Short Note

Melanophore metachrosis response in amphibian tadpoles: effect of background colour, light and temperature

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Abstract. The developmental and biochemical mechanisms of colour change through chromatophore metachrosis in amphibian tadpoles are relatively well studied, but the environmental factors driving colour change remain unclear. A cryptic response to background colour in order to reduce predation is an intuitively valid explanation, however, other hypotheses need to be explored. In this study, we aimed to investigate the environmental factors driving the melanophore metachrosis process in *Alytes dickhilleni* tadpoles. First, we tested the response to two backgrounds with clearly distinct reflectance: black and white. The proportion of dark tadpoles became significantly higher when they were located on the black background, and pale tadpole proportion was dominant on the white background, as expected from the crypsis hypothesis. Secondly, we added two new factors, temperature and photoperiod, maintaining the background variation. Our results suggest that lower temperatures, and short photoperiods were significantly driving a change to dark colouration in tadpoles, possibly allowing a more efficient thermoregulation, and in consequence, development and growth. Next we tested whether dark and pale tadpoles selected backgrounds that matched their colouration (black and white background), and found no evidence for behavioral selection. The apparent response in colour change to background appears to be mediated by the background reflectance of light, that there does not seem to be behavioral selection of matching background by the tadpoles, and therefore it suggests that color change is more likely to be a physiological response with thermoregulatory implications.

Keywords: behavioral, melanin, melanosome, phenotypic plasticity, thermoregulation.

Information on environmental factors affecting the colouration of amphibian tadpoles is scarce and incomplete. This information is better developed, including genetics and evolution, in several model organisms like birds or fishes. We thus need a better understanding of ontogeny,

diversity and functions of colouration in amphibian tadpoles (Thibaudeau and Altig, 2012). Excluding descriptive notes, studies about the functions, including crypsis, thermoregulation, ultraviolet protection, and modification of social interactions of colouration of amphibian larvae, are rare (Caldwell et al., 1982)

Amphibians, unlike other vertebrates (e.g. mammals), have melanophores (containing melanin that produce browns and blacks), xanthophores (containing purine and pteridine pigments), and iridophores (containing crystalline purines). These cells arise from some trunk neural-crest cells that migrate dorsolaterally during development (Olsson and Löfberg, 1992). In amphibians, the pigment is located in organelles called melanosomes, pterinosomes, and reflecting platelets, found in each of these cell types respectively (Frost et al., 1984).

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These organelles have the ability of being rearranged within the cells, allowing metachrosis (i.e. change colour and pattern) (Thibaut et al., 2012).

Melanophores are mainly responsible of pigment patterns observed during the first larval stages (Parichy, 1996). A larger quantity of organelles per cell or pigment per organelle results in a higher intensity of black in melanophores. In addition, melanosomes can aggregate within the cell body or disperse radially by means of microtubules (Nascimento et al., 2003) when stimulated by melanocyte stimulating hormone, epinephrine or melatonin (Odenthal et al., 1996).

Excluding studies of biochemistry and cellular mechanism of pigmentation, there is not enough information to understand the influence of colouration on behavioral ecology of tadpoles. Behavioral consequences of ontogenic colour changes are known in only a few cases (Nilsson et al., 2013). Some forms of sociality can often be involved in colouration, promoting the behavioral changes. Several studies show that tadpoles can preferentially recognize and associate with others of their own species, using colour for conspecific recognition (Foster and McDiarmid, 1982; Blaustein, 1998).

Sometimes colour differences among tadpoles of the same species in adjacent ponds are obvious and until recently, these differences were assumed to be caused by light regimes, turbidity and tannin content (Oshima, 2001). The inducible colour response varies among individuals, and variations can also be mediated by the presence of predators and competitors, tadpoles with modified colours proving to have a selective advantage in predatory encounters (McClure and McCune, 2003). Our aim in this study is to assess the effect of some factors: substratum albedo influence (white or black background), light period, temperature, and behavioral selection of background. For this purpose, we used the primitive family Alytidae (Pough

et al., 2016) in order to get an example of implications of melanophore metachrosis in basal anuran species.

