

## RESEARCH ARTICLE

# Does dietary $\beta$ -carotene influence ontogenetic colour change in the southern corroboree frog?

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## 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  $\beta$ -carotene supplementation on colouration in southern corroboree frogs (*Pseudophryne corroboree*) during early post-metamorphic development. Frogs were reared on four dietary treatments with four  $\beta$ -carotene concentrations (0, 1, 2 and 3 mg g<sup>-1</sup>), with frog colour measured every 8 weeks for 32 weeks.  $\beta$ -Carotene was not found to influence colouration at any dose. However, 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, (1)  $\beta$ -carotene may contribute little or nothing to colouration, (2) frogs can manufacture their own colour, (3) colour development is a continual process and (4) 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.

**KEY WORDS:** Carotenoid, Colour, Aposematic, Diet, Ontogeny

## 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 and 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 and 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 and 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 and Schöneberg, 2010; Mills and Patterson, 2009; Suga and 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 regularly play an important role in sexual signalling (Baeta et al., 2008; Davis and 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 and McGraw, 2009). Pteridines are synthesised by vertebrates during purine production (Ziegler, 2003) and may 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 and Olson, 1989; Fernandes et al., 2018).

In species in which 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 and Ruxton, 2010). As such, a common manifestation of ontogenetic colour change is a switch in defensive strategies, most often seen when drab juveniles become more colourful as they mature (Higginson and 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 anti-oxidants and remove reactive oxygen species 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

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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 (CDBs) in a carotenoid increases, so does its mean wavelength. Thus, carotenoids such as  $\beta$ -carotene and lycopene (11 CDBs) are perceptibly more red than carotenoids such as violaxanthin and neoxanthin (nine CDBs), which are more yellow (Meléndez-Martínez et al., 2007). Carotenoids that 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  $\beta$ -carotene (11 CDBs) are located at shorter wavelengths than those of lycopene (11 CDBs), as two of the CDBs of  $\beta$ -carotene are located in its rings, making it noncoplanar (Meléndez-Martínez et al., 2007). Owing 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 (Paloza, 1998; Young and 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., 2013; Prado-Cabrero et al., 2020; Toomey and McGraw, 2011; Weaver et al., 2018, 2020; Yasir and 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 and Hero, 2016), each containing unique pigment granules responsible for brown–black, blue–green and red–yellow colour, respectively (Suga and Munasada, 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 and 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 and 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. *Pseudophryne 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 showed that the main carotenoid compounds present were lutein and  $\beta$ -carotene (P.G.B. and A.J.S., unpublished data). However, the extent to which each compound influenced colouration remains unknown. Dietary  $\beta$ -carotene has been shown to make the skin of false tomato frogs (*Dyscophus guineti*) more yellow (Brenes-Soto and 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  $\beta$ -carotene on colour expression in *P. corroboree*. The specific aims were to investigate (1) the influence of different concentrations of  $\beta$ -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.

