow);
    frogimg=double(frogimg);
    fr=frogimg(:,:,1); fg=frogimg(:,:,2); fb=frogimg(:,:,3);
    frf=fr(yellow==1);  fgf=fg(yellow==1);  fbf=fb(yellow==1);
%entire frog
    frh=fr(yellowhead==1);  fgh=fg(yellowhead==1);
fbh=fb(yellowhead==1);  %head
    frt=fr(yellowtorso==1); fgt=fg(yellowtorso==1);
fbt=fb(yellowtorso==1); %torso
    frv=fr(yellowvent==1);  fgv=fg(yellowvent==1);
fbv=fb(yellowvent==1);  %vent

    clear yellow img;
    %VARIABLES
*********************************************************************
    %  fn      file name
    %  scale   %pixels/mm, derived from 50mm in scale
    %  rgbg(24,4) mean RGB and Gray values for the 24 colour & gray
standards
    %  frogimg(rows,cols,3) frog colour image, cropped for greater
efficiency
    %  frf,fgf,fbf  RGB values for all yellow stripe pixels in frog
    %  frh,fgh,fbh  RGB values for all yellow stripe pixels in frog's
head
```

```
    %  frt,fgt,fbt  RGB values for all yellow stripe pixels in frog's
torso
    %  frv,fgv,fbv  RGB values for all yellow stripe pixels in frog's
vent
    %    to view frogimg imshow(uint8(frogimg))

%*********************************************************************
*********
    rfl=zeros(6,1);  %gray standards
    rfl(1)=0.0310; rfl(2)=0.0910; rfl(3)=0.1950;
    rfl(4)=0.3720; rfl(5)=0.6090; rfl(6)=0.9480; %actual values for Xrite
grayscale
    rst=rgbg(19:24,1); gst=rgbg(19:24,2); bst=rgbg(19:24,3);
btst=rgbg(19:24,1);
  %   %plot raw data vs actual
  %   figure;
  %   plot(rfl,rst,'r',rfl,rst,'or'); hold on;
  %   plot(rfl,gst,'g',rfl,gst,'og');
  %   plot(rfl,bst,'b',rfl,bst,'ob');
  %   plot(rfl,btst,'k--',rfl,btst,'ok'); hold off; %log shape, log(rfl)
not quite linear
  %   xlabel('actual reflectance'); ylabel('R,G,B,BT');
    %get calibration
    %get function to correct RGB to white (R=G=B), first fit lines to all
points
    x=(0.03:0.005:1)';
    [xData,yData]=prepareCurveData(rfl,rst); ft=fittype('exp2');
    opts=fitoptions('Method','NonlinearLeastSquares');
    opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
    fitted=fit(xData,yData,ft,opts); yr=feval(fitted,x);
    [xData,yData]=prepareCurveData(rfl,gst); ft=fittype('exp2');
    opts=fitoptions('Method','NonlinearLeastSquares');
    opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
    fitted=fit(xData,yData,ft,opts); yg=feval(fitted,x);
    [xData,yData]=prepareCurveData(rfl,bst); ft=fittype('exp2');
    opts=fitoptions('Method','NonlinearLeastSquares');
    opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
    fitted=fit(xData,yData,ft,opts); yb=feval(fitted,x);
    clear xData ydata opts fitted ft;
    % yr,yg,yb are the continuous best fits to the grayscale data
  %   figure;
  %   plot(rfl,rst,'or',rfl,gst,'og',rfl,bst,'ob'); hold on;
  %   plot(x,yr,'r',x,yg,'g',x,yb,'b'); xlabel('actual total
reflectance');
  %   ylabel('R,G,B,Brt'); title('fitted data for irradiance
correction');
    % excange x y and get fits from RGB to actual reflectance
    [xData,yData]=prepareCurveData(yr,x); ft=fittype('exp2');
    opts=fitoptions('Method','NonlinearLeastSquares');
    opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
    eqR=fit(xData,yData,ft,opts);
    [xData,yData]=prepareCurveData(yg,x); ft=fittype('exp2');
    opts=fitoptions('Method','NonlinearLeastSquares');
```

```
    opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
    eqG=fit(xData,yData,ft,opts);
    [xData,yData]=prepareCurveData(yb,x); ft=fittype('exp2');
    opts=fitoptions('Method','NonlinearLeastSquares');
    opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
    eqB=fit(xData,yData,ft,opts);
    clear xData ydata opts fitted ft;
  %     figure; plot(yr,x,'r',yg,x,'g',yb,x,'b'); hold on;
  %     xlabel('R,G,B,Bt'); ylabel('reflectance+light');
  %     title('Fitted data to convert to light+reflectance');
  %     yR=feval(eqR,rst); plot(rst,yR,'or'); yG=feval(eqG,rst);
plot(rst,yG,'og');
  %     yB=feval(eqB,rst); plot(rst,yB,'ob');

