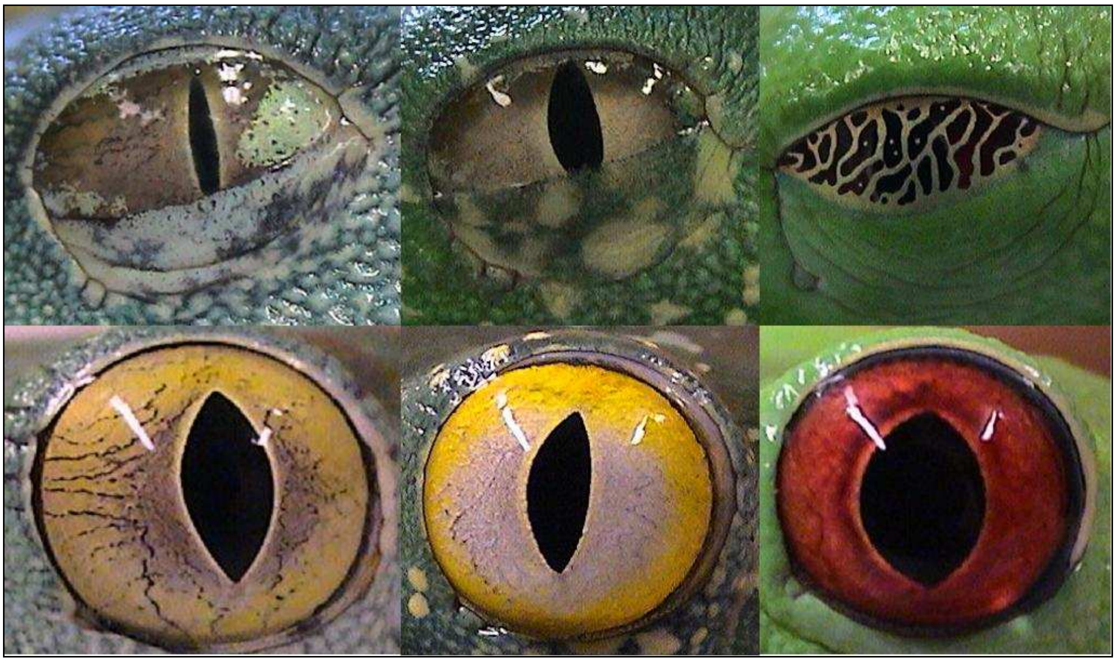
An Investigation of Apparent Iris Metachrosis, and Comparative Morphology of the Eye of *Agalychnis* Tree Frogs (Anura: Hylidae: Phyllomedusinae)



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ABSTRACT

The eye morphology of the tree frogs *Agalychnis calcarifer* and *A. craspedopus*, is unusual within the genus *Agalychnis*. The iris of these two species displays apparent metachrosis (colour change) as the eye opens, a phenomenon which is unreported in amphibians. This study uses a new technique involving digital image analysis to quantitatively assess this process, and to determine the mechanism by which it occurs. Observations suggest that the visible colour of the iris is modulated by revealing different areas of the iris as opposed to redistributing pigment. The eye morphology of *A. calcarifer* and *A. craspedopus* was also described in relation to other *Agalychnis* frogs. The advantages and limitations of digital image analysis as a morphometric tool are discussed.

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3.1 INTRODUCTION

3.1.1 The Eye

Vision is enormously important to the survival many animals. It is often the major sensory modality used for hunting prey and avoiding predation, and consequently the eye is of great importance to the survival of numerous animals including amphibians. The eyes of frogs (anurans) vary greatly in size, with those of arboreal species proportionately the largest (Duellman and Trueb, 1994). This may infer that vision is more important in these species, or perhaps indicate that eyes are used as a signal in visual communication.

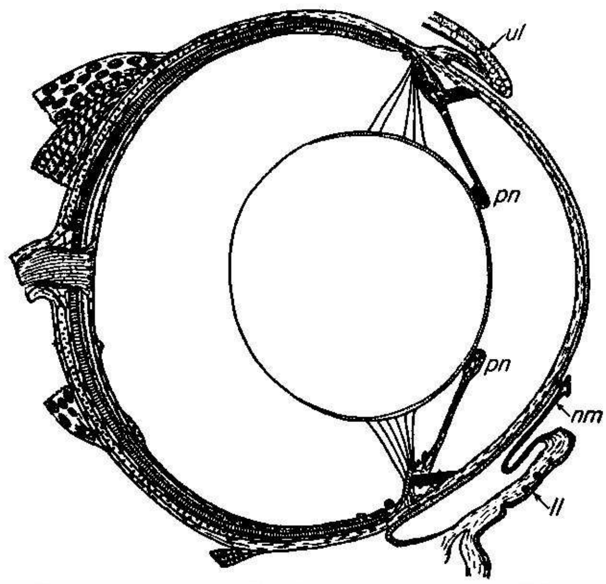

Fig. 3.1. Semi-diagrammatic cross section of the anuran eye (based mainly on the leopard frog, *Rana pipiens*). nm – nictitating membrane; ll – lower eyelid (lower portion); ul; upper eyelid; pn – papillary nodules (Adapted from Walls, 1942).

The amphibian eyeball is almost spherical (Fig. 3.1). It can be retracted into the orbit by contracting the retractor bulbi muscle, and is returned back to its normal position by the levator bulbi muscle (Williams and Whitaker, 1994). No other movement of the globe occurs, as the six remaining extraocular muscles are vestigial (Stebbins and Cohen, 1995, Williams and Whitaker, 1994). The retraction of the eyeball into the orbit is important in swallowing, because the orbit and the buccal cavity are only separated by a thin membrane. This action also provides the eye with mechanical protection (Williams and Whitaker, 1994). The retraction of the eyeball also allows the lower eyelid to be drawn over the eye, providing further protection (Fig. 3.1 and 3.2). This is achieved by contraction of the membrana nictitans muscle, which is attached to either end of the upper edge of the lower eyelid by a tendon (Duellman and Trueb, 1994, Noble, 1931). The lower eyelid is withdrawn principally by the action of the m. levator bulbi muscle, but also by protrusion of the eyeball (Duellman and Trueb, 1994).

The lower eyelid is divided into an upper and a lower portion (Duellman and Trueb, 1994, Noble, 1931). The upper portion of the lower eyelid is known as the nictitating membrane, and usually consists of a thin elastic translucent conjunctival fold (Fig. 3.1 and 3.2) (Duellman and Trueb, 1994, Williams and Whitaker, 1994). It has little or no pigmentation in some frogs. In frogs with a pigmented nictitating membrane, the pigmentation probably serves to conceal the potentially conspicuous eye below, while allowing the frog to see (Duellman and Trueb, 1994). Anurans also have an upper eyelid (Fig. 3.l), but this has very limited movement and consists of merely an integuementary fold (Duellman and Trueb, 1994).

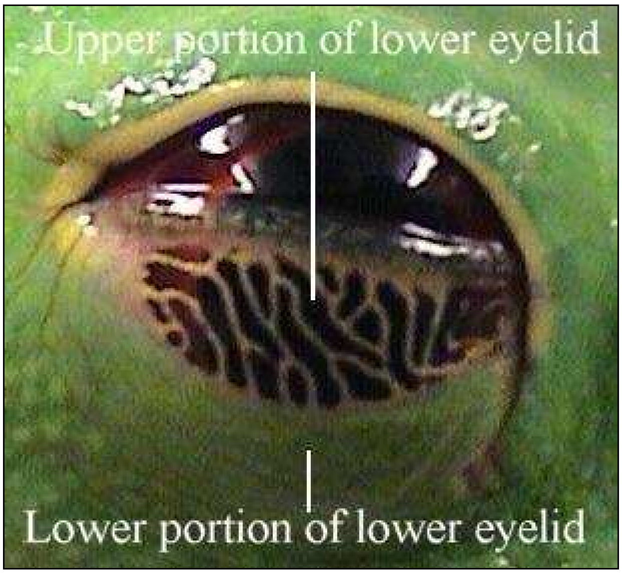

Fig. 3.2. Half-closed lower eyelid of *A. callidryas*. Note the upper portion of the lower eyelid is composed of translucent areas and a network of opaque reticulation. The black pupil and red iris can be seen through the translucent areas.

3.1.2 Colour and Predators

In amphibians the iris is coloured with the pigment-containing cells (chromatophores) melanophores, iridophores and in some anurans, xanthophores. Carotenoid pigments and crystals of guanine also colour the iris (Duellman and Trueb, 1994, Williams and Whitaker, 1994). Iris colouration does not affect the vision of animals (Walls, 1942), so perhaps the significance of the variety of iris colours displayed by anurans lies in an anti-predator role.

Some animals make use of bright colours to increase their visibility as an anti-predator mechanism. Bright aposematic (warning) colouration is used by the poison-dart fogs (Dendrobatidae) of tropical America (Duellman and Trueb, 1994, Stebbins and Cohen, 1995). More commonly, however, bright colouration is confined to areas of the body that are usually concealed so as not to interfere with any concealing colouration, such as the brightly coloured flanks of many frogs in Central America (Duellman, 1970, Stebbins and Cohen, 1995). These so-called flash markings are normally vivid and contrast with the dorsal colouration. When the frog is threatened it leaps, suddenly temporarily exposing the flash markings to startle the predator. When the frog lands it assumes its resting posture, concealing the bright colouration and presenting only the possibly cryptically coloured dorsal surfaces (Duellman and Trueb, 1994, Stebbins and Cohen, 1995).

In contrast, animals more commonly use colour to make them more difficult for predators to detect. This frequently takes the form of camouflage. Cott (1940) noted that the iris colour of animals often matches that of the head as a whole, enhancing camouflage. This has been taken to extremes in some species of animal such as the lionfish, *Pterois volitans*, and the western painted turtle, *Chrysemys picta marginata*, where uninterrupted black and yellow cross the head, including the eye (Fig. 3.3).