## MATERIALS AND METHODS

### Ethics statement

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

### Study animals

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

### Husbandry and diet treatments

Upon arrival at UOW, metamorphs were separated into individual enclosures (21×12×12 cm) and randomly assigned to one of four experimental diet treatments: 0 mg g<sup>-1</sup>  $\beta$ -carotene (T0); 1 mg g<sup>-1</sup>  $\beta$ -carotene (T1); 2 mg g<sup>-1</sup>  $\beta$ -carotene (T2); and 3 mg g<sup>-1</sup>  $\beta$ -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, one cup (~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 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 in bulb; Pet Pacific, Emu Plains, Australia) were suspended ~20 cm above enclosures and were maintained on a 9 h:15 h 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 5 weeks until experimental diets commenced on 15 March 2019. Experimental diets were administered twice weekly and were prepared by dusting a standardised weight of crickets (~15 g per treatment) with one of four treatment powders, corresponding to each experimental diet: 0 mg g<sup>-1</sup>  $\beta$ -carotene (T0); 1 mg g<sup>-1</sup>  $\beta$ -carotene (T1); 2 mg g<sup>-1</sup>  $\beta$ -carotene (T2); and 3 mg g<sup>-1</sup>  $\beta$ -carotene (T3) (Table 1). Cellulose microcrystalline powder (435 236; Sigma-Aldrich, Castle Hill, Australia) 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<sup>-1</sup>). 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,  $\beta$ -carotene doses tested ranged from 0.1 to 10 mg g<sup>-1</sup> and from 0.1 to 1 mg g<sup>-1</sup>, respectively, and the optimal doses identified in both studies was 1 mg g<sup>-1</sup>. 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 to 10 days old at the beginning of the experimental period and was increased to 9 to 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 15 March 2019 until 21 October 2019. The dorsal surface of each frog was photographed immediately prior to the commencement of experimental diets and every 8 weeks thereafter (weeks 0, 8, 16, 24 and 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) 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 and Haworth, 2014). At each sampling time (weeks 0, 8, 16, 24 and 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 were then transformed into hue, chroma and luminance (HCL) values using another MATLAB

**Table 1. Composition of experimental diets fed to *Pseudophryne corroboree* metamorphs**

Diet treatment	$\beta$ -carotene mass (g)	$\beta$ -carotene concentration per g of feed (mg g <sup>-1</sup> )	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

N=34 for each treatment group. Diet supplements were dusted onto ~15 g of crickets per treatment, per feed.



**Fig. 1. Arrangement of the internal photo-staging area for colour quantification of *Pseudophryne corroboree*.** Shown is the placement of the XRite ColorChecker, identification tag and a southern corroboree frog.



programme (for MATLAB code used for the analyses, see Supplementary Materials and Methods). Hue is commonly represented as degrees on a circular scale, in which red=0 deg (or 360 deg), green=120 deg and blue=240 deg. To translate our data to this scale, hue ( $H$ ) was transformed according to the formula:

$$H = H_0 + 120, \quad (1)$$

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 (LMMs) 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, 1, 2 and 3 mg g<sup>-1</sup>), test week (0, 8, 16, 24 and 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–Wilk test was conducted on hue, chroma and luminance variables to test for distribution normality. Chroma data were transformed using an arcsine square root transformation [ $\sin^{-1}(\sqrt{x})$ ;  $x$  = chroma] to improve normality and homogeneity. In models in which significant effects were detected, *post hoc* comparisons were made using Tukey's HSD tests. During the study, 18 individuals (three to eight frogs per treatment) died from unexpected adverse events or failure to thrive and were excluded from all analyses. The final numbers 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, Cary, NC, USA). Results were considered significant at  $P<0.05$ .

## RESULTS

### Hue

Overall, there was no effect of diet treatment on hue (LMM:  $F_{3,122.6}=1.5708$ ,  $P=0.1999$ ; Fig. 2), and no significant interaction between time and diet treatment (LMM:  $F_{12,470.5}=1.4277$ ,  $P=0.1494$ ; Fig. 2). There was a significant effect of time on hue (LMM:  $F_{4,485.6}=8.1314$ ,  $P<0.0001$ ; Fig. 2), with hue becoming slightly more yellow shifted over the 32 week experimental period

(week 0 mean range, 68.36–69.10 deg; week 32 mean range, 69.14–70.46 deg; Fig. 2). Hue was not significantly associated with body weight (LMM:  $F_{1,538.8}=1.8606$ ,  $P=0.1731$ ).

