



## Light-induced changes in pigmentation through ontogeny in cane toad tadpoles (*Rhinella marina*)

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Light-induced pigmentation changes are widespread among tadpole species. In this study we characterised light-induced changes in melanin dispersion through development in cane toad tadpoles (*Rhinella marina*). We describe a pattern of light-induced pigmentation change in which tadpoles are darker in the presence of light and lighter in darkness until the onset of metamorphosis. This pattern contrasts with that of other non-bufoiid tadpole species, but mimics the pattern of pigment change exhibited by embryonic anurans. We discuss our results in light of the ontogenetic changes in toxicity to evaluate the photoresponse of cane toad tadpole pigments as a potential aposematic signal, neotenic trait, and thermal adaptation.

*Key words:* aposematic, *Bufo marinus*, camouflage, melanophore, ontogeny, pigment changes, toxicity

Colour and pigmentation are used by animals in a variety of contexts. While many organisms develop colouration patterns that are fixed throughout their life, individuals of some species have the ability to change their colouration in response to, for example, higher predation risk (reviewed in Caro, 2005) and changing environmental conditions (Garcia et al., 2003, 2004; Kats & van Dragt, 1986). Among vertebrates, colour change can be achieved physiologically by actively moving the pigment within chromatophores (known as metachrosis) or morphologically by altering the amount of pigment in chromatophores or the number of chromatophores present in the integument (Oshima, 2001). Within physiological colour change, the movement of pigments may be driven by photo receptors such as eyes (secondary colour response; Oshima, 2001), or by photosensitive pigments found within chromatophores (primary colour response; Laurens, 1915; Bagnara, 1965). Melanin, a brown-black pigment, is a common light-sensitive pigment involved in colour changes of ectothermic vertebrates. The aggregation of melanin into the centre of melanophores results in skin lightening, while its

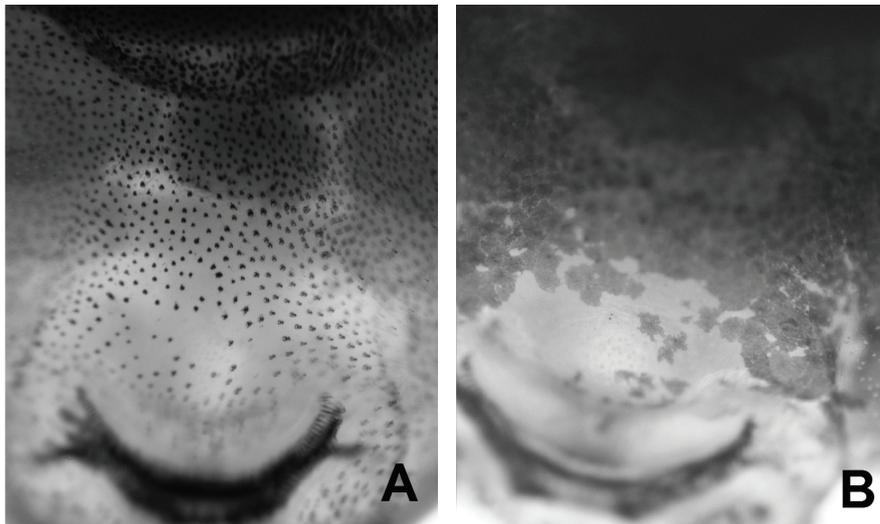
dispersal makes the skin appear darker (Visconti & Castrucci, 1993).

Light-induced change in tadpole pigmentation, a primary colour response, corresponds with diel light cycles and is widespread throughout anuran taxa (Bagnara & Hadley, 1973; Altig & McDiarmid, 1999a; Viertel & Richter, 1999). Such light-induced pigmentation changes, however, are not constant throughout development. In many anuran species, embryos exposed to light appear dark given the induced changes in melanin dispersion (e.g., *Xenopus laevis*, Obika & Bagnara, 1963), while later-stage tadpoles from the same species appear dark in the absence of light (Bagnara, 1974; Binkley et al., 1988). This pigmentation change is thought to prevent the reflection of moonlight off the tadpoles' lower layer of iridophores at night, increasing crypticity (van der Lek, 1967).

