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

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

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20   Abstract

21   Colouration is an important trait for social communication in amphibians, being used in intra-

22   and intersexual signaling to express information about individual body condition, health state,

23   amongst other things. The striking colour pattern exhibited by some anuran species are also

24   used in "aposematic" signals to advertise unpalatability to predators. The aim of this study was

25   to investigate how the captive environment affects the colour of golden mantella frogs by

26   comparing captive reared frogs with wild conspecifics. A USB-2000 portable diode-array

27   spectrometer and a xenon strobe light source were used to perform spectrophotometric

28   measurements on captive and wild populations. Hue, chroma and brightness of skin colour

29   were analyzed as well as body condition using the scaled mass index. Analyses showed

30   variation among populations, but significant differences were only found between captive and

31   wild populations. Generalized linear mixed models were used to evaluate the effects of body

32   condition on colour variation and showed that animals with lower body condition from one

33   captive population had significantly different colouration than their wild counterparts.

34   Importantly, one captive population was not greatly different in colouration from their wild

35   counterparts – demonstrating that this problem is not inevitable in captivity. These results can

36   have important implications for reintroduction programmes.

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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

80   aposematism confers can lower the costs of otherwise risky behaviours, including foraging and

81   sexual displays (Dugas et al., 2015).

82   Divergent antipredator strategies such as aposematism not only require integration of

83   physiology, morphology and behaviour; they also alter the way selection acts on other suites

84   of traits (Stankowich & Blumstein, 2005). It is expected that, in some scenarios, an increase in

85   the toxicity should also coincide with a greater visual contrast of the warning signal, with

86   "nastier" animals "shouting loudest" (Mann & Cumming, 2012; Speed & Ruxton, 2007). This

87   is because the greater risk of detection and attack on highly conspicuous prey can be

88   compensated for by the stronger predator deterrence induced by high toxicity (Darst et al.,

89   2006). A positive relationship may also emerge from physiological or energetic trade-offs

90   between the two traits (Blount et al., 2012).

91   Colour refers to a sensory experience, not an objective quantity, and how animals

92   perceive this information can vary quite considerably according to their visual system and how

93   they process colour (Maia et al., 2013). The vertebrate retina contains two types of

94   photoreceptor: rods that function at low light levels, and cones that function in daylight and

95   provide the basis for colour vision. Colour vision requires at least two spectrally distinct classes

96   of cone cells combined with a nervous system that can compare the quantum catch of one class

97   of cone with the quantum catch of another. (Bowmaker and Hunt, 2006)

98   Animals have evolved their visual sensitivity to match aspects of their photic

99   environment (Bowmaker and Hunt, 2006), with modern teleosts, reptiles and birds possessing

100   rods and four spectral classes of cones each representing one of the five visual pigment families

101   giving these species the potential for tetra chromatic colour vision. In contrast, mammals, due

102   to their nocturnal ancestry, have rod-dominated retinas with colour vision reduced to a basic

103   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 is 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,

138   2008). This is an ideal species to test the effects of captivity on colouration because the species

139   is naturally only one consistent orange colour.

140   Study sites:

141   Mangabe area (Madagascar wild): Most breeding ponds for the golden mantella frogs are found

142   in this area according to recent studies concerning conservation priority sites for mantella frogs.

143   Also known as, the "blue forest", Mangabe is a site of international biodiversity importance,

144   divided between two administrative districts, Moramanga in the north and Anosibe An'ala to

145   the south. Data sampling (15 males and 15 females) for this study was done in a protected area

146   of the Moramanga region.

147   Ambatovy Mining Site (Madagascar wild): Ambatovy's Mine is located within a species-rich

148   region of Madagascar at the southern end of the remaining Eastern Forest Corridor at

149   Moramanga region. As part of the Environmental Management Plan, there is a Conservation

150   zone of native forest kept under pristine conditions by the mining company. During this study

151   animals from the Conservation zone (15 males and 15 females) were sampled.

152   Mitsinjo Association Captive Breeding Centre (Madagascar captive): Mitsinjo Association is

153   a community-run conservation organization. This is Madagascar's first biosecure facility to

154   safeguard amphibians from extinction, currently maintaining a genetically viable population of

155   the golden mantella frog taken from the Ambatovy mining site (i.e., genetic founders), their

156   offspring (F1) that are intended for reintroductions at artificially created breeding and natural

157   ponds. Animals are kept in tanks with aquarium gravel as substrate, a potted plant, coconut

158   shells for hiding. Animals were fed a variety of live invertebrates (fruit flies- *Drosophila sp.*,

159   isopods – *Trichorhina sp.,* springtails - Collembolas). We sampled 8 males and 8 females

160   founder frogs (i.e. wild caught) and the same number from their F1 frogs.