### Chroma

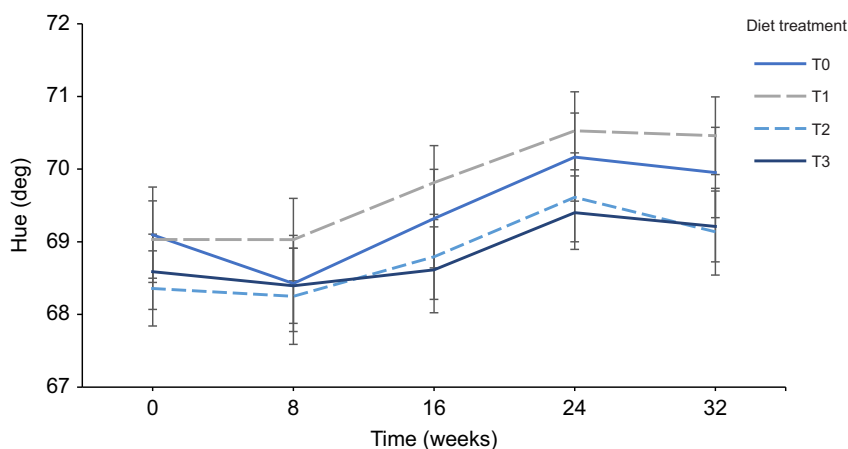
Overall, there was no effect of diet treatment on chroma over the 32 week experimental period (LMM:  $F_{3,104.4}=1.6820$ ,  $P=0.1751$ ; Fig. 3). There was a significant effect of time on chroma (LMM:  $F_{4,489.5}=18.6314$ ,  $P<0.0001$ ; Fig. 3) and a significant treatment-by-time interaction (LMM:  $F_{12,463.9}=2.2824$ ,  $P=0.0080$ ; 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 (LMM:  $F_{1,219}=2.3576$ ,  $P=0.1260$ ).

### Luminance

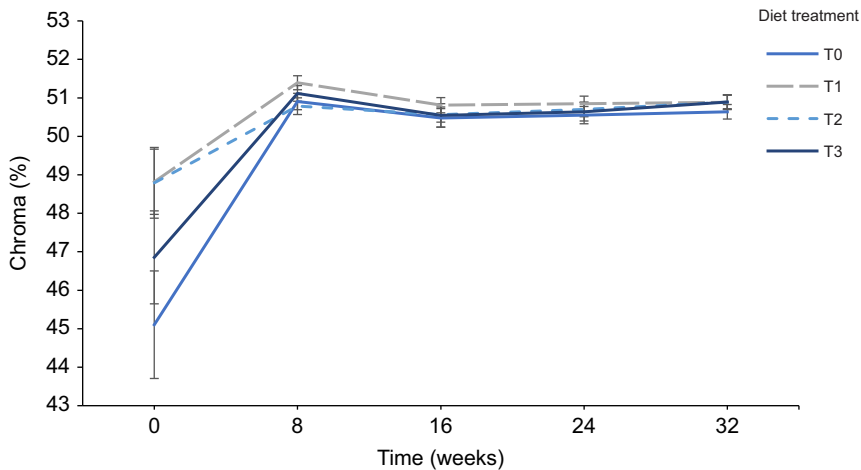
Overall, there was no significant effect of diet treatment on luminance (LMM:  $F_{3,123.1}=0.7974$ ,  $P=0.4976$ ; Fig. 4), and no significant interaction between time and treatment (LMM:  $F_{12,472.6}=1.7251$ ,  $P=0.0586$ ; Fig. 4). There was a significant effect of time on luminance (LMM:  $F_{4,500.1}=4.2999$ ,  $P=0.0020$ ; 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 (LMM:  $F_{1,585.4}=6.6655$ ,  $P=0.0101$ ), whereby luminance was positively correlated with body weight.

## DISCUSSION

The present study shows that dietary  $\beta$ -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  $\beta$ -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.



**Fig. 2. Effect of dietary  $\beta$ -carotene supplementation on *P. corroboree* hue over the 32 week experimental period.** Data shown are untransformed means  $\pm$  s.e.m. Sample sizes for each diet treatment were as follows: T0 (0 mg g<sup>-1</sup>,  $N=30$ ); T1 (1 mg g<sup>-1</sup>,  $N=31$ ); T2 (2 mg g<sup>-1</sup>,  $N=29$ ); T3 (3 mg g<sup>-1</sup>,  $N=26$ ).