In this study, we investigate how light-induced pigmentation changes of cane toad (*Rhinella marina*) tadpoles vary through ontogeny. The black colouration of cane toad tadpoles has been proposed to be an aposematic signal linked to their unpalatability to most predators (Wassersug, 1971; Peterson & Blaustein, 1991). Conspicuous black colouration in the presence of light, however, is unexpected given that other non-bufoiid tadpole species appear lighter in the presence of light. We experimentally investigate the photoresponse of cane toad tadpole colouration. We report an unusual pattern of light-induced colouration changes, and discuss potential causes underlying this pattern within aposematic, neotenic and thermal frameworks.

Cane toads are a large (up to 1.25 kg, 230 mm) neotropical bufoiid whose native range extends from central Brazil to southern Texas (Zug & Zug, 1979), and have been introduced to many locations in the Pacific and Caribbean (Lever, 2001). Tadpoles for this study were originally bought from Carolina Biological Supply, bred from adults collected from introduced populations in Florida. Female cane toads are highly fecund, with each female producing up to 84,000 eggs (Lampo & Medialdea, 1996). The eggs hatch within 24–72 hours, and tadpoles

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**Fig. 1.** Gular region of *Rhinella marina* tadpoles at Gosner stage 33 in the A) dark-adapted and B) light-adapted treatments, representing melanophore index values of 1 and 5, respectively.

metamorphose in 142–148 days (Tyler, 1989; Hearnden, 1991; Bayliss, 1995). All life stages of the cane toad are toxic and contain bufadienolides. However, eggs contain a high concentration of bufadienolides, which then decreases during tadpole development and increases again after metamorphosis (Hayes et al., 2009).

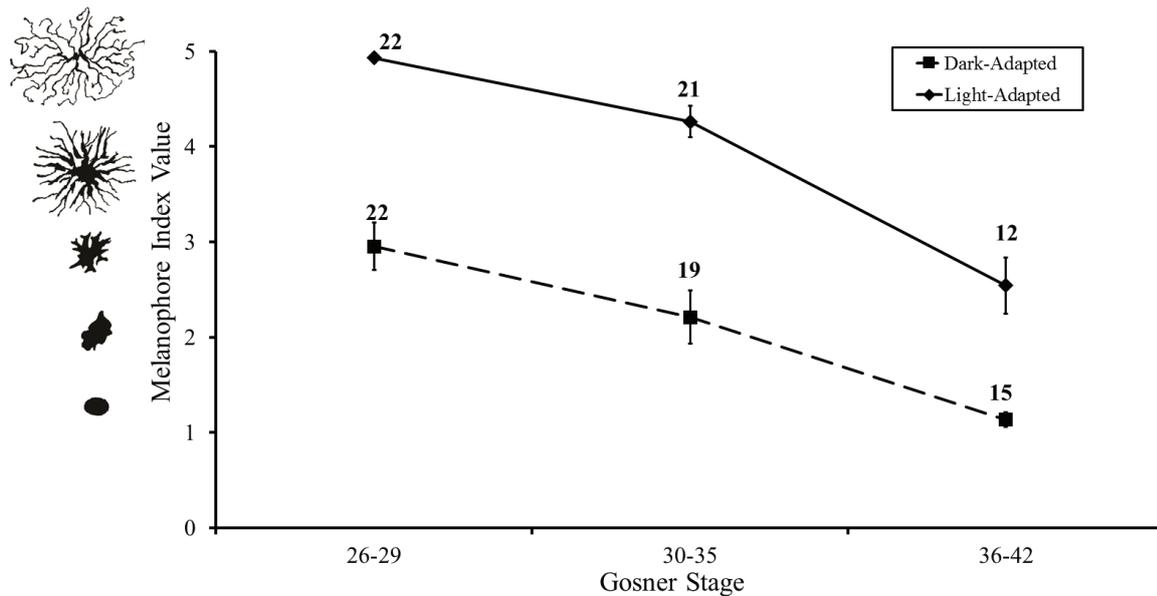
To investigate light-induced pigment changes in cane toad tadpoles throughout ontogeny, we examined melanin dispersion of light- and dark-adapted tadpoles from Gosner stage 26 (immediately after gill absorption) through 42 (emergence of forelimb buds; Gosner, 1960). We divided the tadpoles into three groups (Gosner stages 26–29, 30–35 and 36–42) to match previous work (Hayes et al., 2009). Tadpoles were housed in 40 L glass aquaria under a 10:14 light (945 lux): dark (0.05 lux) photoperiod and were fed with algae (*Spirulina*) flakes *ad libitum*.

