

**Full title: How does captivity affect skin colour reflectance of golden mantella frogs?**

**Running Title: Golden mantellas' skin colouration**

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## Abstract

Colouration is an important trait for social communication in amphibians, being used in intra- and intersexual signaling to express information about individual body condition, health state, amongst other things. The striking colour pattern exhibited by some anuran species are also used in “aposematic” signals to advertise unpalatability to predators. The aim of this study was to investigate how the captive environment affects the colour of golden mantella frogs by comparing captive reared frogs with wild conspecifics. A USB-2000 portable diode-array spectrometer and a xenon strobe light source were used to perform spectrophotometric measurements on captive and wild populations. Hue, chroma and brightness of skin colour were analyzed as well as body condition using the scaled mass index. Analyses showed variation among populations, but significant differences were only found between captive and wild populations. Generalized linear mixed models were used to evaluate the effects of body condition on colour variation and showed that animals with lower body condition from one captive population had significantly different colouration than their wild counterparts. Importantly, one captive population was not greatly different in colouration from their wild counterparts – demonstrating that this problem is not inevitable in captivity. These results can have important implications for reintroduction programmes.

## 39 Introduction

58           The current global amphibian crisis has resulted in an unprecedented rate of amphibian-  
59 biodiversity loss, resulting in a growth of captive breeding as a conservation tool for  
60 amphibians (Griffiths & Pavajeau, 2008). Maintaining captive populations is important in  
61 terms of species conservation for potential reintroduction into the wild (Harding, Griffiths &  
62 Pavajeau, 2016).

63           One of the biggest challenges faced while keeping amphibians in captivity is mimicking  
64 their diet and nutritional needs (Livingston et al., 2014) this can directly impact many aspects  
65 of the individual's ecology, including skin pigmentation (Brenes-Soto & Dierenfeld, 2014). It  
66 is common to observe amphibians kept in captivity displaying a faded colouration in  
67 comparison to their wild counterparts (Brenes-Soto & Dierenfeld, 2014). In amphibians, skin  
68 colouration influences courtship and mate preference, recognition of breeding partners and  
69 perception of fitness, consequently affecting breeding success, resulting in advantages for  
70 selective females and for strikingly coloured males (Brenes-Soto et al. 2017).

71           Skin colour may also inform about physiological conditions, such as oxidative and  
72 immune status (McGraw 2005;), and parasite load (Molnár et al. 2013). Carotenoid based  
73 colourations can be used to assess physiological and health status of an individual while  
74 colouration associated with melanin informs about hormonal levels and social dominance  
75 (Candolin 2003).

76           The remarkable colour patterns displayed by many anuran species (Hoffman & Blouin,  
77 2000) are also used as conspicuous “aposematic” indicators, warning signals to advertise  
78 unpalatability to potential predators (Hegna et al., 2013; Mann & Cumming, 2012; Ruxton et  
79 al., 2004). By reducing the frequency of costly encounters with predators, the protection that

aposematism confers can lower the costs of otherwise risky behaviours, including foraging and sexual displays (Dugas et al., 2015).

Divergent antipredator strategies such as aposematism not only require integration of physiology, morphology and behaviour; they also alter the way selection acts on other suites of traits (Stankowich & Blumstein, 2005). It is expected that, in some scenarios, an increase in the toxicity should also coincide with a greater visual contrast of the warning signal, with “nastier” animals “shouting loudest” (Mann & Cumming, 2012; Speed & Ruxton, 2007). This is because the greater risk of detection and attack on highly conspicuous prey can be compensated for by the stronger predator deterrence induced by high toxicity (Darst et al., 2006). A positive relationship may also emerge from physiological or energetic trade-offs between the two traits (Blount et al., 2012).

Colour refers to a sensory experience, not an objective quantity, and how animals perceive this information can vary quite considerably according to their visual system and how they process colour (Maia et al., 2013). The vertebrate retina contains two types of photoreceptor: rods that function at low light levels, and cones that function in daylight and provide the basis for colour vision. Colour vision requires at least two spectrally distinct classes of cone cells combined with a nervous system that can compare the quantum catch of one class of cone with the quantum catch of another. (Bowmaker and Hunt, 2006)

Animals have evolved their visual sensitivity to match aspects of their photic environment (Bowmaker and Hunt, 2006), with modern teleosts, reptiles and birds possessing rods and four spectral classes of cones each representing one of the five visual pigment families giving these species the potential for tetra chromatic colour vision. In contrast, mammals, due to their nocturnal ancestry, have rod-dominated retinas with colour vision reduced to a basic dichromatic system (Bowmaker, 2015).