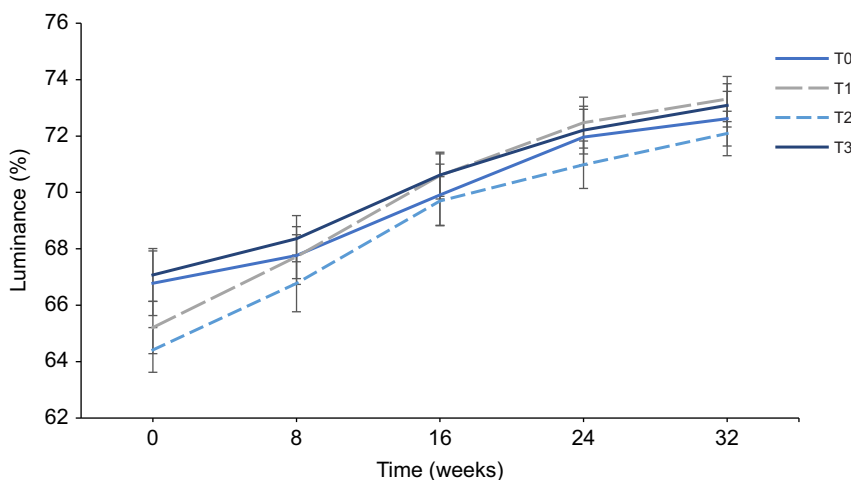


**Fig. 3. Effect of dietary  $\beta$ -carotene supplementation on *P. corroboree* chroma over the 32 week experimental period.** Data shown are untransformed means  $\pm$  s.e.m. Sample sizes for each diet treatment were as follows: T0 (0 mg g<sup>-1</sup>),  $N=30$ ; T1 (1 mg g<sup>-1</sup>),  $N=31$ ; T2 (2 mg g<sup>-1</sup>),  $N=29$ ; T3 (3 mg g<sup>-1</sup>),  $N=26$ .

Our finding that  $\beta$ -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  $\beta$ -carotene) exhibited significantly different colour (more saturated chroma and orange-shifted hue) compared with the control group. It is possible that  $\beta$ -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  $\beta$ -carotene (e.g. lutein). Alternatively, it is possible that certain aspects of our methodology restricted our capacity to detect effects of  $\beta$ -carotene on colouration. Because  $\beta$ -carotene is a light-sensitive molecule, the lighting conditions we imposed may have altered our findings. In the present study, frogs were exposed to ultraviolet (UV) radiation for 9 h per day, which was much higher than the exposure level provided by Umbers et al. (2016) (1 h 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  $\beta$ -carotene supplementation. Specifically, elevated UV levels may have either degraded  $\beta$ -carotene prior to consumption, making the supplement ineffective, or caused the destruction of pigment granules in the frogs' skin, restricting colour expression (McNett and Marchetti, 2005; Surmacki, 2008). At temperatures similar to those used in our experiment, complete UV degradation of  $\beta$ -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  $\beta$ -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  $\beta$ -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<sup>-1</sup>) 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  $\sim 0.00007$  to 0.25 mg g<sup>-1</sup>, although as high as 291 mg g<sup>-1</sup>), or circulating levels of carotenoids in the blood (Cantarero et al., 2020a,b; Koch and Hill, 2018; Koch et al., 2018, 2019; Mahler et al., 2003; McCoy et al., 2021; McGraw et al., 2003; Olson and Owens, 2005; Powers and 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 with our previous study in which frogs received a mixed carotenoid at 20 mg g<sup>-1</sup>, the colour of



**Fig. 4. Effect of dietary  $\beta$ -carotene supplementation on *P. corroboree* luminance over the 32 week experimental period.** Data shown are untransformed means  $\pm$  s.e.m. Sample sizes for each diet treatment were as follows: T0 (0 mg g<sup>-1</sup>),  $N=30$ ; T1 (1 mg g<sup>-1</sup>),  $N=31$ ; T2 (2 mg g<sup>-1</sup>),  $N=29$ ; T3 (3 mg g<sup>-1</sup>),  $N=26$ .