Trials were held twice weekly, during which ten tadpoles were randomly selected from aquaria and only tested once. Tadpoles were equally divided into two treatment groups (dark-adapted and light-adapted), and each individual was placed in a small plastic pill container (3 cm x 4 cm) containing 1 cm of dechlorinated water. Dark-adapted tadpoles were placed in an opaque dark chamber (16.3 cm x 16 cm x 9.7 cm), without light source (0.15 lux). Light-adapted tadpoles were placed in an identical chamber under ambient, laboratory light (723 lux), including both natural light from a window and an artificial source of light (GE #F32T8-25W-SPEX41-ECO). All tadpoles were tested during daylight between 1400 and 1800 hours. Tadpoles were left in each light environment to acclimate for 15 min following Daniolos et al. (1990), and then rapidly moved one at a time under a Nikon SMZ-745T dissecting microscope. Images from the dissecting microscope were transferred onto a computer screen via a Nikon DS camera control unit DS-L2. Preliminary observations of melanin dispersion were conducted on non-focal cane toad tadpoles. Pigment movements on the gular region, where individual melanophores can easily be distinguished, were representative of pigmentation changes on the dorsum and tail. Therefore, only photos of the gular region of the tadpoles were captured and used for analysis.

For each tadpole, we quantified the degree of melanin dispersion using the Hogben-Slome melanophore index (Hogben & Slome, 1931), and recorded Gosner stage. All photos were evaluated by a blind observer and ten photos were scored twice to estimate repeatability. While the intracellular movement of melanin is influenced by temperature and predation risk in other amphibian larvae (Garcia & Sih, 2003; Garcia et al., 2004), our experimental tadpoles experienced a constant temperature and predator-free environment. Thus, our results solely reflect the influence of light and ontogeny on melanin dispersion. Differences between the light- and dark-adapted treatments were analysed using a bootstrapped *t*-test in R (R Core Team, 2013). To account for a possible interaction between light treatment and developmental stage, we ran a nonparametric alternative to an ANCOVA that converted melanophore index scores to ranks, averaging the rank of tied values (Conover & Iman, 1982).

There was a marked difference in melanin dispersion between the light- and dark-adapted treatments throughout development. Melanin pigments in dark-adapted tadpoles were more aggregated (dark-adapted  $N_{\text{total}}=56$ , light-adapted  $N_{\text{total}}=55$ ; Fig. 1A), resulting in lower melanophore index values. Conversely, melanin pigments of light-adapted tadpoles were highly dispersed (Fig. 1B) and scored higher on the melanophore index. Melanophore index values from images were highly repeatable ( $R=0.84$ , 95% CI=0.83–1.0). There was a significant effect of light treatment on melanophore index values for all stage groups until the emergence of forelimbs at stage 43 ( $p<0.001$ ; Fig. 2). The rank-transformed ANCOVA revealed that both developmental stage and treatment had a significant effect on melanophore index score (stage:  $F_{1,1}=90.8$ ,  $p<0.0001$ ; treatment:  $F_{1,1}=144.6$ ,  $p<0.0001$ ) with a marginally significant interaction effect among them ( $F_{1,1}=3.9$ ,  $p=0.0502$ ).