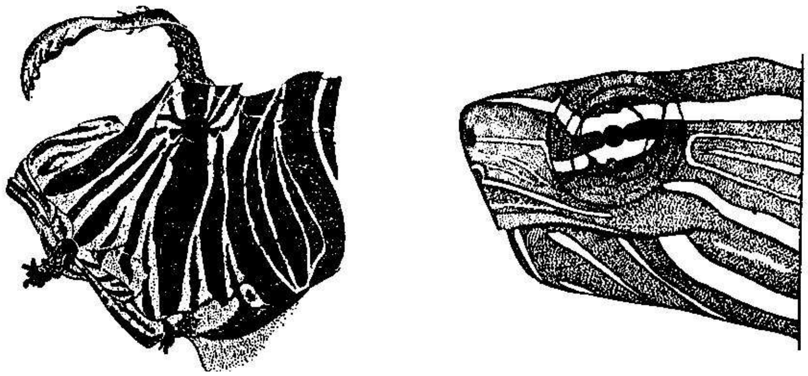

Fig. 3.3. Continuous colouration patterns across the skin and eyes of the lionfish (left), and the western painted turtle (right) (after Cott, 1940 and Walls, 1942).

The eyes are not the only area of the body that use concealing colouration. The skin colours of many frogs matches the substrates on which they live, such as the leaf-green dorsum of many species of arboreal frogs (Duellman, 1970, Norris and Lowe, 1964). Concealing patterns are also sometimes used in the skin, such as in *Hyla arenicolor* (Fig. 3.4) (Duellman and Trueb, 1994).

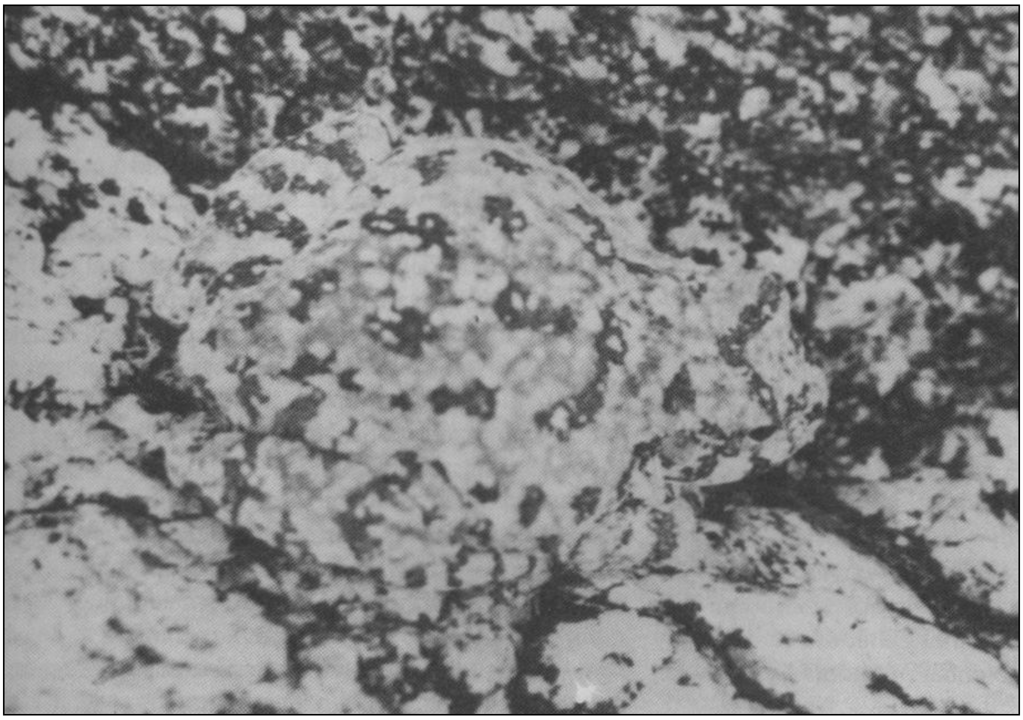

Fig. 3.4. Concealing colouration of *Hyla arenicolor* (Duellman and Trueb, 1994).

Another strategy involves disruptive colouration, which interferes with the perception of the true outline of the animal such as the dorsum pattern of species of the California tree frog, *Pseudacris cadaverina* (Bradbury and Vehrencamp, 1998, Stebbins and Cohen, 1995). In addition, many anurans are capable of modulating their skin colour (metachrosis) in response to changing light levels and background colours, which may also make them less conspicuous to predators (Duellman, 1970, Iga and Bagnara, 1975, King and King, 1991). One such group of frogs that display this ability are the hylids.

3.1.3 Hylid Frogs

Frogs belonging to the family Hylidae form part of a group of anurans known as tree frogs. Hylids share several morphological adaptations to their arboreal habitat including modified fingers and toes that allow them to climb more effectively. There are approximately 450 species of hylid, which are currently classified into five sub families (Frost, 2002). The morphological characteristics, along with behavioural, physiological and chromosomal characteristics of one hylid sub family, Phyllomedusinae, indicate an early divergence from other tree frogs (Duellman, 1968, Maxson, 1976). Their evolutionary biology and relations to other taxa is controversial and not fully understood (Bagnara, 2003). Recent revision splits the 40 species of the sub family into 6 genera (formerly 3) (Duellman, 1993). Phyllomedusines occur exclusively in the Neotropics, and are the only frogs in the region with a vertical slit pupil. The morphology of the eye has been used as a key taxonomic character to define hylid species and groups, although the phylogeny of this group is not fully understood (Duellman, 1970, Cruz, 1991).

Within the Phyllomedusinae, frogs of the genus *Agalychnis* also have vivid colouration: they often have brilliant flash markings on their flanks and thighs, and also have large, brightly coloured eyes (Duellman, 1970). *A. callidryas* has both of these characters and is typical of the genus. The nictitating membrane is reticulated in all eight *Agalychnis* species (such as in Fig. 3.2), with the exception of *A. calcarifer* and *A. craspedopus*, in which it is unpigmented (Duellman, 1970, Hoogmoed and Cadle, 1991). The iris of these two species also differs from the other *Agalychnis*. The iris of *A. calcarifer* and *A. craspedopus* consists of two colours; a dull grey inner area, and a bright yellow peripheral border, whereas the eyes of other *Agalychnis* are a single solid colour of red (Fig. 3.5) (*A. callidryas*, *A. saltator*, *A. spurelli*, *A. litodryas* and *A. moreletti*) or orange (*A. annae*) (Duellman, 1970, Gray, 2001, Hoogmoed and Cadle, 1991). The eye morphology of these species has not previously been described in detail, and it is not known how these characteristics vary between sexes or developmental stages. However, eye colouration is an important characteristic used to assess phylogenetic relationships, so its study may help to clarify the taxonomic status of these frogs (Cruz, 1991). The reason for the interspecific difference between *A. calcarifer* and *A. craspedopus* is it is not understood.

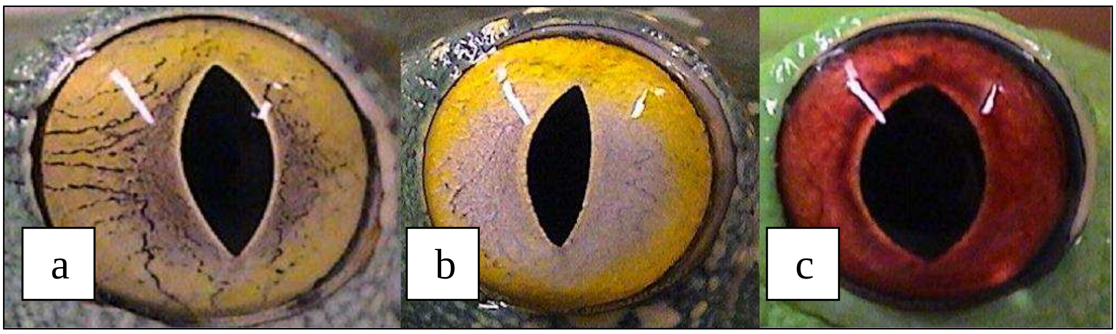

Fig. 3.5. Iris colouration and patterns in a) *A. calcarifer*, b) *A. craspedopus*, c) *A. callidryas*.

The yellow area of the iris of *A. calcarifer* and *A. craspedopus* appears to only become visible as the eye opens, an observation that has only been acknowledged in the unpublished work of Gray (2001). The mechanism by which this occurs could be effected by several mechanisms. Firstly, the grey chromatophores in the iris could change colour and become yellow (Bagnara, 1976, Bagnara and Hadley, 1973). Secondly, the yellow chromatophores could migrate towards the centre of the iris. Both of these hypotheses involve a redistribution of pigment throughout the iris. Thirdly, the pigment could remain in a constant pattern, but the animal could simply reveal more of the area in which they are positioned. However the mechanism used is as yet unknown.

Traditionally, morphometric measurements are taken manually with callipers. This makes measurement of two-dimensional features difficult, particularly if they are irregularly shaped. This may be the reason that measurements of lengths rather than areas are conventionally used in morphological studies (Duellman, 1970). However, given recent advances in digital technology, it would be relatively simple and cost-effective to use digital photography and image analysis software to measure both one- and two-dimensional features, such as the proportions of the iris with a particular pigmentation. As it does not require physical contact, this technique would also permit measurements of features that are difficult measure manually, such as sensitive areas of the body like the eye. Dangerous animals could also be measured safely, as no handling is necessary. The digital image analysis technique would also allow a number of dimensions to be measured from a single picture, which would be less stressful to live animals as it reduces the time spent handling them. Several previous studies have used digital photography techniques to study iris colour, but this does not yet seem to have been applied to morphometrics (Bee *et al.*, 1997, Niggemann, 2000, Niggemann, *et al.*, 2003).