161   Chester Zoo (UK): Chester Zoo is actively involved in the conservation of the golden mantella

162   frogs in Madagascar. The zoo currently maintains two *ex situ* groups of *M. aurantiaca*, one is

163   on public display at the Zoo's Tropical Realm exhibit and a second group is kept off show in a

164   biosecurity container specifically for conservation-related research. Animals have been in

165   captivity for more than 5 generations. Animals are kept in naturalistic tanks with different live

166   species of plans, moss for substrate, water, hiding places under rocks and UV light. Animals

167   are fed different live invertebrates (fruit flies- *Drosophila sp.*, isopods – *Trichorhina sp.,*

168   springtails - Collembolas). We sampled 8 males and 8 females from the Chester Zoo off-show

169   colony.

170

171   Spectrophotometric measurements

172   We used a USB-2000 portable diode-array spectrometer and a PX-2 xenon strobe light

173   source (both from Ocean Optics, Dunedin, USA), probe positioned at an angle of 90°, to

174   perform spectrophotometric measurements. To exclude ambient light and standardize

175   measuring distance a cylindrical plastic tube was mounted on the fibre optic probe. The

176   equipment permitted that the spectral analyses were conducted in the 300 and 700 nm range.

177   Spectral reflectance measurements were always taken of each individual from the dorsum,

178   three consecutive measurements per frog, only adult frogs during breeding season were used

179   during this study. Colour measurements should sample the most visible surfaces to obtain a

180   representative sample (within an individual) of the spectral shape of the entire body. Golden

181   mantellas do not show any sexual dichromatism, allowing the use of both male and females.

182   Summary variables for the colour measurements were calculated. Spectralon white standard

183   measurements were taken between each individual to account for lamp drift. This methodology

184   was based on previous studies measuring colour variation in different species (Crothers, et al.,

185   2011; Maan & Cummings, 2008; Siddiqi et al., 2005).

186   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 (Qt) may be defined as the total intensity of light (Endler, 1990). Qt was calculated by summing the percentage reflectance (R) across the entire spectrum (R300 and R700).

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 (Rmax – Rmin)/Qt).

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.

211   In general, when JND < 1, the spectral pair is barely distinguishable under ideal conditions,

212   and as JND becomes greater, discrimination can be made more rapidly and under increasingly

213   unfavourable viewing conditions (Siddiqi et al., 2004). Due to the lack of data on golden

214   mantella photoreceptor sensitivity, sensitivity data used in the analysis were those from a

215   species with similar activity pattern (i.e. aposematic diurnal).

216   Body Condition

217   Body condition is a valuable index that can be assessed using reliable, non-invasive

218   techniques, and it can identify the health condition of a population before any deleterious

219   effects can be observed (MacCracken & Stebbing, 2012). Body condition (BC) was assessed

220   using the scaled mass index proposed by Peig & Green (2009). This method is independent of

221   size and can be used for comparison between different populations; those characteristics

222   potentially make it superior to the traditional residual indices and, reportedly have worked well

223   in amphibian studies (MacCracken & Stebbing, 2012, Michaels et al., 2014. The scaled mass

224   index of condition (SMI) was calculated as follows:

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

226   Where M and SVL are the mass and snout-ventral length of the individual, SVL_0 is the

227   arithmetic mean SVL of the population, and bSMA is the standardized major axis slope from

228   the regression of ln mass on ln SVL for the population (Peig & Green, 2009). Each individual

229   SVL was measured (±0.01mm) using a digital caliper (Lujii 150mm, Omiky) mass was

230   measured using a precision scale (accurate to 0.01g, Smart Weigh ACC200 AccuStar). Body

231   condition was calculated for each individual and groups were compared using an ANOVA test

232   followed up by a *post-hoc* test.

233   Results

234   The colorimetric variables analysis showed no differences between wild and captive

235   animals for brightness, but significant differences (p<0.0001) for hue and chroma were found.

