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Circadian rhythm of physiological color change in the amphibian *Bufo ictericus* under different photoperiods

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Abstract

Ectothermic vertebrates can exhibit chromatic adaptation to the environment. The aim of this work was to characterize the rhythm of color change of the amphibian *Bufo ictericus*, submitted to different photoperiodic regimens, as quantified by skin reflectance values. Adult males were maintained under a 12 : 12 Light/Dark (LD) cycle during seven days before every experiment. During the experiments, animals were kept in individual boxes for 8 days, under the following photoperiodic regimens: LD 12 : 12, LD 14 : 10, DD and LL. In the last 3 days of the treatments, the reflectance of the toad dorsal skins was measured at 3-h intervals, with the aid of a reflectometer. A 3-day time series consisting of 8 data points per day was obtained, which was analyzed by the Cosinor method. The analysis demonstrated that the reflectance values exhibited significant circadian oscillations in the regimens LD 12 : 12, LD 14 : 10 and DD, suggesting that the specie *B. ictericus* shows an effective circadian rhythm of color change. The reflectance values did not exhibit a significant circadian rhythm in the LL regimen showing that this is a condition not permissive for the expression of the color change rhythm.

Keywords: Amphibian; Circadian rhythm; Color change; Melanophores; Photoperiod; Melatonin; α-MSH; Toad

1. Introduction

Ectothermic vertebrates can exhibit chromatic adaptation to the environment, mainly due to the light, background color and social behaviour. This process allows the occurrence of processes such as mimetism, thermoregulation, protection of the skin from radiation and expression of behavioral signs such as fear or anger (Bagnara and Hadley, 1973). Chromatic adaptation or color change depends on chromatophores, which are pigment cells from the embryonic neural crest, later located in the adult animal's integument (Parker, 1948; Bagnara and Hadley, 1973).

Color change can be classified as morphological or physiological. All vertebrates exhibit morphological color change (in days or weeks), which depends on quantitative changes in pigments and/or of the number of pigment cells. On the other hand, only ectothermic vertebrates exhibit physiological color change (in seconds or hours), which depends on migration of pigments into the dendritic cell processes of the chromatophores (Hadley, 1996). In these animals, melanophores, which synthesize melanin, are the most conspicuous chromatophores. The dispersion of melanosomes into the dendritic cells processes results in skin darkening, whereas granule aggregation around the perinuclear region leads to skin lightening (Sherbrooke et al., 1988; Visconti and Castrucci, 1993).

Melanosome translocation is regulated by a variety of endogenous agonists that may be related with the rhythmic color change investigated in this study, such as the α-melanocyte-stimulating hormone (α-MSH), melatonin, catecholamines, prolactin and endothelins (Binkley, 1988; Hadley, 1996; Camargo et al., 1999; Karne et al., 1993; Sherbrooke et al., 1988; Visconti et al., 1999). The hormone α-MSH, for example, seems to be the most physiologically relevant melanotropin in the control of vertebrate pigment patterns. It has been held responsible for skin *darkening* in *Rana pipiens*, *R. berlandieri*, *R. catesbeiana*, *Xenopus laevis* and *Bufo ictericus* (Castrucci et al., 1984; Ferroni and Castrucci, 1987;

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Filadelfi and Castrucci, 1994; Hruby et al., 1987; Rollag et al., 1989; Sherbrooke et al., 1988; Visconti and Castrucci, 1993). Secretion of α-MSH is determined by the balance between inhibitory and stimulatory brain inputs, according to the background color (Hadley, 1996). On the other hand, melatonin (*N*-acetyl-methoxytryptamine) is produced by the pineal gland, exhibiting a peak during the dark phase and usually inducing *lightening* of teleost, amphibian and reptile skin (Binkley, 1988; Binkley et al., 1987, 1988; Filadelfi and Castrucci, 1994; Kavaliers et al., 1980; Reiter, 1991). In *X. laevis* tadpoles, both the light-sensitive tail melanophores and the meninge light-insensitive melanophores are responsive to melatonin (Rollag, 1988; Rollag et al., 1989). Although the melatonin effects can exhibit interspecific, ontogenetic and autodesensitizing variations (Filadelfi and Castrucci, 1994, 1996; Fujji and Oshima, 1986; Rollag and Lynch, 1993) the pineal seems to be one of the oscillators controlling the circadian rhythm of vertebrate color change. The main evidence for this role of the pineal is that pinealectomy or constant light exposure abolishes the daily rhythm of color change (melatonin-induced dark paling) in the amphibian *X. laevis* and in the reptile *Anolis carolinensis* (Binkley et al., 1987, 1988; Heward and Hadley, 1975).