3.2 AIMS

This study aims to:

1. Digital image analysis to investigate the apparent colour change that takes place as the eye of *A. calcarifer* and *A. craspedopus* opens, and determine the mechanism by which this occurs.
2. Use digital image analysis to describe in detail the morphological characteristics relating to the eye of *A. calcarifer*, *A. craspedopus,* and compare them to other *Agalychnis* frogs. This will be used to assess the phylogeny of the genus. In *A. calcarifer* the differences between males and females, and juvenile and adult specimens will also be investigated. This will be accomplished by studying the following characters:
    a. The lower eyelid
        i. The relative proportions of the nictitating membrane and the lower portion of the lower eyelid.
        ii. The relative proportions of the transparent and the opaque regions of the nictitating membrane.
    b. The eyeball
        i. The total area of the eye when open.
        ii. The extent to which the eyeball protrudes from the head.
        iii. The area occupied by the colours that make up the iris when the eye is both closed and open.

3.3 MATERIALS AND METHODS

Captive specimens of the required species were available from the hylid research collection at the Vivarium and Aquarium, The Manchester Museum, The University of Manchester. Research was possible because the specimens were maintained at The Manchester Museum under The Zoo Licensing Act (1981), and as such non-invasive research is permitted under The Home Office Animals (Scientific Procedures) Act 1986.

3.3.1 Study Animals

Four adult male *A. calcarifer*, five adult female *A. calcarifer*, and four young *A. calcarifer* were studied. Four adult male *A. craspedopus* were also studied. Four un-sexed adult *A. callidryas* specimens were used as a representative species of the other *Agalychnis* frogs, as they have a solid iris colour and a reticulated nictitating membrane like all other species in the genus. The specimens of *A. calcarifer* and *A. callidryas* originate from Costa Rica, those of *A. craspedopus* originate from Ecuador. The sizes of each group studied are given in Table 3.1.

Table 3.1. Mean snout-vent lengths of each group studied. Values in parenthesis represent standard deviations.

Group	**Mean snout-vent length ± standard deviation (mm)**
Male *A. calcarifer*	67.7 ± 1.5
Female *A. calcarifer*	78.2 ± 2.0
Young *A. calcarifer*	33.0 ± 2.3
A. craspedopus	61.6 ± 2.8
A. callidryas	60.2 ± 3.2

3.3.2 Measurements

In order to quantify the extent of apparent iris metachrosis as the eye opens, and the morphological differences between the eyes of the three study species, between male and female *A. calcarifer*, and between adult and young *A. calcarifer*, a series of digital photographs were taken. These were taken using a 3-CCD Sony DCR-TRV30E digital video camera recorder that was fixed into position using a tripod. A rule was included in each shot to set the scale. Light levels varied between 81 and 209 lux. It was necessary to take photographs of each frog from several angles to measure all the necessary features. A summary of the photographs taken and the measurements made from each shot is given in Table 3.2 and selections of the measurements made from them are given in Fig. 3.4-3.6.

Table 3.2. Summary of the photographs taken of the frogs, the angles from which they were taken, the measurements made from them, and the figures that relate to them.

Area of body photographed	Photograph angle	Measurements made	Fig.
Lower eyelid	Straight on	Area of: total eye, lower portion of the lower eyelid, nictitating membrane, transparent section of nictitating membrane, opaque section of nictitating membrane, central iris colour, peripheral iris colour, pupil	3.6a
Eyeball	Straight on	Area of: total eye, peripheral iris colour, central iris colour, pupil	3.6b
Eyeball	Along plane of face	Maximum protrusion of eye from head, eye diameter	3.7
Head and body	Above	Head width, snout-vent length	3.8

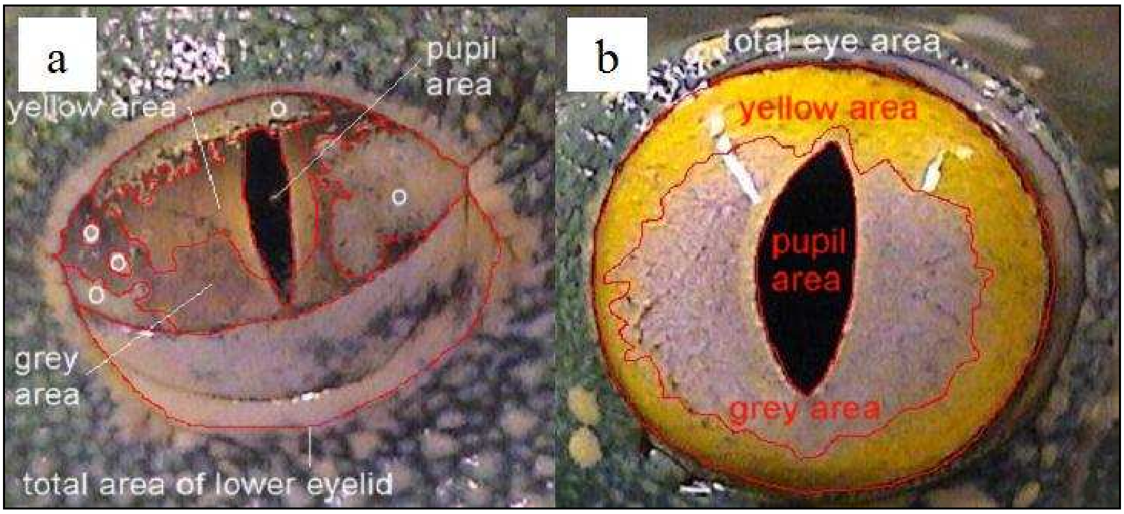

Fig. 3.6. A selection of areas measured from the eye. a) Closed eye; b) Open eye. Areas marked with an 'o' made up the opaque section of the nictitating membrane. These photographs will be used to investigate apparent iris metachrosis and to describe eye morphology.

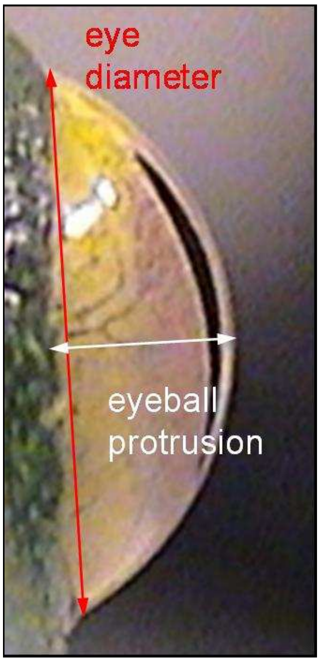

Fig. 3.7. Measurements made from the shots of fully open eyes photographed along the plane of the face. In each shot the nostril was in line with the edge of the face and was positioned below the eye.

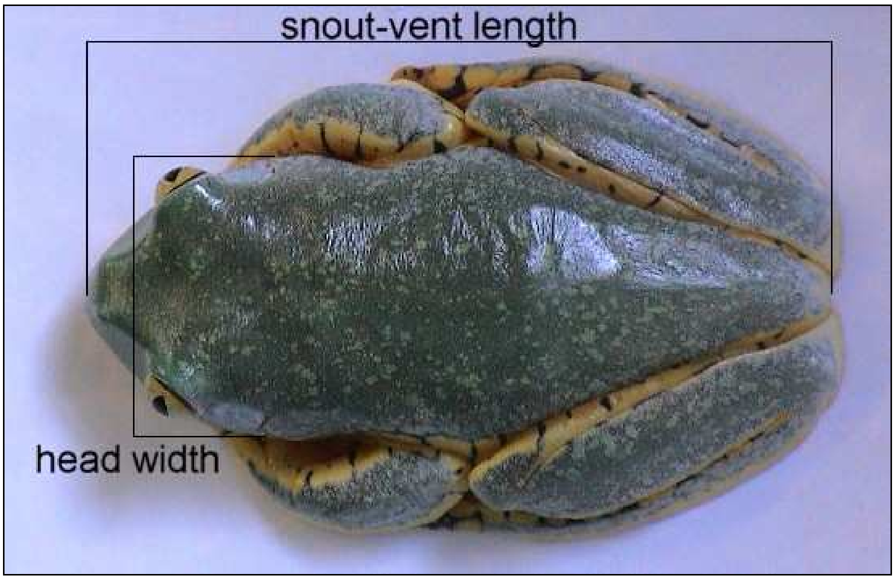

Fig. 3.8. Body morphology measurements. Head width was taken from in line with the posterior edge of the tympanum.

The process of apparent iris metachrosis occurred very quickly. As a result, no clear photographs could be taken of this phenomenon, so no quantitative data on this aspect of the morphology could be collected. The mechanism of apparent iris metachrosis was therefore investigated by visually observing the boundary of the bright peripheral colour of the iris as the eye opens and as the pupil dilates. The distance between the boundary of bright yellow pigment and the centre of the pupil was estimated visually, providing an indication of whether or not the distribution of pigment cells was changing.

3.3.3 Data Analysis

Lengths and areas of morphological features were measured using the image analysis program ImageJ (Rasband, 2001). The morphological measurements taken were used to test for differences between groups. Proportions rather than absolute measurements are more useful for comparing morphological measurements between samples of hylids, because individuals grow continuously (Duellman, 1970). This was particularly important in this study, because the frogs tested exhibited a range of different sizes (Table 3.1).

For this reason, the lower portion and the nictitating membrane areas of the lower eyelid were expressed as a proportion of the total area of the lower eyelid. Opaque and transparent regions of the nictitating membrane were expressed as a proportion of the total area of the nictitating membrane. In species with two different iris colours, the visible areas of these two coloured regions were expressed as a proportion of the total area of the nictitating membrane when the eyelid is closed, or as a proportion of the total area of the eye when the eyelid is open. Because proportions rather than absolute values were generated for analysis, they were transformed to make them appropriate for parametric statistical analysis by taking the arcsine of their square root. This meant that rather than being expressed as a proportion of 1.00 they were expressed as a proportion of 1.57. Eye protrusion was calculated as a ratio of the eye diameter to give the protrusion index. In order to account for the different absolute sizes of the frogs, measurements of the total eye area were calculated as a ratio to the head width. The relationship between these two parameters was tested by taking logs of each and performing regression analysis (Fig. 3.9). There was a highly significant positive relationship between log head width and log eye area ($t = 12.305$, $P < 0.001$). Growth was considered isometric, as the gradient of the regression line of these two variables was 1.916, which is close to the expected value of 2.0 for isometric growth. The total eye area was therefore expressed as a proportion of the head width squared.