While several studies have shown that anuran tadpoles have light-induced changes in colouration (e.g. Binkley et al., 1988; Iga & Bagnara, 1975; Moriya et al., 1996), here we report that cane toad tadpoles exhibit melanin dispersion in the presence of light and the aggregation of melanin in darkness throughout the majority of their larval development – a pattern similar to only the



**Fig. 2.** Melanophore index values ( $\pm 1$  SE) for each stage group of *Rhinella marina* tadpoles in light- and dark-adapted treatments. Numbers above points indicate the sample size for that treatment group in each treatment. The images to the left of the melanophore index value axis represent the degree of melanin dispersion indicative of each melanophore index value. Images adapted from Hogben & Slome, 1931.

embryos of other anuran species. To our knowledge, this is the first study to reveal such a pattern of light-induced changes in pigmentation in anuran larvae.

Given that cane toad tadpoles demonstrate the same light-induced patterns of pigment change as the embryos of other anuran species, the absence of an ontogenetic switch in the pattern of pigment change may represent a neotenic trait. Neotenic traits – the retention by adults of traits previously only seen in young (see Reilly et al., 1997) – in anurans are limited, and restricted to changes in rates of growth and development. Examples include delayed metamorphosis in *Pseudis paradoxa* (Fabrezi, 2011) and tadpoles of *Lithobates catesbeianus* and *L. clamitans* (Ingram, 1929), as well as non-metamorphosing *Xenopus laevis* tadpoles which appear sporadically in laboratory populations for individuals that lack thyroid glands (Rot-Nikcevic & Wassersug, 2004). The dispersion of melanin in response to light in late-stage cane toad tadpoles may represent the ontogenetic extension of a trait restricted to embryos in other anuran species. Diurnal black colouration is characteristic of the tadpoles of many toads (Altig & McDiarmid, 1999b), but it is unclear how widespread this ontogenetic extension may be across this family. Future studies using a phylogenetic perspective to examine the ontogenetic patterns of light-induced changes in pigmentation are necessary to formally test for the presence of a neotenic trait.

A developmental delay in light-induced melanin aggregation, if associated with toxicity, may have promoted the evolution of the characteristic black colouration of toad tadpoles as an aposematic signal during the day (Wassersug, 1971; Peterson & Blaustein, 1991). It is also possible that melanin aggregation at night increases toad tadpole conspicuousness by allowing moonlight to reflect off the tadpole's iridophore

layer (van der Lek, 1967). Aposematism is rare in anuran larvae (Toledo & Haddad, 2009; Thibaudeau & Altig, 2012). Although this phenomenon has been suggested in some species (e.g., Rödel et al., 2009), the role of conspicuous tadpole colouration in avoiding predation has been seldom tested (but see van Buskirk et al., 2004). Experiments that integrate light-induced melanophore changes, toxin concentration, and behavioural responses of predators to cane toad tadpoles of different stages in a single population are required to directly test the aposematic colouration hypothesis in this species.

Colouration changes can also lead to different thermal properties, and the black colouration in cane toad tadpoles might also be a thermal adaptation. Given that higher temperature increases development in tadpoles (e.g., Smith-Gill & Berven 1979), the dark colouration of toad tadpoles could serve as a heat sink during larval development, resulting in shorter time to metamorphosis. Future work that examines the influence of temperature on light-induced tadpole pigmentation changes and developmental rate across species is needed to evaluate this hypothesis.

This study is the first to reveal variation among anuran species in patterns of light-induced colour change in tadpoles, offering new possibilities to investigate the role of phylogenetic and ecological factors that affect this trait. Further work that examines the ontogeny of light-induced changes in tadpole pigmentation using a phylogenetic framework would contribute to our understanding of the evolutionary history of this trait.

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