The role of colouration and colour vision in animal communication has been a fundamental question in evolutionary biology for many decades (Maia et al., 2013). Colour is involved in a wide range of biological phenomena such as thermoregulation, crypsis, mimicry, communication as well as indicating health status of an individual (Endler, 1993; Forsman et al., 2002; Robertson & Rosenblum, 2009). Therefore, the quantification of animal colour variation is a crucial component of conservation and ecological studies. If captive animals are bred for conservation purposes and a reintroduction is a future goal, these issues are of major concern. The aim of this study was to investigate if the captive environment is affecting the colour of golden mantella frogs and, if so, to quantify this difference.

## Methodology

### Ethical Approval

All the research reported in this study was approved by the Ethics Commission of Chester Zoo, UK, and from the Research Ethics Committee from the University of Salford and it conforms to all regulations and laws in all relevant countries in relation to care of experimental animal subjects. To collect data from wild individuals, permission (permit) was obtained from the government of Madagascar. Furthermore, we can confirm, from our post-experimental monitoring, that no animals suffered any injuries, became ill or had their survivorship negatively affected because of this study.

### Study subject:

The golden mantella frog (*Mantella aurantiaca*) is a critically endangered species (Vence & Raxworthy, 2009), found only in Madagascar with a distribution restricted to a fragment of forest that is under severe threat from mining, agriculture, timber extraction and over-collecting for the pet trade (Randrianavelona et al., 2010). According to the Amphibian Ark, *ex situ* assistance is vital for the long-term survival of the golden mantella frog (Johnson,

2008). This is an ideal species to test the effects of captivity on colouration because the species is naturally only one consistent orange colour.

Study sites:

Mangabe area (Madagascar wild): Most breeding ponds for the golden mantella frogs are found in this area according to recent studies concerning conservation priority sites for mantella frogs. Also known as, the “blue forest”, Mangabe is a site of international biodiversity importance, divided between two administrative districts, Moramanga in the north and Anosibe An'ala to the south. Data sampling (15 males and 15 females) for this study was done in a protected area of the Moramanga region.

Ambatovy Mining Site (Madagascar wild): Ambatovy’s Mine is located within a species-rich region of Madagascar at the southern end of the remaining Eastern Forest Corridor at Moramanga region. As part of the Environmental Management Plan, there is a Conservation zone of native forest kept under pristine conditions by the mining company. During this study animals from the Conservation zone (15 males and 15 females) were sampled.

Mitsinjo Association Captive Breeding Centre (Madagascar captive): Mitsinjo Association is a community-run conservation organization. This is Madagascar’s first biosecure facility to safeguard amphibians from extinction, currently maintaining a genetically viable population of the golden mantella frog taken from the Ambatovy mining site (i.e., genetic founders), their offspring (F1) that are intended for reintroductions at artificially created breeding and natural ponds. Animals are kept in tanks with aquarium gravel as substrate, a potted plant, coconut shells for hiding. Animals were fed a variety of live invertebrates (fruit flies- *Drosophila sp.*, isopods – *Trichorhina sp.*, springtails - Collembolas). We sampled 8 males and 8 females founder frogs (i.e. wild caught) and the same number from their F1 frogs.

Chester Zoo (UK): Chester Zoo is actively involved in the conservation of the golden mantella frogs in Madagascar. The zoo currently maintains two *ex situ* groups of *M. aurantiaca*, one is

on public display at the Zoo's Tropical Realm exhibit and a second group is kept off show in a biosecurity container specifically for conservation-related research. Animals have been in captivity for more than 5 generations. Animals are kept in naturalistic tanks with different live species of plants, moss for substrate, water, hiding places under rocks and UV light. Animals are fed different live invertebrates (fruit flies- *Drosophila sp.*, isopods – *Trichorhina sp.*, springtails - Collembolas). We sampled 8 males and 8 females from the Chester Zoo off-show colony.