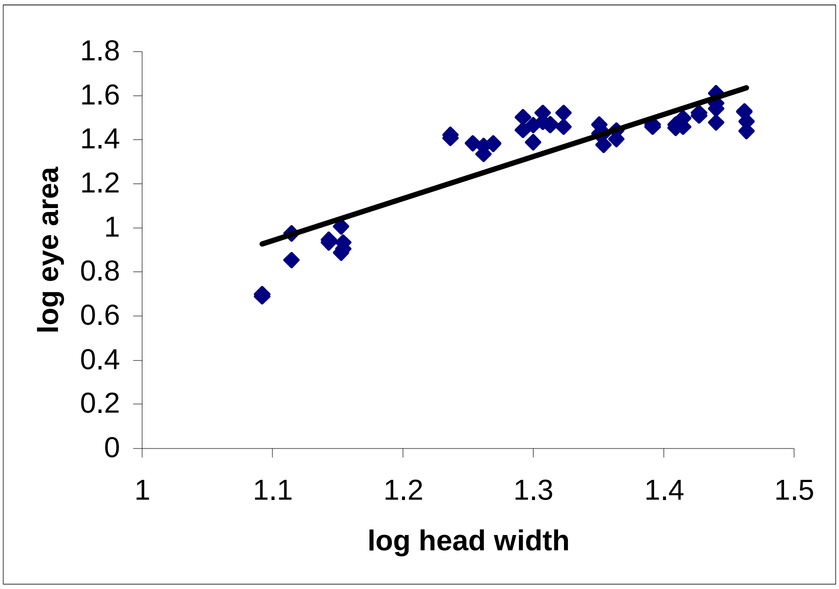

Fig. 3.9. Graph of log head width against log eye area.

When testing for differences between different *Agalychnis* species or between adult and young *A. calcarifer*, the data for adult male and adult female *A. calcarifer* were pooled. Differences in all measurements between species were tested using one-way ANOVA using followed by Tukey post hoc tests. Differences between male and female, and between young and adult *A. calcarifer* were tested using two sample *t* tests. In species with two colours to their iris, paired *t* tests were used to investigate differences in the proportions of these colours between the closed and the open eye. Significance was set at the 5% level.

3.4 RESULTS

3.4.1 Iris Metachrosis

The proportion of the bright peripheral colours visible when the eye was open was significantly greater than when the eye was closed in both *A. calcarifer* (t = -28.012, P < 0.001) and *A. craspedopus* (t = -21.243, P <0.001) (Fig. 3.10, 3.11). The proportion of the dull central colour visible was significantly smaller when the eye was open than when the eye was closed in *A. craspedopus* (t = 3.549, P = 0.002, 3.11). In *A. craspedopus*, however, the portion of dull central colour was significantly greater when the eye was open than when it was covered by the eyelid (t = -5.047, P = 0.001, Fig. 3.11).

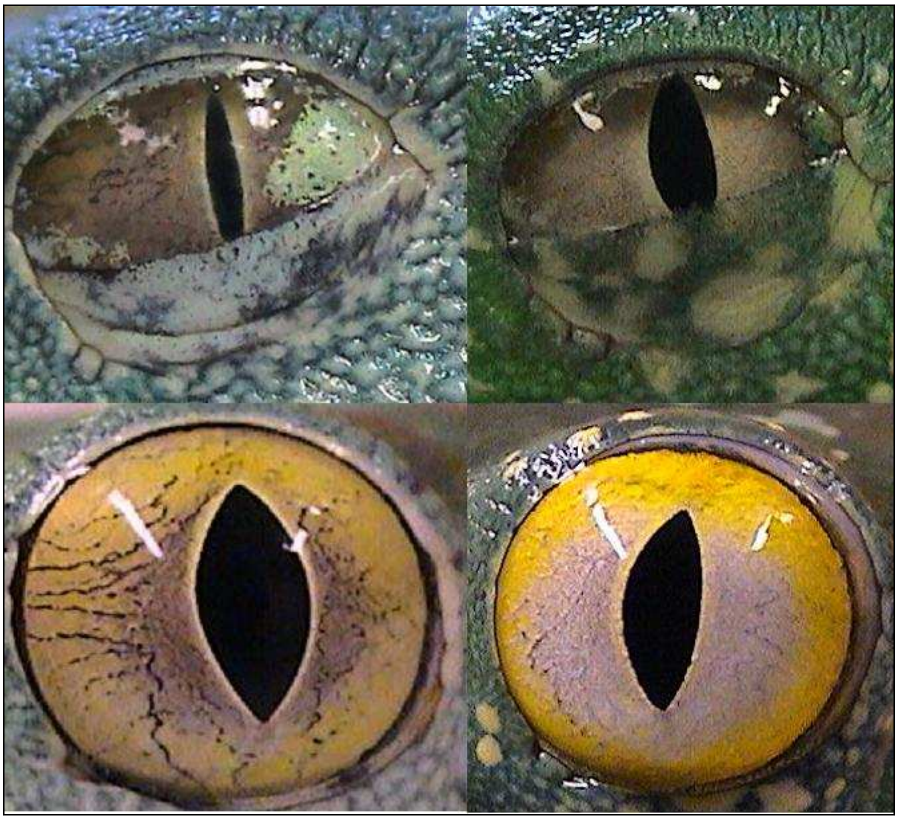

Fig. 3.10. The eye of *A. calcarifer* (left) and *A. craspedopus* (right) when closed (above) and open (below). Note that a much greater proportion of bright peripheral colour is visible when the eye is open than when it is closed.

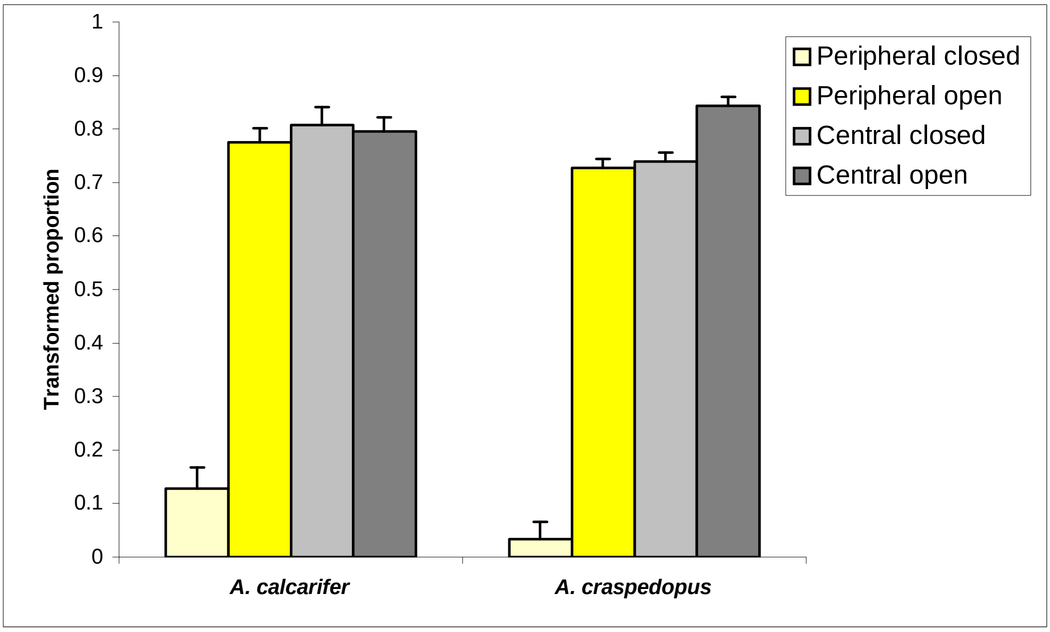

Fig. 3.11. Proportion of the total eye area occupied by the bright peripheral and dull central iris colours when the eye was both closed and open. Error bars represent standard errors.

Observations indicated that the border of the bright and dull coloured pigment remained static in relation to the centre of the pupil as the eye opened. This had to be estimated visually, as the phenomenon was rapid, and no clear photographs could be taken. Therefore, no quantitative data could be collected on the mechanism of metachrosis.

3.4.2 Differences Between Species

The lower portion of the lower eyelid is opaque in all species examined (Fig. 3.12). It is also patterned in *A. calcarifer* and *A. craspedopus*, as is the skin surrounding the eye and covering the rest of the body. These patterns have been termed lichen markings by some authors (Duellman, 1970, Hoogmoed and Cadle, 1991). The markings tend to form more distinct shapes in *A. craspedopus* than *A. calcarifer*. These markings are absent on the lower portion of the lower eyelid in *A. callidryas*, as they are absent from the rest of its skin. The proportions of the nictitating membrane and the lower portion of the lower eyelid are given in Fig. 3.13.