Biological rhythms are events in biological systems that recur systematically in regular intervals, and are related with biological variables that oscillate in organisms. These rhythms provide the animals adaptability to cyclic environmental factors, such as light–dark or temperature cycles. These cyclic factors are denominated "zeitgebers" because they synchronize or entrain the endogenous rhythms. The organs or cells that posses timing ability are called biological clocks or oscillators (Binkley, 1988; Illnerová, 1991). The most conspicuous biological rhythms are the circadian rhythms or biological cycles that continue to oscillate even under constant conditions (free-running rhythm) with periods close to 24 h (Binkley, 1988). Some studies show a circadian vertebrate dark paling due to melatonin secretion (Binkley, 1988; Binkley et al., 1987, 1988; Kavaliers et al., 1980). However, in *R. catesbeiana* no endogenous rhythm of color change was detected (Camargo et al., 1999). In any case there is a lack of systematic studies establishing the endogenous/circadian rhythm of color change in amphibians using chronobiological approaches. The aim of this work was to characterize the rhythm of color change in the amphibian *B. ictericus* submitted to different photoperiodic regimens.

2. Materials and methods

Adults males of *B. ictericus* (≈100 g) were obtained from local suppliers in Curitiba, and were acclimated for 7 days inside light-brown tanks (with pebbles and water on the bottom), under control of room temperature (21±3 °C) and under a LD 12:12 cycle (lights on at 7:00 h), before every experiment. Two 40 W fluorescent bulbs remained turned on during the light phase of the day, 2 m above the tanks. An infrared light (150 W) was turned on the whole time to allow readings during the dark phase. For reflectance readings taken during the dark phases, animals received a black cap on their heads, to avoid the incidence of photons from the equipment photosensor light on their retinas. During these 7 days of acclimation toads were fed ad libitum with newborn white Wistar rats every 2 or 3 days.

The toads were housed and treated according to the recommendations for animal care by the "Brazilian Federation of Societies of Experimental Biology" (FESBE), and all the experiments comply with current Brazilian laws.

2.1. Experiments

Adults males of *B. ictericus* ($n=6$ to 13) were kept in light-brown individual boxes covered with glass plates 3 mm-thick for 8 days, under one of the following photoperiodic regimens: (1) LD 12:12; (2) LD 14:10; (3) DD; (4) LL. Light up always occurred at 7:00 h. The reflectance of the toad dorsal skin was measured at 3-h intervals, for 72 h during the last 3 days of the 8-day treatments, and was determined using a reflectometer (Photovolt, USA) according to Castrucci and co-authors (1984). Reflectance values are proportional to the color of the toad skin: darker skins exhibit less light reflection than lighter skins, thus yielding a lower reflectance value. Three successive reflectances values were obtained from each animal (and averaged) to provide a more accurate reading. The dorsal side of the toad was placed against the glass plate of the reflectance sensor. The results were displayed in plexogram graphics and in a table.

2.2. Statistical analysis

Average reflectance of the three successive measurements (obtained for each animal in each time) was calculated for all the photoperiodic regimens. Then, a 3-day time series consisting of 8 data points per day (a total of 24 data points per toad) was analyzed by the Cosinor method. The Cosinor software program fits a 24:00 h cosine curve by the method of the least squares to generate estimates of the 24:00 h rhythm-adjusted mean (term MESOR — Midline Estimating Statistic of Rhythm), amplitude (one-half the peak-to-trough variation), and acrophase (peak time referenced to local 0:00 h), as derived by the best approximating cosine curve function. Circadian rhythmicity was verified by *F*-test of the variance accounted for by the 24:00 h cosine curve vs. that accounted for the straight-line approximation to the data at $p<0.05$.

3. Results and discussion

The statistical analysis of the data demonstrated that reflectance values exhibited significant circadian oscillations in the LD 12:12 ($p<0.05$, Fig. 1), LD 14:10 ($p<0.001$, Fig. 2) and DD ($p<0.005$, Fig. 3) regimens (Table 1). These results suggest that the toad *B. ictericus* shows an effective circadian rhythm of color change. Other studies have demonstrated a melatonin-induced dark paling in ectothermic vertebrates, such as in the lamprey *Geotria* sp. (Binkley, 1988), the teleost *Fundulus heteroclitus* (Kavaliers et al., 1980), the frog *X. laevis* and the lizard *A. carolinensis* (Binkley et al., 1987,