#### Spectrophotometric measurements

We used a USB-2000 portable diode-array spectrometer and a PX-2 xenon strobe light source (both from Ocean Optics, Dunedin, USA), probe positioned at an angle of 90°, to perform spectrophotometric measurements. To exclude ambient light and standardize measuring distance a cylindrical plastic tube was mounted on the fibre optic probe. The equipment permitted that the spectral analyses were conducted in the 300 and 700 nm range. Spectral reflectance measurements were always taken of each individual from the dorsum, three consecutive measurements per frog, only adult frogs during breeding season were used during this study. Colour measurements should sample the most visible surfaces to obtain a representative sample (within an individual) of the spectral shape of the entire body. Golden mantellas do not show any sexual dichromatism, allowing the use of both male and females. Summary variables for the colour measurements were calculated. Spectralon white standard measurements were taken between each individual to account for lamp drift. This methodology was based on previous studies measuring colour variation in different species (Crothers, et al., 2011; Maan & Cummings, 2008; Siddiqi et al., 2005).

#### Colour analyses

Colour may be described by three essential parameters: Hue, Chroma, and Brightness and all three variables were analysed as they are customarily used in studies of animal coloration, thereby facilitating comparisons between studies. Brightness ( $Q_t$ ) may be defined as the total intensity of light (Endler, 1990).  $Q_t$  was calculated by summing the percentage reflectance ( $R$ ) across the entire spectrum ( $R_{300}$  and  $R_{700}$ ).

Hue represents the common meaning of colour, for example, violet, blue, orange, green (Endler, 1990); In general, the hue of a spectrum is a function of its shape. Hue is correlated with the wavelength of the maximum slope, as well as the sign of the slope (Endler, 1990). It is the wavelength within the visible-light spectrum at which the energy output from a source is greatest Hue (nm) was measured as the wavelength of maximum reflectance.

Chroma is a measure of the ‘purity’ or ‘saturation’ of a colour and is a function of how rapidly intensity changes with wavelength (Endler, 1990). Chroma was calculated as relative medium wavelength chroma (MC, calculated as  $(R_{max} - R_{min})/Q_t$ ).

Brightness, Hue and Chroma differences between populations were analysed with a mixed model with origin (wild or captive) as fixed factors and populations as random factors. Data were analysed using the Pavo (Maia et al., 2013) package from R studio (R Studio Team 2015). The data from each population were plotted on the same graph to confirm standardization of sampling, no error from the sampling design was found. Data from different populations were compared based on colour distance and colorimetric variables. Four different visual systems: human, a snake (Boidae, Bowmaker, 2015), a Scincidae lizard (New et al., 2012), representing potential predators, and a diurnal poison frog (*D. pumilio*, Siddiqi et al., 2004) under an ideal illumination condition, were used to calculate colour distance. The colour distance analyses in Just Noticeable Distance units (JND) (Wen, 2012) - were used to infer if different visual systems would be able to notice differences between the different populations.



In general, when  $JND < 1$ , the spectral pair is barely distinguishable under ideal conditions, and as JND becomes greater, discrimination can be made more rapidly and under increasingly unfavourable viewing conditions (Siddiqi et al., 2004). Due to the lack of data on golden mantella photoreceptor sensitivity, sensitivity data used in the analysis were those from a species with similar activity pattern (i.e. aposematic diurnal).

## Body Condition

Body condition is a valuable index that can be assessed using reliable, non-invasive techniques, and it can identify the health condition of a population before any deleterious effects can be observed (MacCracken & Stebbing, 2012). Body condition (BC) was assessed using the scaled mass index proposed by Peig & Green (2009). This method is independent of size and can be used for comparison between different populations; those characteristics potentially make it superior to the traditional residual indices and, reportedly have worked well in amphibian studies (MacCracken & Stebbing, 2012, Michaels et al., 2014). The scaled mass index of condition (SMI) was calculated as follows:

$$SMI = M * [SVL_0 / SVL]^{bSMA}$$

Where  $M$  and  $SVL$  are the mass and snout-ventral length of the individual,  $SVL_0$  is the arithmetic mean  $SVL$  of the population, and  $bSMA$  is the standardized major axis slope from the regression of  $\ln$  mass on  $\ln$   $SVL$  for the population (Peig & Green, 2009). Each individual  $SVL$  was measured ( $\pm 0.01$ mm) using a digital caliper (Lujii 150mm, Omiky) mass was measured using a precision scale (accurate to 0.01g, Smart Weigh ACC200 AccuStar). Body condition was calculated for each individual and groups were compared using an ANOVA test followed up by a *post-hoc* test.