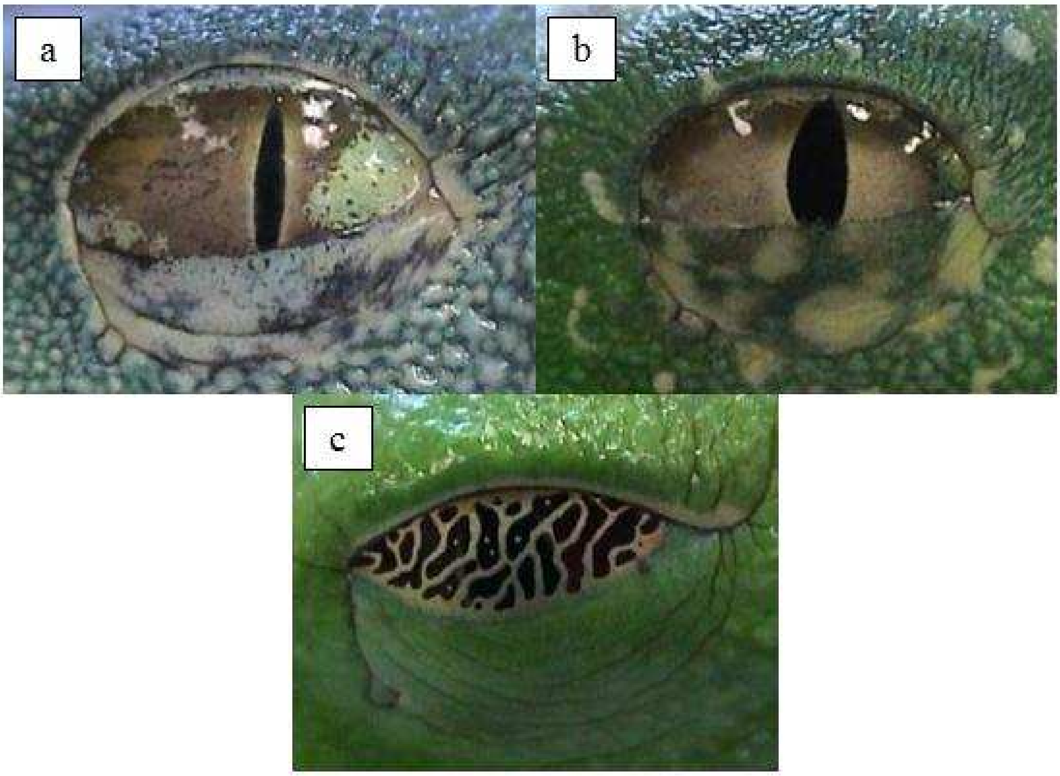

Fig. 3.12. The lower eyelid and skin surrounding the left eye of a) *A. calcarifer*, b) *A. craspedopus*, and c) *A. callidryas*.

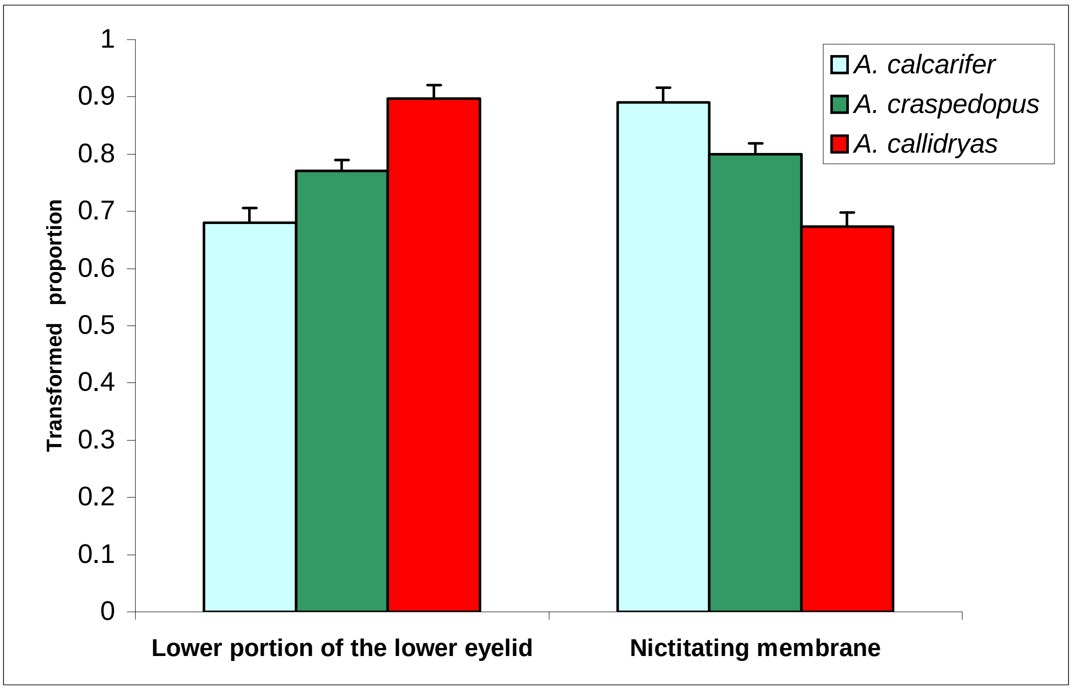

Fig. 3.13. Proportion of the lower eyelid made up of the lower portion and the nictitating membrane, in the three different species. Error bars represent standard errors.

There was a significant difference between the proportions of the lower eyelid occupied by the nictitating membrane in the three species ($F = 26.068$, $P < 0.001$). *A. calcarifer* had a significantly larger nictitating membrane than *A. craspedopus* ($P = 0.016$) and than *A. callidryas* ($P < 0.001$), and *A. craspedopus* had a significantly larger nictitating membrane than *A. callidryas* ($P = 0.003$). The lower portion of the lower eyelid displayed the inverse trend.

The nictitating membrane consisted of transparent and opaque portions. There was a significant difference in the extent of these portions between the species ($F = 18.485$, $P < 0.001$, Fig. 3.14).

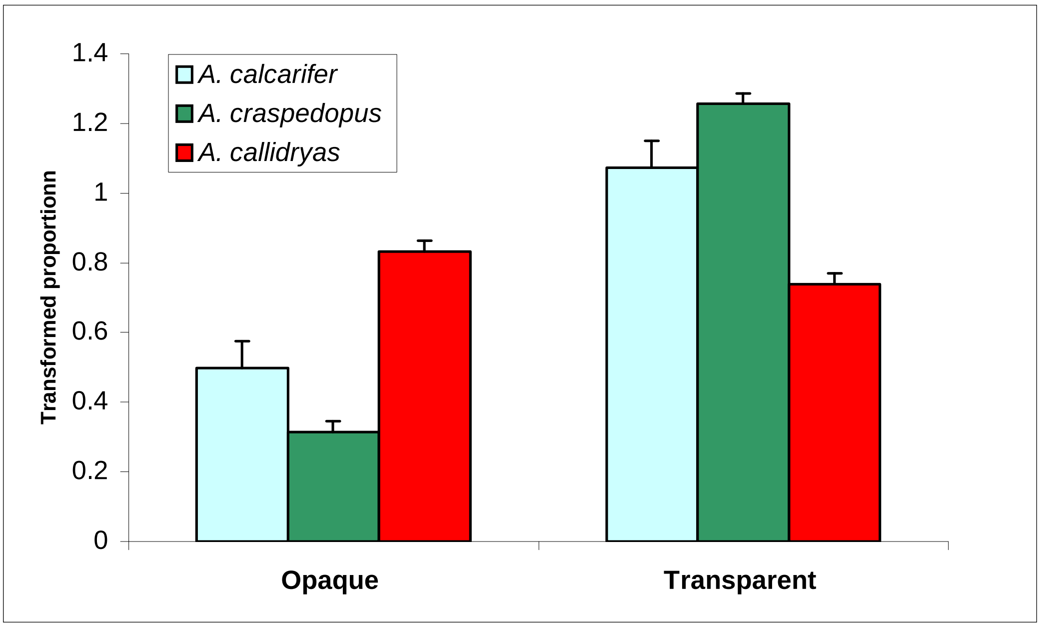

Fig. 3.14. Proportions of the nictitating membrane made up of opaque and transparent portions for the three different species. Error bars represent standard errors.

The transparent portion was more extensive in *A. craspedopus* than *A. calcarifer* ($P = 0.049$) and than *A. callidryas* ($P < 0.001$), and more extensive in *A. calcarifer* than *A. callidryas* (P <0.001). The opaque portion shows the inverse trend.

There was a significant difference in eye area index between species ($F = 16.908$, $P < 0.001$, Fig. 3.15). *A. callidryas* had a significantly larger eye area index than *A. craspedopus* ($P < 0.001$) and *A. calcarifer* ($P < 0.001$), although there was no significant difference between *A. calcarifer* and *A. craspedopus* ($P < 0.005$). *A.*

calcarifer had a significantly smaller eye area index than both *A. craspedopus* (*P* < 0.001) and *A. callidryas* (*P* < 0.001), but there was no significant difference between *A. craspedopus* and *A. callidryas* (*P* < 0.05).

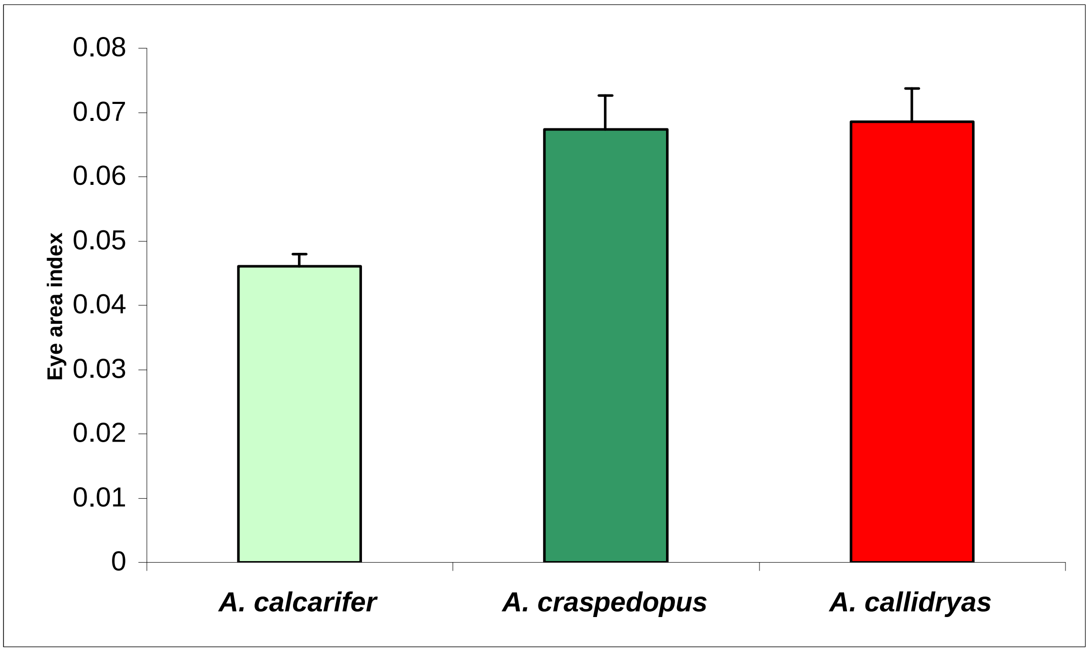

Fig. 3.15. Eye area indices of the three different species. Error bars represent standard errors.