    %convert to estimated reflectance in each channel and luminance
    %  frf,fgf,fbf  RGB values for all yellow stripe pixels in frog
    %  frh,fgh,fbh  RGB values for all yellow stripe pixels in frog's
head
    %  frt,fgt,fbt  RGB values for all yellow stripe pixels in frog's
torso
    %  frv,fgv,fbv  RGB values for all yellow stripe pixels in frog's
vent


    r=feval(eqR,frf); g=feval(eqG,fgf); b=feval(eqB,fbf);
    rgb=[r g b]; tot=sum(rgb,2); rgb=rgb./tot; %toral relative
intensities for RGB
    mnsf=mean(rgb); sdsf=std(rgb); mlumf=mean(tot); slumf=std(tot);  %all
frog

    r=feval(eqR,frh); g=feval(eqG,fgh); b=feval(eqB,fbh);
    rgb=[r g b]; tot=sum(rgb,2); rgb=rgb./tot; %toral relative
intensities for RGB
    mnsh=mean(rgb); sdsh=std(rgb); mlumh=mean(tot); slumh=std(tot);
%head

    r=feval(eqR,frt); g=feval(eqG,fgt); b=feval(eqB,fbt);
    rgb=[r g b]; tot=sum(rgb,2); rgb=rgb./tot; %toral relative
intensities for RGB
    mnst=mean(rgb); sdst=std(rgb); mlumt=mean(tot); slumt=std(tot);
%torso

    r=feval(eqR,frv); g=feval(eqG,fgv); b=feval(eqB,fbv);
    rgb=[r g b]; tot=sum(rgb,2); rgb=rgb./tot; %toral relative
intensities for RGB
    mnsv=mean(rgb); sdsv=std(rgb); mlumv=mean(tot); slumv=std(tot);
%vent


    fprintf(fid,'%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f',...
        mnsf(1),mnsf(2),mnsf(3),mlumf,sdsf(1),sdsf(2),sdsf(3),slumf);
    fprintf(fid,',%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f',...
        mnsh(1),mnsh(2),mnsh(3),mlumh,sdsh(1),sdsh(2),sdsh(3),slumh);
    fprintf(fid,',%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f',...
        mnst(1),mnst(2),mnst(3),mlumt,sdst(1),sdst(2),sdst(3),slumt);
    fprintf(fid,',%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f,%6.4f',...
```

```
        mnsv(1),mnsv(2),mnsv(3),mlumv,sdsv(1),sdsv(2),sdsv(3),slumv);
    fprintf(fid,',%s\n',fname);
    fprintf(1,'Photo %s finished\n',fname);
  end; %if v>0
 end; %file selected or v=1 while loop
 fclose(fid);
 fprintf(1,'Finished, results in %s\n',oname);
end %function



function [dirs,nds]=GetDIRList(dirname);
% [dirs,nds]=GetDIRList(dirname);
% INPUT (string in single-quotes)
%   dirname is a directory name, such as 'C:\Active\Heinsohn\'  % Must
end '\'
% OUTPUT
%   dirs is a list of the directories within dirn (without dirn in front)
%   nds    is the number of directories (can be 0)
drl=dir(dirname); [sz,d]=size(drl);
dirs=[]; nds=0;
for d=1:sz
   temp=drl(d).name;
   typ =drl(d).isdir;
   k1=findstr('.',temp); if isempty(k1) k1=0; end;
   k2=findstr('..',temp); if isempty(k2) k2=0; end;
   if ((typ==1) & (k1==0) & (k2==0))
       dirs=[dirs; cellstr(temp)];
       nds=nds+1;
   end;
end;
end %function

function [files,nf]=GetFileList(dirn,stype)
% [files,nf]=GetFileList(dirn,stype);
% INPUT (strings in single-quotes)
%   dirn is a directory name, such as 'C:\Active\Heinsohn\'  % Must end
'\'
%    to read in the current directory, use '' (empty)
%   stype is the kind of file, such as '.ttt' or '.Master.transmission'
% OUTPUT
%   files is a list of the files (without the directory in front)
%   nf    is the number of files (can be 0)
% Note: will recognize .Master.tra with stype='.tra'
drp=[dirn '*' stype];
drl=dir(drp); [nf,f]=size(drl);
files=[];
for f=1:nf
  temp=drl(f).name;
  files=[files; cellstr(temp)];
end;
end %function

function [pcx,pcy]=polycenter(px,py);
% [pcx,pcy]=polycenter(px,py); finds the geometric center (pcx,pcy)
%   of a polygon whose points are in (px,py) column vectors
[np,i]=size(px);
for j=1:np
```