## Results

The colorimetric variables analysis showed no differences between wild and captive animals for brightness, but significant differences ( $p < 0.0001$ ) for hue and chroma were found.

The colour distance analyses (Table 1) showed that all visual system tested would be able to detect a noticeable difference when comparing wild individuals from Mangabe and with individuals from Mitsinjo, both founder and F1, on all visual systems used. The individuals from Chester Zoo and the animals from Mangabe had a low or non-detectable difference in the colour distance analyses.

Body condition analyses showed a significant difference between the groups ( $F = 7.109$ ,  $df = 7$ ,  $p < 0.001$ ). The *post-hoc* analyses confirmed a significant difference ( $p < 0.0001$ ) between the Mitsinjo group and all other groups. Frogs kept at Mitsinjo had a significantly lower body condition, both founder and F1.

Generalized linear mixed models were used to evaluate the effects of body condition on the chroma, and hue variation. Location was included as a random factor (chroma: variance 0.38, St. Dev.  $\pm 0.62$ , hue: variance 118.13, St. Dev.  $\pm 10.86$ ). The selected model with an Akaike information criterion (AIC) of 1195.1 for chroma and AIC of 332.80 for hue, showed that body condition had a strong impact on both chroma ( $F = 7.17$ ,  $df = 1$ ,  $p < 0.001$ ) and hue ( $F = 25.83$ ,  $df = 1$ ,  $p < 0.001$ ).

## Discussion

In this study, we showed that different populations of golden mantella frogs vary in colour, most notably between captive and wild conditions. In general, wild frogs were brighter, more colourful and were a different shade of orange/red in comparison to captive frogs, especially those from the captive populations in Madagascar (Figure 1). A relationship between lower body condition and duller colouration was also observed. The implication of

the differences observed could be negative survival or lower reproductive success if captive frogs were to be released to the wild (Rojas, 2016).

The hue comparison results showed that the golden mantella frogs' skin coloration has been affected by captivity with a significant difference when compared to wild conspecifics. However, during the colour distance analysis, the visual systems used would only have the ability to distinguish between animals kept at Mitsinjo and the other populations. The results showed that, even though, there were significant differences between all populations, most of them would be low or non-detectable by the visual systems tested, with the exception of the Mitsinjo colony. Both founders and F1 at Mitsinjo presented a colouration that differed significantly from their wild counterparts. This shows that the change in the skin coloration is not a generalized effect of captivity since frogs kept at Chester Zoo did not display such a dramatic change.

Results showed that the individuals kept at the Mitsinjo breeding centre had a much lower body condition than any other group. Body condition is a result of many variables including nutritional status, stress levels and abiotic variables (MacCracken & Stebbings, 2012). Replicating diverse diets in captivity creates a range of challenges including issues of environment, economics and practicality of insect husbandry (the main food item - Livingston et al., 2014). Animals tend to have nutritional imbalances, due to deficiencies of one or several nutrients; for example, low quantities of carotenoids (which are known to affect frog colouration) are common in commercially-reared insects (Finke, 2015).

A relationship between body condition and loss of skin coloration was also detected; animals with lower body condition also had a greater difference in skin colouration according to the colour distance analyses. Animal coloration is a product of different variables, including pigments obtained from the diet, such as carotenoids. Dietary carotenoids are associated with yellow, orange, and red coloration and increased levels can lead to brighter coloration and

changes in hue (Umbers et al., 2016). The consequences of limited carotenoid availability on ornamental coloration have been shown in both field and captive conditions (Hill, 2009; Brenes-Soto & Dierenfeld, 2014). Healthier animals in good body condition, after meeting their physiological needs of pigments for immune and antioxidant response, can accumulate enough pigments to increase colouration, maximizing sexual display (Hill, 2009). The preference of females for males with brighter colouration shows the choice for phenotypic quality connected with direct or indirect genetic benefits (Brenes-Soto et al., 2017). Striking colouration reveals an individual animal's ability to provide material advantages, such as fertility, high quality territory, nutrition and the maintenance of the genetic variation (Andersson & Simmons, 2006, Zamora-Camacho & Comas, 2019).