The eye protrusion index also differed significantly between the species (*F* = 19.031, *P* < 0.001, Fig. 3.16). The eye of *A. calcarifer* protruded significantly less than that of *A. craspedopus* (*P* < 0.001) and *A. callidryas* (*P* < 0.001). There was no significant difference between the protrusion indices of *A. craspedopus* and *A. callidryas* (*P* > 0.005).

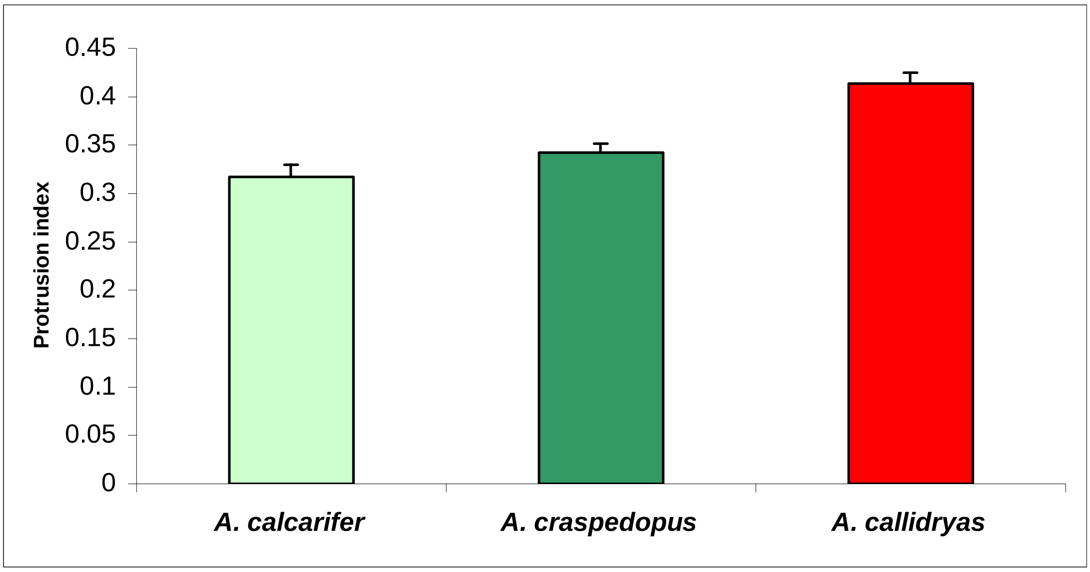

Fig. 3.16. Protrusion indices of the three different species. Error bars represent standard errors.

The iris of *A. calcarifer* and *A. craspedopus* was composed of two colours: a peripheral bright yellow colour and a central dull grey colour. There was no significant difference between *A. calcarifer* and *A. craspedopus* in the proportion of bright peripheral colour visible when the eyes were closed (t = -1.428, P > 0.05) or when the eyes were fully open (t = 1.026, P > 0.05). However, the proportion of dull central colour did differ between the two species. When the eyes were closed a significantly greater proportion of dull central colour was visible in *A. craspedopus* than *A. calcarifer* (t = -8.551, P < 0.001), but when the eyes were open the proportion of central colour was significantly greater in *A. calcarifer* than *A. craspedopus* (t = 8.551, P < 0.001) (Fig. 3.17).

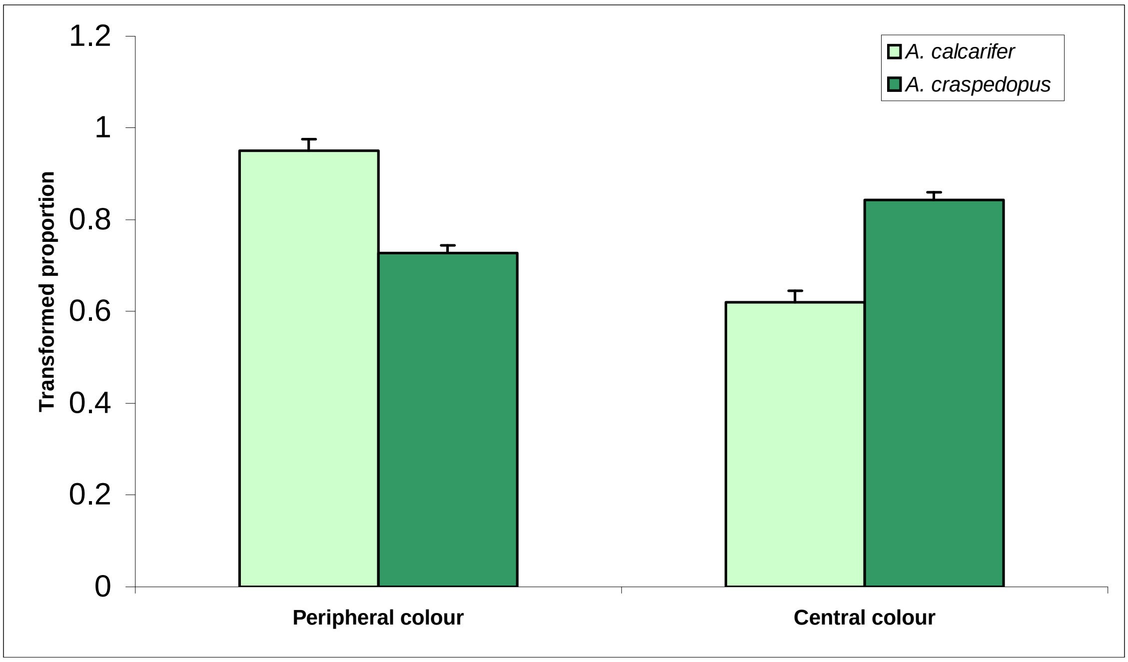

Fig. 3.17. Proportions of the total eye area occupied by peripheral and central colour in the eyes of *A. calcarifer* and *A. craspedopus* when the eye is open. Error bars represent standard errors.

3.4.3 Differences Between Sexes of A. calcarifer

There was no significant difference in the proportions of the lower eyelid occupied by the lower portion (t = -1.258, P > 0.05) or the nictitating membrane (t = 1.258, P > 0.05) between male and female *A. calcarifer*. Similarly, there were no significant differences between the proportions of the nictitating membrane that were opaque (t = 0.088, P > 0.05) or transparent (t = -0.088, P > 0.05). The eye area index was, however, significantly greater in male than female specimens (t = 2.444, P = 0.036). There was no significant difference in the protrusion index of the sexes (t = -1.887, P > 0.05). Similarly, there were no significant differences between the sexes in the proportions of the bright peripheral colour when the eyes were closed (t = -1.995, P > 0.05) or open (t = -1.518, P > 0.05), or dull central colour when the eyes were closed (t = 1.518, P > 0.05) or open (t = -1.518, P > 0.05). Both males and females displayed a significantly greater proportion of bright yellow colour visible when the eyes were open than when the eyes were closed (males: t = -25.236, P < 0.001; females: t = -22.832, P < 0.001). The proportion of dull central colour visible was significantly smaller when the eyes were open than when the eyes were closed (males: t = 3.264, P = 0.014; females: t = 2.661, P = 0.026).

3.4.4 Differences Between Developmental Stages of A. calcarifer

There was no significant difference between adult and young *A. calcarifer* in the proportion of the lower eyelid occupied by the lower portion (t = -2.045, P > 0.05) or the nictitating membrane (t = 2.045, P > 0.05). However, the proportion of the nictitating membrane that was transparent was significantly greater in young than adult *A. calcarifer* (t = 3.088, P = 0.005, Fig. 3.18). The proportion of the nictitating membrane that was opaque followed the inverse trend (t = -30.88, P = 0.005).

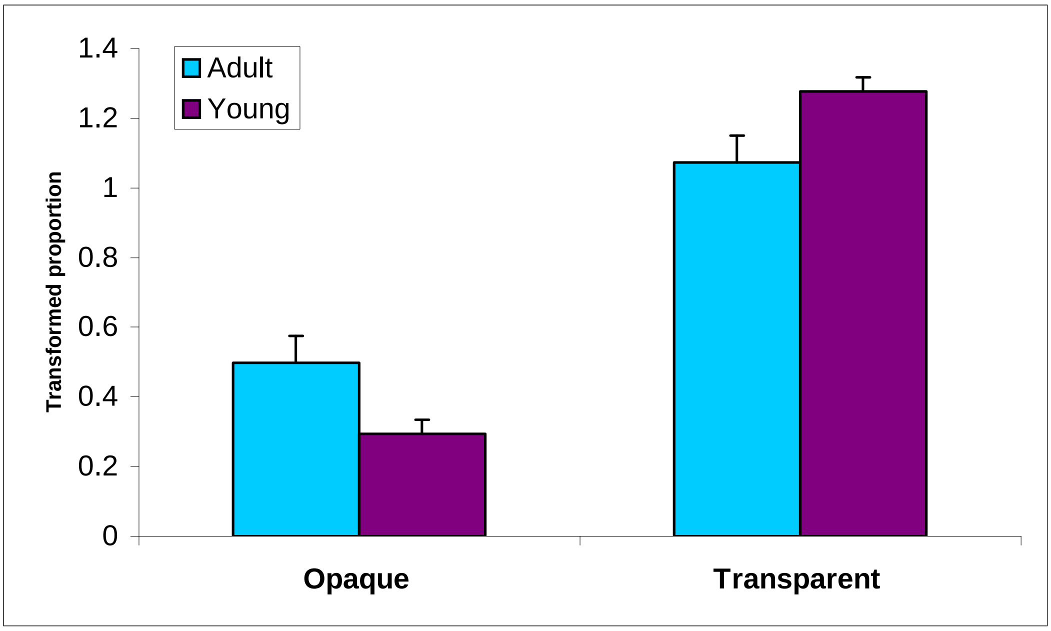

Fig. 3.18. Proportion of the nictitating membrane that is transparent and opaque in adult and young *A. calcarifer*. Error bars represent standard errors.