236   The colour distance analyses (Table 1) showed that all visual system tested would be

237   able to detect a noticeable difference when comparing wild individuals from Mangabe and with

238   individuals from Mitisnjo, both founder and F1, on all visual systems used. The individuals

239   from Chester Zoo and the animals from Mangabe had a low or non-detectable difference in the

240   colour distance analyses.

241   Body condition analyses showed a significant difference between the groups (F=7.109, df=

242   7, p<0.001). The *post-hoc* analyses confirmed a significant difference (p<0.0001) between the

243   Mitsinjo group and all other groups. Frogs kept at Mitsinjo had a significantly lower body

244   condition, both founder and F1.

245   Generalized linear mixed models were used to evaluate the effects of body condition

246   on the chroma, and hue variation. Location was included as a random factor (chroma: variance

247   0.38, St. Dev. ± 0.62, hue: variance 118.13, St. Dev. ± 10.86). The selected model with an

248   Akaike information criterion (AIC) of 1195.1 for chroma and AIC of 332.80 for hue, showed

249   that body condition had a strong impact on both chroma (F=7.17, df= 1, p<0.001) and hue

250   (F=25.83, df= 1, p<0.001).

251

252   Discussion

253   In this study, we showed that different populations of golden mantella frogs vary in

254   colour, most notably between captive and wild conditions. In general, wild frogs were brighter,

255   more colourful and were a different shade of orange/red in comparison to captive frogs,

256   especially those from the captive populations in Madagascar (Figure 1). A relationship

257   between lower body condition and duller colouration was also observed. The implication of

258   the differences observed could be negative survival or lower reproductive success if captive

259   frogs were to be released to the wild (Rojas, 2016).

260   The hue comparison results showed that the golden mantella frogs' skin coloration has

261   been affected by captivity with a significant difference when compared to wild conspecifics.

262   However, during the colour distance analysis, the visual systems used would only have the

263   ability to distinguish between animals kept at Mitsinjo and the other populations. The results

264   showed that, even though, there were significant differences between all populations, most of

265   them would be low or non-detectable by the visual systems tested, with the exception of the

266   Mitsinjo colony. Both founders and F1 at Mitsinjo presented a colouration that differed

267   significantly from their wild counterparts. This shows that the change in the skin coloration is

268   not a generalized effect of captivity since frogs kept at Chester Zoo did not display such a

269   dramatic change.

270   Results showed that the individuals kept at the Mitsinjo breeding centre had a much

271   lower body condition that any other group. Body condition is a result of many variables

272   including nutritional status, stress levels and abiotic variables (MacCracken & Stebbings,

273   2012). Replicating diverse diets in captivity creates a range of challenges including issues of

274   environment, economics and practicality of insect husbandry (the main food item - Livingston

275   et al.,2014). Animals tend to have nutritional imbalances, due to deficiencies of one or several

276   nutrients; for example, low quantities of carotenoids (which are known to affect frog

277   colouration) are common in commercially-reared insects (Finke, 2015).

278   A relationship between body condition and loss of skin coloration was also detected;

279   animals with lower body condition also had a greater difference in skin colouration according

280   to the colour distance analyses. Animal coloration is a product of different variables, including

281   pigments obtained from the diet, such as carotenoids. Dietary carotenoids are associated with

282   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.

332   The colour distance analyses done using the human visual system (Bowmaker, 2015)

333   demonstrated that keepers would be able to detect the different colouration in the animals they

334   manage from wild conspecifics. This could be used as a measurement to select animals with

335   greater colour similarities to the wild populations for reintroduction purposes. Colour charts

336   are commonly used to evaluate colour scores of animals in zoos (Brenes-Soto & Dierenfeld,

337   2014); although this is a qualitative measurement, a species-specific colouration chart, could

338   be produced and used as a health/management parameter. Frogs kept under optimal condition

339   would have a more similar colouration to their wild counterparts; that is, a health check with

340   less need to handle animals.

341   The *M. aurantiaca* is a critically endangered frog with reintroduction as part of its Species'

342   Action Plan to help mitigate the environmental impacts on the species' natural distribution

343   (Edmonds et al., 2015). It is important to consider the present results when thinking about

344   releasing *M. aurantiaca* back to the wild. The aposematic coloration plays an important role

345   on the behaviour and ecology of many species, making the results presented here important to

346   consider when planning reintroductions. Pre-release assessment should also take into account

347   the physical condition, and include a colour assessment as part of the process.

348   Acknowledgements

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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) Mitisinjo F1

496   individuals.

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