experimental frogs in the present study (given  $\beta$ -carotene at 1–3 mg g<sup>-1</sup>) was less saturated (previous study, 0.78; present study, 0.50–0.51). Second, for a subset of frogs ( $N=32$ ) that were recaptured ~3.5 months post-release, the colour of individuals spanning all treatment groups became significantly brighter and more orange shifted (P.G.B. and A.J.S., unpublished data). This change may have resulted from the natural diet supplying either a higher concentration of  $\beta$ -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 and 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 in which 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 (drosoperin) 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 in which 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 and Yamada, 1998). Nevertheless, based on available information, it appears that the DCU is not fully formed

until after metamorphosis (Mahalwar et al., 2014; Thibaudeau and 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 4 weeks. Despite these initial differences, frogs in all treatment groups converged on similar mean values within a period of 2 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 and 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 and 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 and 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 and 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 in which 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 and Blouin, 2000; Rudh and 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  $\beta$ -carotene on the putative aposematic colouration of corroboree frogs. Varying  $\beta$ -carotene supplementation had no detectable effect on hue, chroma or luminance over the course of frog maturation. This indicates that  $\beta$ -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

Conceptualization: A.J.S., P.G.B.; Methodology: S.J.W., A.J.S., J.A.E., P.G.B.; Software: J.A.E.; Validation: P.G.B.; Formal analysis: J.A.E., P.G.B.; Investigation: S.J.W., A.J.S., P.G.B.; Resources: A.J.S.; Data curation: S.J.W., A.J.S.; Writing - original draft: S.J.W., P.G.B.; Writing - review & editing: A.J.S., J.A.E.; Visualization: A.J.S.; Supervision: A.J.S., P.G.B.; Project administration: A.J.S., P.G.B.

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## Supplementary Materials and Methods

**MATLAB script used for the conversion of RGB 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};
Brn=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 extract 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=fitttype('exp2');
opts=fioptions('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=fitttype('exp2');
opts=fioptions('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=fitttype('exp2');
opts=fioptions('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=fitttype('exp2');
opts=fioptions('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');
%exchange x y and get fits from RGB to actual reflectance
[xData,yData]=prepareCurveData(yr,x); ft=fitttype('exp2');
opts=fioptions('Method','NonlinearLeastSquares');
opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
eqR=fit(xData,yData,ft,opts);
[xData,yData]=prepareCurveData(yg,x); ft=fitttype('exp2');
opts=fioptions('Method','NonlinearLeastSquares');
opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
eqG=fit(xData,yData,ft,opts);
[xData,yData]=prepareCurveData(yb,x); ft=fitttype('exp2');
opts=fioptions('Method','NonlinearLeastSquares');
opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
eqB=fit(xData,yData,ft,opts);
[xData,yData]=prepareCurveData(ybt,x); ft=fitttype('exp2');
opts=fioptions('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(online,'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(online,'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);
rbg=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));
    rbg(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
% rbg(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 t1s 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
ftr=fr(yellowtorso==1); fgt=fg(yellowtorso==1);
fbr=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=rgb(19:24,1); gst=rgb(19:24,2); bst=rgb(19:24,3);
btst=rgb(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=fioptions('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=fioptions('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=fioptions('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');
%      % exchange x y and get fits from RGB to actual reflectance
    [xData,yData]=prepareCurveData(yr,x); ft=fittype('exp2');
    opts=fioptions('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=fioptions('Method','NonlinearLeastSquares');

```

```

    opts.StartPoint=[0.0329939089339003 0.0136159137399716 -
0.0983277644336392 0.00351296038818105];
    eqG=fit(xData,yData,ft,opts);
    [xData,yData]=prepareCurveData(yb,x); ft=fitttype('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 %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, %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, sqrt(3)/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
```