There was no significant difference between adult and young *A. calcarifer* in the eye area index (t = -1.223, P > 0.05) or the protrusion index (t = 0.877, P > 0.05). There was no significant difference between young and adult *A. calcarifer* in the proportion of bright peripheral colour visible when the eye was closed (t = 0.1227, P > 0.05). In contrast, young *A. calcarifer* displayed a significantly greater proportion of bright peripheral colour visible when the eye was open (t = 3.225, P = 0.004). Similarly, the proportion of dull central colour is significantly greater in young than adult *A. calcarifer* both when the eye is closed (t = -7.119, P < 0.001) and open (t = 7.119, P < 0.001). These differences are illustrated in Fig. 3.19.

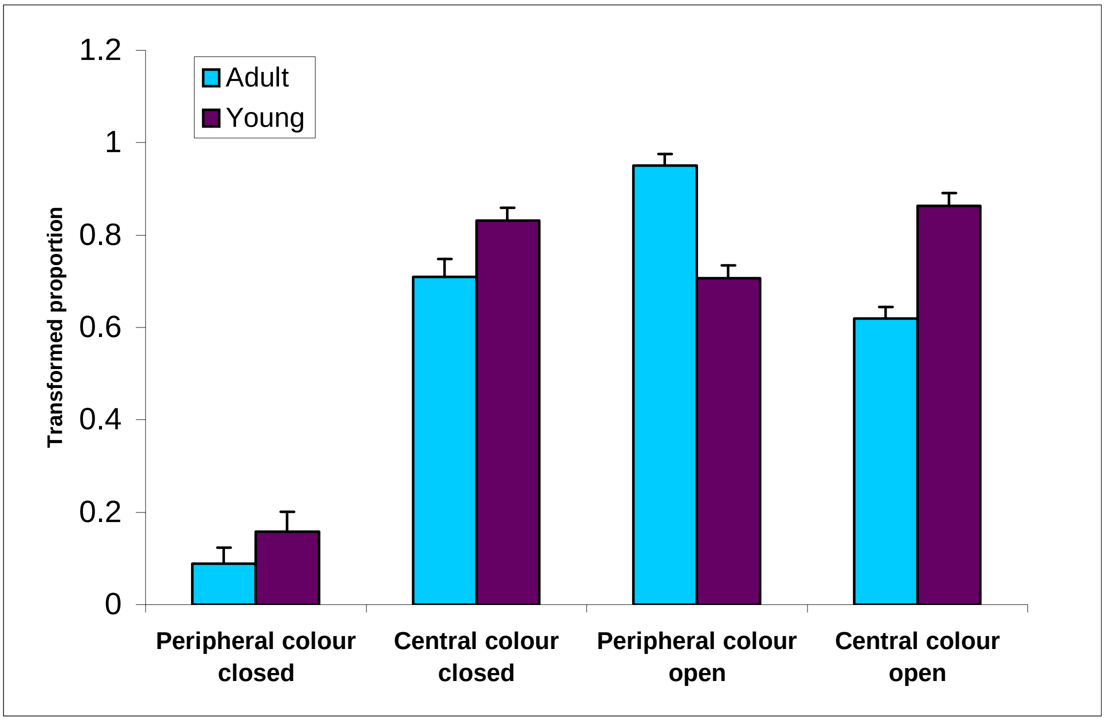

Fig. 3.19. Proportions of the total eye area occupied by peripheral and central colours when the eye is closed and when the eye is open in adult and young *A. calcarifer*. Error bars represent standard errors.

The proportion of the bright peripheral colour that is visible is significantly greater when the eye is open than when the eye is closed in both adult (t = -28.012, P < 0.001) and young (t = -15.755, P < 0.001) *A. calcarifer*. The proportion of dull central area is significantly smaller in the open eye than the closed eye of adults (t = 3.549, P = 0.002), but is not significantly different between the open and the closed eye in young *A. calcarifer* (t = -1.008, P > 0.05). This is illustrated in Fig. 3.20.

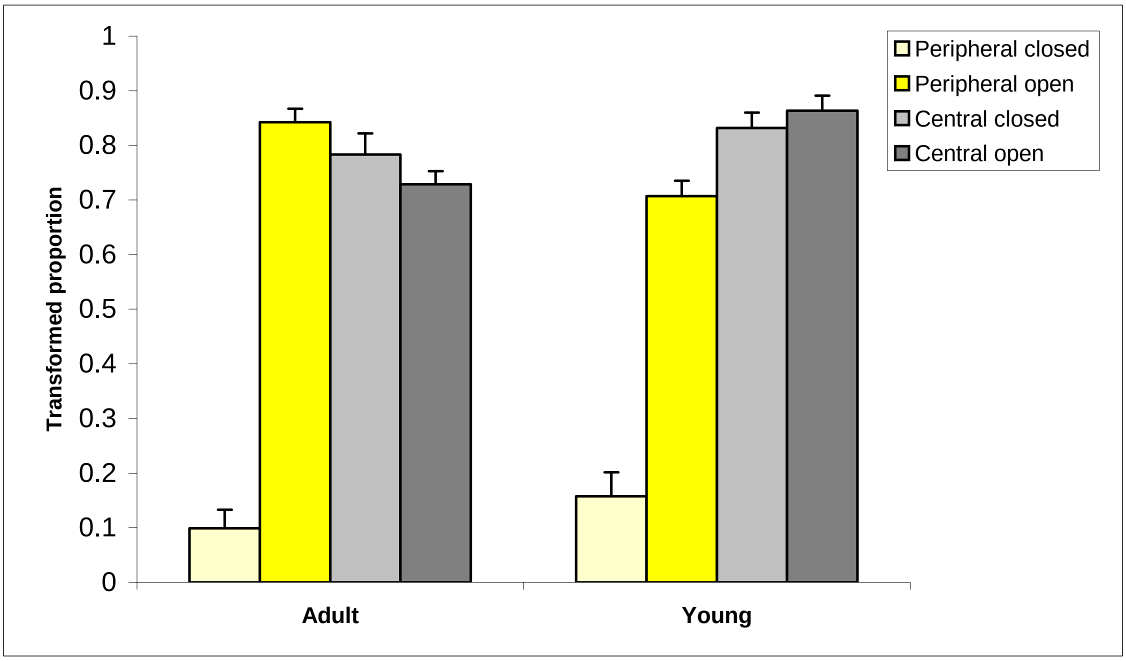

Fig. 3.20. Proportions of total eye area occupied by the peripheral and central colours when the eye is closed and when the eye is open for adult and young *A. calcarifer*. Error bars represent standard errors.

3.4 DISCUSSION

3.4.1 Apparent Iris Metachrosis

The apparent visible changes in iris colouration of *A. calcarifer* and *A. craspedopus* that were made in preliminary observations were quantified by the data collected in this study. The proportion of the total eye area occupied by the bright peripheral colour shows a significant increase when the eye is open relative to when it is closed (*A. calcarifer*: t = -28.012, P < 0.001, *A. craspedopus*: t = -21.243, P < 0.001; Fig. 3.10, 3.11). This was also observed in both sexes and developmental stages of *A. calcarifer* (male: t = -25.336, P < 0.001; female: t = 3.264, P = 0.014; young: t = -15.775, P < 0.001). This gives the appearance of iris metachrosis, but in fact the distribution of iris pigment appears to remain static regardless of how open the eye or the pupil is. If this is the case, the hypotheses that the pigment cells either migrate or change colour to bring about the apparent metachrosis as the eye opens seem unlikely. This indicates that the peripheral iris colour is present in a constant pattern, but is simply concealed when the eye is closed. It seems that as the eye opens, the peripheral region of the eye is revealed, displaying a greater area of the bright colour. As such, this phenomenon cannot be classified as metachrosis as such, as the pigment cells themselves are not modulated in any way.

3.4.2 Differences Between Species

Qualitative analysis of the features of the eyes of the three species suggests that the eyes of *A. calcarifer* and *A. craspedopus* are more similar to each other than to *A. callidryas*. *A. calcarifer* and *A. craspedopus* both share a number of features that differ from *A. callidryas* and other *Agalychnis* species: they have a grey central iris colour with a bright yellow periphery (Fig. 3.5). They also have markings on their nictitating membranes, and the lower portions of their lower eyelids (Hoogmoed and Cadle, 1991, Fig. 3.5). This contrasts with the other six members of the genus, who all have a solid iris colour and lack markings on the lower portions of their lower eyelids (Duellman, 1970, Fig. 3.5 Fig. 3.12). The mottled white to grey pattern of the opaque regions of the nictitating membrane of *A. calcarifer* and *A. craspedopus* is also unique in comparison to the gold pigmented reticulations of other *Agalychnis* frogs (Fig. 3.2 and Fig. 3.12). *A. calcarifer* and *A. craspedopus* are also the only members of the genus that have dermal flaps and black vertical bars on their flanks and barred thighs and upper arms (Duellman, 1970).