```
    i=j+1;  if j==np i=1; end;
    a(j)=((py(i)+py(j))*(px(j)-px(i)))/2;
    my(j)=(((py(i)^2)+py(i)*py(j)+(py(j)^2))*(px(j)-px(i)))/6;
    mx(j)=(((px(i)^2)+px(i)*px(j)+(px(j)^2))*(py(j)-py(i)))/6;
end;
sx=0; sy=0; sa=0;
for j=1:np
    sa=sa+a(j);
    sx=sx+mx(j);
    sy=sy+my(j);
end;
pcx=-sx/sa;
pcy=sy/sa;
end %function


function GetHueChrLum;
%calculate Hue, Chroma and Luminance from calibrated RGB from photos
clear;
[fname,path,f]=uigetfile('.csv','Select a CSV RGB file');

oname=fname; k=strfind(oname,'.'); if isempty(k) k=0; end;
oname=[oname(1:(k-1)) 'WithHueChrLum' oname(k:end)];
ofid=fopen(oname,'wt');


fid=fopen(fname);
rd=textscan(fid,'%s',1,'whitespace','\n'); head1=char(rd{1});
rd=textscan(fid,'%s %s %s %s %s %s %s %s %s',1,'delimiter',',');
heads=[rd{1:9}];
rd=textscan(fid,'%f %f %f %f %f %f %f %f %s','delimiter',',');
fclose(fid);
head1=regexprep(head1,'"','');
head1=regexprep(head1,',','');
R=rd{1}; G=rd{2}; B=rd{3}; Lum=rd{4}; sdR=rd{5}; sdG=rd{6}; sdB=rd{7};
sdTot=rd{8};
photo=rd{9}; n=length(R); clear fid rd;
Hue=zeros(n,1); Chr=Hue;
for k=1:n
  r=R(k); g=G(k); b=B(k); [cx,cy]=PXYZtoTRI(1/3,1/3,1/3);
  [tx,ty]=PXYZtoTRI(r,g,b); tx=tx-cx; ty=ty-cy; %set gray to (0,0)
  [hue,chr]=cart2pol(ty,tx); Hue(k)=rad2deg(hue); %red -120, green 0,
blue +120
  Chr(k)=chr/0.5774;  %maximum chroma set to 1
end;

fprintf(ofid,'%s\n',head1);
fprintf(ofid,'Hue,Chroma,Luminance');
for k=1:9 fprintf(ofid,',%s',heads{k}); end;
fprintf(ofid,'\n');
for k=1:n
   fprintf(ofid,'%8.4f,%6.4f,%6.4f',Hue(k),Chr(k),Lum(k));

fprintf(ofid,',%8.4f,%8.4f,%8.4f,%8.4f,%8.4f,%8.4f,%8.4f,%8.4f,%s\n',...
       R(k),G(k),B(k),Lum(k),sdR(k),sdG(k),sdB(k),sdTot(k),photo{k});
end;
fclose(ofid);
fprintf(1,'Data read from %s\n',fname);
```

```
fprintf(1,'Results in     %s\n',oname);

end %main function

function [tx,ty]=PXYZtoTRI(p1,p2,p3);
%[tx,ty]=PXYZtoTRI(p1,p2,p3);  converts proportions p1,p2,p3
%   to triangular coordinates tx,ty        (sets p1+p2+p3=1)
%   Triangle edge lengths 1.0   [height sqrt(3)/2]
%   Triangle vertices: (0, 0), (1/2, sqrt3/2), (1, 0)

% first ensure p2+p3+p1 = 1
[n,i]=size(p2);
for i=1:n
    tot=p1(i)+p2(i)+p3(i);
    if tot>0  p1(i)=p1(i)/tot; p2(i)=p2(i)/tot; p3(i)=p3(i)/tot; end;
end;
% convert to triangle
s3=sqrt(3);
for i=1:n
    tx(i)=(p2(i)+2*p3(i))/s3; ty(i)=p2(i);
end;
%rescale so that triangle edges have length=1
for i=1:n
    tx(i)=tx(i)*s3/2; ty(i)=ty(i)*s3/2;
end;
tx=tx'; ty=ty'; %convert to column vectors;
end %triangle
```