Our first hypothesis is that albedo of substrate is influencing aggregation of melanosomes of anuran larvae, and thus, influencing the colour of the *Alytes* tadpoles. As for our next hypothesis, we aim to test two points: the role of environmental temperature in colour change, and to evaluate if there is a possible effect of colour in body temperature (in case of same environmental temperature) and thus, in larvae thermoregulation. We also test whether photoperiod has a significant role in color change (a longer photoperiod means a longer exposition to radiation). In addition, we tested whether different coloured tadpoles selected different substrate background in relation with their colour.

The model species selected for this experiment was the Betic midwife toad (*Alytes dickhilleni*). This is a basal anuran (Pough et al., 2016), with a tadpole that can reach relatively large size (max. 6 cm) and with a prolonged larval period before reaching metamorphosis (from 3 to 16 months depending on temperature). Tadpoles ($n = 38$) were obtained from an artificial pool at Calar Alto (-2.52 W, 37.24 N; 1833 m; Almería province, southeastern Spain). Larvae (Stage 30; Gosner, 1960) were maintained in two 10 l containers in the same room, with dechlorinated water and the same background (grey). Changes of water was implemented every two days, maintaining it well oxygenated with an air pump. Larvae were fed *ad libitum* once per day with rabbit vegetable pellets (protein: 11.10%; fibre: 18.14%; fat: 7.05%; calcium: 0.25%, Iron: 0.07%), and acclimatized to 18°C (Guerrero-Gómez et al., 2019) in order to slow down the metamorphosis, with a 12-12 h photoperiod (elevation of the lab 7 m over sea level).

After a week of acclimatization, larvae were classified to one of two colour morphs: dark or pale. We established a natural, biological marker as criteria for the assignation: Tadpoles

with the characteristic “V” of the species visible on the top of the body were classified as pale (see supplementary fig. S1), and tadpoles with a colouration that was not pale enough to see the “V” were classified as dark (supplementary fig. S1). This colouration classification can be complemented with observation of melanophores with stereomicroscope, so colouration can be easily assigned to one or other stage.

Then, tadpoles were randomly assigned to one of two treatments: One container ($n = 19$ tadpoles; 10 pale and 9 dark) was located over a black background (albedo = 0.18) and the other ($n = 19$ tadpoles; 9 pale and 10 dark) over a white background (albedo = 0.75). In the laboratory, tadpole containers were kept 25 cm apart on the same shelf. For one week, the number of pale or dark larvae (see supplementary fig. S1 for classification criteria) were recorded in each treatment allowing us to obtain the temporal change of colouration each day. At the end of this week pictures of pigment patches in the tail were taken using a Leica Stereo Microscope MZ10 F. In order to confirm our results, as a control, these experiments were repeated inverting the background of the containers after a week, repeating the same procedure (hereinafter, we will call this experiment reversion experiment).

With the aim of understanding the role of tadpole colour on thermoregulation, first, we measured superficial body temperature of each tadpole (using a no-contact Fluke® Precision Infrared Thermometer 572). To avoid the bias of reflectance in infrared measurements we also tested temperature with a cloacal mercury thermometer (Miller and Weber Inc., New York, USA) placed in the back of the body in a subsample of the animals, and reflectance was fitted for dark or pale colourations. In addition, in order to ensure we were measuring animal temperature and not the superficial water temperature, the temperature of water in the tanks was verified. Later, we implemented a gradual 6°C room temperature reduction (2°C

per day to avoid possible thermal shock) until reaching 12°C (final temperature). Temperature of the tadpoles maintained in the same background treatments were recorded as described above. With this final test, we aimed to observe if within the same substratum treatment there were differences in the response under different temperature conditions. The temperature of this last test was selected considering the average day temperature of a natural pond with presence of the species (HOBO Pendant G temperature Data Logger-UA-004-64 placed in natural water bodies of origin).

In order to investigate the role of light in the process of colour change, we implemented a final test dividing each background treatment in two photoperiod treatments, one with 12D-12L h lighting and one with 24 h lighting, resulting in four treatments (white background 12 h of light, $n = 10$; white background 24 h of light $n = 9$, black background 12 h of light, $n = 10$; and black background 24 h of light, $n = 9$). The source of light was artificial (fluorescent tube). Room and water temperature were maintained at 18°C during all the experiments (water temperature was reset to 18°C in the four days preceding this experiment in order to allow acclimatization), and containers under 12 h lighting were covered by panels to avoid influence of any residual light of the 24 h treatment lamp.