This distinctiveness of the morphology of *A. calcarifer* and *A. craspedopus* within the genus in which they are currently classified is reflected in other aspects of their biology, such as their breeding biology. Most phyllomedusines breed only during the rainy season, and oviposit on vegetation above ponds, temporary pools or streams (Caldwell, 1994, Hoogmoed and Cadle, 1991). They also fold vegetation around their eggs and sometimes deposit water capsules within the clutch to prevent desiccation. In contrast, *A. calcarifer* and *A. craspedopus* are the only *Agalychnis* that deposit small egg clutches exclusively above small pools of water formed in the trunks of fallen trees in primary forests, and do not deposit water capsules or practice leaf folding (Caldwell, 1994, Hoogmoed and Cadle, 1991). The breeding biology of *A. calcarifer* and *A. craspedopus* is so similar, in fact, that the two species have been interbred to produce fertile eggs and offspring that were viable until the tadpole stage (A. Gray, pers. comm.).

Several unpublished studies also imply that *A. calcarifer* and *A. craspedopus* are distinct within *Agalychnis*. Their skin secretions include several proteins that are not produced by other *Agalychnis* frogs (Gray, unpublished). Furthermore, analysis of the mitochondrial 16s rRNA gene of five species of *Agalychnis* frogs found strong support for the pairing of *A. calcarifer* and *A. craspedopus* within their own monophyletic group, separate from the remaining *Agalychnis* species, which formed a monophyletic group together (Kerfoot, unpublished).

Based on this body of evidence, it is suggested that *A. calcarifer* and *A. craspedopus* should be reclassified into a monophyletic genus, separate to other *Agalychnis* frogs. This is consistent with Duellman's (1970) hypothesis that these two species are believed to be evolved through geographical isolation from other *Agalychnis* stock, and Hoogmoed and Cadle's (1991) description of *A. calcarifer* and *A. craspedopus* being 'sister species'. This suggested revision is based on similar data to that used in the last major revision of the phyllomedusines, which used morphology including iris colour and pattern to reassign certain species into new taxa (Cruz, 1991).

The quantitative morphological data do not distinguish the three species so clearly, and the degree of similarity between the species is different for many characteristics measured.

3.4.3 Geographic Variation in Morphology

Minor differences in body colour and pattern is variable not only between individuals but between geographical regions. An example of this is the variation of the colour of the flanks and thighs of *A. callidryas*, which exhibits pale blue flanks in Mexico and Guatemala, and bark blue to purple flanks in Costa Rica and western Panama (Duellman, 1970). There may also be some geographic variation in the iris colouration of the study species. As the specimens were only collected from a single locality for each species, the morphological evidence given in this study should therefore be interpreted with caution. It alone may not be sufficient to suggest the taxonomic revisions proposed here, but because the eye morphology evidence is corroborated by further evidence based on external morphology, molecular biology, protein secretions and breeding biology, this suggested revision seems valid.

3.4.4 Digital Image Analysis as a Morphometric Tool

The use of digital image analysis made it possible to quantify the apparent iris metachrosis, which would have otherwise been extremely problematic. It was also used to investigate the way in which subtle characteristics such as the composition of small body parts such as frog eyelids differed between species. Although the technique was able to quantify these characteristics, they did not prove to be useful criteria for comparing species.

3.4.5 Differences Between Sexes of A. calcarifer

Male and female specimens of *A. calcarifer* were very similar. The only significant difference was that male specimens had a significantly greater eye area ration than females (t = 2.444, P = 0.026). Hylids in general display few morphological differences between the sexes, so this is to be expected (Duellman, 1970).

3.4.6 Differences Between Developmental Stages of A. calcarifer

The proportion of the nictitating membrane that was opaque was significantly greater in adults than young *A. calcarife*r (t = -3.088, P = 0.005, Fig.15). However, the proportion of the eye area that is occupied by bright peripheral colours was significantly greater in young than adult *A. calcarifer* when the eye was open (t = 3.225, P = 0.004, Fig. 3.19). Therefore young *A. calcarifer* are able to expose a relatively greater area of bright colour than adults, any yet they conceal it equally well when the eye is closed despite having a more transparent nictitating membrane.

3.4.7 The Role of Iris Colour In Agalychnis Frogs

The use of brightly coloured areas of the body as flash markings to startle predators has been reported in several species including anurans (Bradbury and Vehrencamp, 1998, Duellman and Trueb, 1994). It is possible that the bright iris of the *Agalychnis* frogs is used in the same way. All species of *Agalychnis* are capable of concealing their bright iris colour when at rest, and exposing the bright colour quickly. A bright iris for use as flash markings may be a useful anti-predator mechanism, but these bright colours must only be revealed when appropriate, to avoid making the animals more conspicuous. If bright eye colouration is used as an anti-predator flash marking system, this could explain why young *A. calcarifer* are able to expose relatively greater area of bright colouration than adults, as young animals are more susceptible to predators than adults. They therefore often have better anti-predator mechanisms than at later developmental stages (Bradbury and Vehrencamp, 1998).

The eye morphology of *A. calcarifer* and *A. craspedopus* appears distinct to that of other *Agalychnis* frogs, and it seems that these two distinct morphological groups represent two different approaches to the concealment of conspicuous bright flash markings of the iris. In *A. callidryas* and all other *Agalychnis* species with a solid eye colour, pigmented reticulation runs throughout the nictitating membrane, concealing the entire iris when at rest (Duellman, 1970, shown partially closed in Fig. 3.2). In contrast, the bright colour of the eye in *A. calcarifer* and *A. craspedopus* is restricted to the periphery of the iris, and it is therefore not necessary for these species to conceal the entire iris in order to conceal the bright colour. Consequently, the nictitating membrane of these species has no opaque reticulation, and a significantly smaller proportion of the nictitating membrane is opaque in comparison with *A. callidryas* (*A. calcarifer*: $P < 0.001$; *A. craspedopus*: $P < 0.001$). This may allow better vision for *A. calcarifer* and *A. craspedopus* than *A. callidryas* when the lower eyelid is covering the eye, so could aid predator detection.

There is, however, some opaque pigmentation on the nictitating membrane of *A. calcarifer* and *A. craspedopus*, although this is not reticulated. The pigmentation of the nictitating membrane of these species may serve to further obscure any bright iris colouration that is not covered by the skin when the eye is retracted. It may also obscure other conspicuous features of the eye, such as the pupil (Walls, 1942). They may alternatively be present on the nictitating membrane merely because lichen markings are present on the dorsum of *A. calcarifer* and *A. craspedopus*, and the absence of such markings on the eyelid would in itself make the eye conspicuous.

It is considered that the use of iris colour as flash markings to startle predators is a form of inter-specific communication (Bradbury and Vehrencamp, 1998, Duellman and Trueb, 1994). Another possibility is that the difference in the visible iris colour as the eye opens could be used for intra-specific communication. *A. calcarifer* is thought to use its bright body markings for intra-specific communication (Gray, 2001). Several other species communicate with conspecifics using temporal modulation of colour by briefly revealing brightly coloured body parts. An example of this is the use of the brightly coloured dew-lap of *Anolis* lizards, or the brightly coloured skin around the eyes of the roadrunner bird, both of which normally conceal these bright areas (Bradbury and Vehrencamp, 1998). Similarly, temporal modulation of the eye colour and pattern for the purpose of intra-specific communication has been described in the Atlantic salmon (*Salmo salar*) (Suter and Huntingford, 2002). However, as the colours visible in the iris of *A. calcarifer* and *A. craspedopus* change every time they open their eyes, it is difficult to see how this could be useful in any communication. Their role in flash colouration therefore remains their most likely role.

3.4.8 Conclusions

Apparent changes in the colour of the iris of *A. calcarifer* and *A. craspedopus* were able to be quantified using digital image analysis. A significant increase in the proportion of bright peripheral colours is observed in *A. calcarifer* and *A. craspedopus* as the eye opens. Observations suggest that this probably occurs by simply revealing more of the brightly coloured peripheral region of the eye as opposed to altering the distribution of the pigment.

Differences in the eye morphology of the three study species are discussed. They support evidence on breeding biology, protein secretions and molecular biology, supporting the suggestion that *A. calcarifer* and *A. craspedopus* should be reclassified into a separate genus to other *Agalychnis* frogs. Differences in the eye morphology between the study species and between the developmental stages of *A. calcarifer* can be explained in terms of their adaptive value. It is suggested that the bright eye colouration of *Agalychnis* frogs may serve as an anti-predator mechanism. Differences in eye morphology may indicate that *A. calcarifer* and *A. craspedopus* have adopted a different approach to the use of flash markings of the iris. Little evidence was found for sexual dimorphism in *A. calcarifer*.

GENERAL CONCLUSIONS

The population density, population size, and habitat preferences of the Buton macaque were assessed using DISTANCE analysis and frame quadrats. Results indicate that overall population density of the macaques in the protected forests of central Buton was as 14.9 individuals/km², and the total population size was 3,752. This population is likely to be viable. No habitat preferences could be identified, but the existing habitat appears adequate to support the species.

Data on morphology and behaviour suggest that the species of howler monkey present in the northwest Honduras is *A. palliata*. Microsatellite analysis was not successful. Group size appears smaller at the Cusuco National Park than reported elsewhere for howler monkeys, although this is difficult to confirm with the limited data available. The overall activity budget and daily activity pattern observed was similar to reports on populations from other countries.

Apparent iris metachrosis in *A. calcarifer* and *A. craspedopus* was able to be quantified using digital image analysis. This process is thought to occur by simply revealing a greater extent of the brightly coloured peripheral region of the eye as opposed to altering the distribution of the pigment. Differences in the eye morphology of the three study species are discussed. It is suggested that the bright eye colouration of *Agalychnis* frogs may serve as an anti-predator mechanism.

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