Previous studies have shown a clear link between skin colouration, body condition and health status in amphibians (Brenes-Soto et al., 2017), with animals with higher levels of glucose and protein concentration in the blood showing a darker yellow and orange colouration (Brenes-Soto et al., 2017). The faded colouration and lower body condition observed on animals kept at Mitsinjo breeding centre could be interpreted as a warning sign of animals' health conditions.

Alteration of pigmentation could hypothetically affect potential recognition of breeding partners, perception of fitness, and could thus have an indirect effect on health and reproductive output (Crothers et al., 2011, Brenes-Soto & Dierenfeld, 2014, Ogilvy et al., 2012). All these factors contribute to the complexity of maintaining the frogs' wellbeing in captivity (Speed & Ruxton, 2007).

Species recognition is a fundamental problem for animals in social contexts (Kraaijeveld-Smit et al., 2006); skin coloration is also involved in the sexual behaviour of many species. The colour distance analyses using the spectral sensitivity of a diurnal poison frog have

shown that frogs would be able to detect colouration differences. Diurnal species of amphibians, such as the golden mantella frogs, use visual signals as an important part of their courtship and mate selection (Mann et al., 2004), and bright colouration is an important one (Bowmaker, 2015). For example, females of different taxa prefer to mate with more colourful or brighter individuals (Maan & Cummings, 2008; Ogilvy et al., 2012). Releasing animals with different skin coloration could, potentially, compromise their breeding opportunities and, for a reintroduction to be successful, individuals released for conservation purposes must not only survive but also must breed (Giligan & Frankham, 2003; Mathews et al., 2005). If reintroduced animals survived, there is a chance that due to this phenotypic difference, captive animals may be more likely to mate with other captive-born animals, which could lead to producing two morphologically separate populations of animals (Slade et al., 2014). A low frequency of breeding between captive-bred and wild animals also means that no improvement of the wild population's genetic diversity and any possible negative genetic changes acquired in captivity would continue to be expressed in their offspring, limiting their fitness in the wild (Slade et al., 2014).

Aposematism is an important anti-predator strategy, which signals unprofitability of prey to a predator via conspicuous traits associated with unpalatability (Dreher et al., 2015;), Captive-raised frogs have no alkaloids detectable in skin extracts, these mantelline frogs are dependent on dietary sources for their skin alkaloids (Daly et al., 1997) making them unpalatable. Potential predators for the golden mantella frogs would be reptile species such as *Zonosaurus madagascariensis* and *Tamnosophis lateralis* (Jovanic et al., 2009). The colour distance analysis using a model of snake and a lizard visual system showed significant and highly detectable differences in the skin coloration of frogs from Mistinjo breeding centre in comparison to wild populations, suggesting that predators would be able to perceive these different colourations.

The colour distance analyses done using the human visual system (Bowmaker, 2015) demonstrated that keepers would be able to detect the different colouration in the animals they manage from wild conspecifics. This could be used as a measurement to select animals with greater colour similarities to the wild populations for reintroduction purposes. Colour charts are commonly used to evaluate colour scores of animals in zoos (Brenes-Soto & Dierenfeld, 2014); although this is a qualitative measurement, a species-specific colouration chart, could be produced and used as a health/management parameter. Frogs kept under optimal condition would have a more similar colouration to their wild counterparts; that is, a health check with less need to handle animals.

The *M. aurantiaca* is a critically endangered frog with reintroduction as part of its Species' Action Plan to help mitigate the environmental impacts on the species' natural distribution (Edmonds et al., 2015). It is important to consider the present results when thinking about releasing *M. aurantiaca* back to the wild. The aposematic coloration plays an important role on the behaviour and ecology of many species, making the results presented here important to consider when planning reintroductions. Pre-release assessment should also take into account the physical condition, and include a colour assessment as part of the process.

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494 Figure 1. Examples of skin colouration from the three groups of golden mantellas. A) Wild  
495 individuals from Mangabe; B) Chester Zoo off-show individuals; C) Mitisingo F1  
496 individuals.

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