A behavioral test for substratum colour preference was conducted after the initial colour change experiment: We placed the tadpoles in a 10 l container with four patches (two black and two white, see supplementary fig. S1), and we tested the colour selection of both groups (dark tadpoles, $n = 19$; pale tadpoles, $n = 19$). We tested the response under two scenarios, response of the whole group and individual response. For each group response, we photographed and counted individually the larvae of each treatment ($n = 19 + 19 = 38$) located in black or white patches during one-hour periods until completing 5 observations. In the case of individual response, we selected individual tadpoles of both treatments and released them in

the container, recording the data after one-hour periods.

Statistical analyses were implemented using R Statistical software (R Core Team, 2013). McNemar's test (a test used on paired nominal data in order to detect changes in time) was used to test for colouration change, reversion, behavioral group and single behavioral test. For this we constructed the 2×2 contingency matrix with the dichotomic response of body colour and time for each treatment. The Mann-Whitney U test (quantitative response in the case of body temperature) were implemented in order to test this last hypothesis. Dependent variables were Colour (proportion of dark tadpoles vs pale tadpoles) for colour change and photoperiod experiments, and predictor variables being background colour treatment, and background colour treatment + photoperiod respectively. In the case of body temperature experiments the dependent variable was body temperature, and the predictor was tadpole colouration. In the behavioral test the predictor was background colour treatment and the dependent variable was colour selection.

In the case of colour change experiments, results show a significant increment of dark tadpole proportion when they are placed on black background and an increment of pale tadpoles on white background (fig. 1). Results of reversion experiments were also significant under both treatments (change to pale colouration with white background and change to dark colouration with black background). We also observed significant differences in body temperature (0.49°C of mean temperature) between tadpoles of different body colour ($p < 0.001$), with a higher temperature in dark tadpoles ($18.75^\circ\text{C} \pm 0.15$; pale tadpoles: 18.25 ± 0.21) with the same water temperature (18°C). Differences for colour change in the decreasing temperature scenario were also significant (fig. 1).

The pictures of tail patches show a clear response in the melanophore patterns (fig. 1). For pale tadpoles we can observe small isolated dots (melanosomes are aggregated at the nucleus),

and for dark tadpoles we can observe larger dots (melanosomes are dispersed throughout the cell body and dendritic processes), creating a colour stain (supplementary fig. S2).

In the case of the photoperiod influence, we found that the proportion of pale tadpoles increased significantly at longer lighting periods ($p\text{-value} < 0.005$). This indicates that the longer photoperiod may cause a shift towards pale colouration in tadpoles at the same temperature and background treatments (fig. 2).

For both, group and individual behavioural substratum selection test we did not find any significant results, with $p\text{-values}$ of 0.09 (group test) and 0.51 (individual test).

Numerous groups have evolved constitutive colour patterns as adaptation to the habitat, however the fixed pigmentation pattern of some organism might be disadvantageous when they face environmental heterogeneity (Magellan and Swartz, 2013). There are several evolutionary responses to this issue, as a colouration constituting a compromise between the requirements of the differing microhabitats (Merilaita et al., 1999), or the evolution of different morphs, each one adapted to a different part of the environment (Bond, 2007). Finally, another evolutionary possibility is the capacity of colouration plasticity (West-Eberhard, 2007).

Our results show a clear plastic response of *A. dickhilleni* tadpoles to background colouration (mediated throughout albedo). This response occurs within days (fig. 1) and at first sight may be considered an efficient cryptic adaptation. Polo-Cavia and Gomez-Mestre (2016) describe a similar response in the urodele *Lissotriton boscai*, but slower than the observed in our experiments. Polo-Cavia et al. (2016) also found background colouration matching in adults of another species of the same genus, *Alytes obstetricans*, but the environmental factors leading to pigmentation change were not studied. The pictures of tail patches of *A. dickhilleni* also show a clear change in melanophores due to the aggregation or dispersion of melanosomes, a process known as metachrosis (Nascimento et

Figure 1. Percentage of dark coloured larvae in each treatment in initial (A), reversion (B) and temperature experiments (C); including average colour change rate per day (CCRD). Initial colour change experiment black background ($\chi^2 = 10$; $p = 0.005$), white background ($\chi^2 = 10$; $p = 0.005$); Reversion experiment white background ($\chi^2 = 15$; $p = 0.005$), black background ($\chi^2 = 15$; $p = 0.005$); temperature experiment ($p = 0.024$). We provide, in addition, section of larval tail crest at white substratum treatment (D) and black substratum treatment (E), with details of black coloured patches. We can observe the effect of melanosome aggregation (D) or dispersion (right) in the melanophore cell. Black scale is 1 mm.

Figure 2. Percentage of colouration response to background and photoperiod treatments at 18°C. Black bars for dark tadpoles and grey bars for pale tadpoles. P-values < 0.05 for both, white background treatment and lack background treatment.

al., 2003). Additionally, in our study we show how temperature, a physical environmental variable, is capable of influencing these physiological processes. We have to consider that our experiments were conducted in only two aquaria, and although aquaria were located in the same room with the same conditions, it is possible that some uncontrolled factor could affect this non-independent sample. It would be interesting to conduct these same experiments with one container for each tadpole. In addition, colour change and reversion experiments were conducted immediately after each other, and this implies that there is a small difference in developmental stage of tadpoles between the experiments. However, these differences were of one Gosner stage at most, and thus, never entering in the metamorphosis process.

Thermal conductivity in water is high and tadpoles are completely immersed in it, therefore, heating and cooling rates tend to be very rapid (Wells, 2007). Traditionally, it has been considered that larval stages of amphibians are not capable of any physiological adjustments that result in an alteration of thermoregulation (Bartholomew, 1982). Consequently, it has been accepted that the only way that an aquatic amphibian can alter its body temperature is to move between microhabitats in response to local differences in water temperature. Our results

contradict in part this observation because tadpoles of our study species can slightly modify their body temperature throughout melanophore metachrosis and thus, colour change. These subtle differences in temperature (0.4°C) may result in significant changes in tadpole growth and development as some studies have concluded (Sanuy et al., 2008; Castano et al., 2010)

Our results also suggest that photoperiod may influence melanophore metachrosis, with a tendency to a paler colouration in the longest period of light. Therefore, we have two main factors affecting this phenomenon of colour change: temperature and photoperiod. Background also is influencing colour change, and we suggest this is a consequence of different light refraction among substrates. Melanophore metachrosis seems to be influencing the thermoregulation of the tadpoles of this species through different light reflection, and in consequence, slight body temperature changes. Large photoperiods (with more radiation received during the day) and increased temperatures induce a pale colouration response and reduces body temperature. Whereas, shorter photoperiods or lower body temperature result in darkening of tadpoles.

Our results suggest that background colour selection can influence body temperature, and that is not a behavioural crypsis response, at least not in the absence of predator pressure.

Future studies should include similar observations with and without the presence of predators in order to evaluate behavioural responses in this scenario. Some studies have assessed behavioural physiological and morphological responses under predatory pressure (Zamora-Camacho et al., 2019). Polo-Cavia and Gomez Mestre (2016) have also assessed the influence of predator presence on background colour selection in newt larvae, with a behavioural response detected. Therefore, we consider it as important variable in future studies.

As conclusions, in addition to crypsis, melanophore metachrosis may have important implications in the thermoregulation of this species. At lower temperatures or short photoperiods, the dark colouration increases the retention of radiation and thus, improve heating of tadpoles. Interestingly, we found that tadpoles had no behavioural preference for white or black background. It is important to test these trends in other species of the same genus and tadpoles of other species. The effect of temperature changes associated with change in coloration on metabolism or developmental rate remain to be studied.

Acknowledgements. We want to acknowledge Mercedes Conradi Barrena for allowing us to use the stereomicroscope of her lab. This magnificent tool was not only critical for producing figure 1, but also helped in the inspiration in the study. Juan M. Pleguezuelos made comments to an earlier version that helped to improve the manuscript.

Ethical standards. Authors have all permits for capture, maintenance, and experimentation with these organisms. Permits were provided by Dirección General de Medio Natural, Biodiversidad y Espacios Protegidos (Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible. Junta de Andalucía; Ref: SGYB/AF 12-09-2019).

Supplementary material. Supplementary material is available online at:
<https://doi.org/10.6084/m9.figshare.12927461>

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Submitted: May 7, 2020. Final revision received: August 28, 2020. Accepted: September 3, 2020.
Associate Editor: Julian Glos.