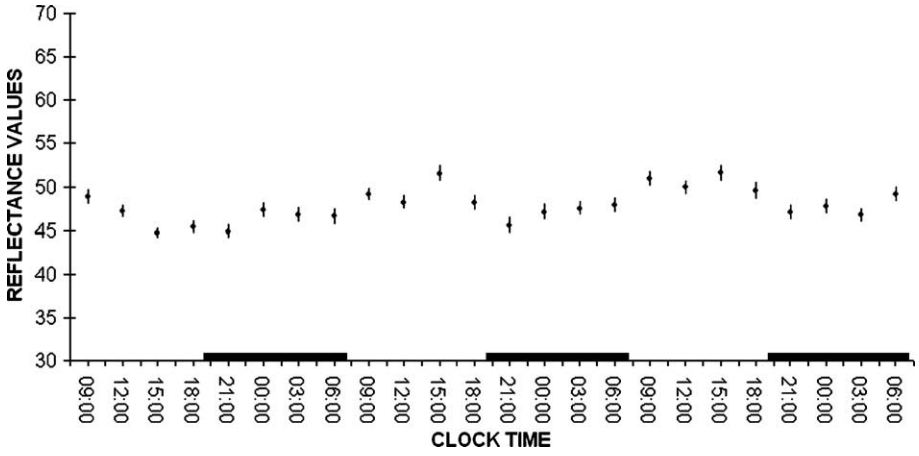

Fig. 1. Plexogram of the data of the experiment with toads maintained in LD 12 : 12. Changes of reflectance values are the mean of 12 toads, ± 1 standard error. The dark phase is shaded.

1988). However, as far as we know, this is the first characterization of a color change rhythm in amphibians using an effective chronobiological approach. Actually, this approach has previously been performed on *R. catesbeiana*, with the demonstration of a color change rhythm, but with unconfirmed endogenicity (Camargo et al., 1999).

In agreement with the statistical results, the plexogram graph of the animals maintained in LD 12 : 12 (Fig. 1) showed a visible circadian oscillation of reflectance values that was maintained during the 3 days of recording. The acrophase values were situated near the end of the morning (10:48 h — Table 1), mainly in the two first days of data collection (Fig. 1) a fact that may suggest that the reflectance values exhibited a gradual increase during the dark phase.

Reflectance values of animals maintained in the LD 14 : 10 condition (Fig 2) showed a similar behavior to the one detected in LD 12 : 12 (Fig. 1). The circadian oscillation of the data was also verified, with a gradual increase of the reflectance values seeming to occur after lights were turned off. In fact, acrophase values were 04:12, 05:06 and 10:48 h in DD, LD 14 : 10 and LD 12 : 12 regimens (Table 1), respectively. All the acrophase values were concentrated at the second half of the night phase, and at the first half of the light phase. These results might indicate that the maximal toad skin lightening depends on a previous exposure to melatonin, since this hormone probably peaks at night. However, according to the statistical results (Table 1), in LD 14 : 10 (Fig. 2) and in DD (Fig. 3), the acrophase value was reached earlier than in the LD 12 : 12 condition (Fig. 1). This fact may suggest that the light/dark hour proportion is involved in the determination of the maximal lightening of the skin and that the species specific color change characteristics are a complex matter. These characteristics probably depend on the relevance of color change for the animal, besides the distribution and types of skin chromatophores (Binkley, 1988).

As the *B. ictericus* color change rhythm was maintained in DD condition (Fig. 3, Table 1), this rhythm can be in fact considered as an endogenous one. Despite the persistence of the rhythm in DD, it exhibited a damped amplitude (Table 1, Fig. 3) when compared to the amplitude in both LD conditions (LD 12 : 12; LD 14 : 10 — Table 1, Figs. 1 and 2). It is possible to verify that the increase in reflectance values during the subjective dark phase has not been so evident in the DD regimen. This flattening of the free-running rhythm under DD

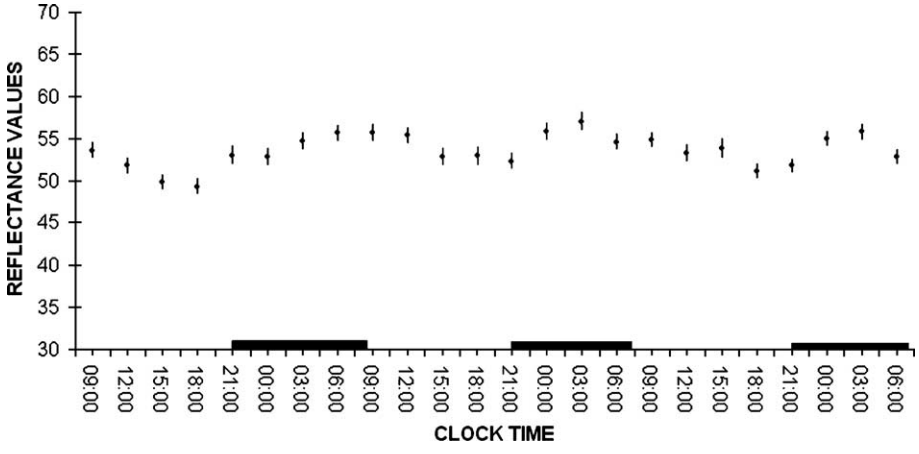

Fig. 2. Plexogram of the data of the experiment with toads maintained in LD 14 : 10. Changes of reflectance values are the mean of 12 toads, ± 1 standard error. The dark phase is shaded.

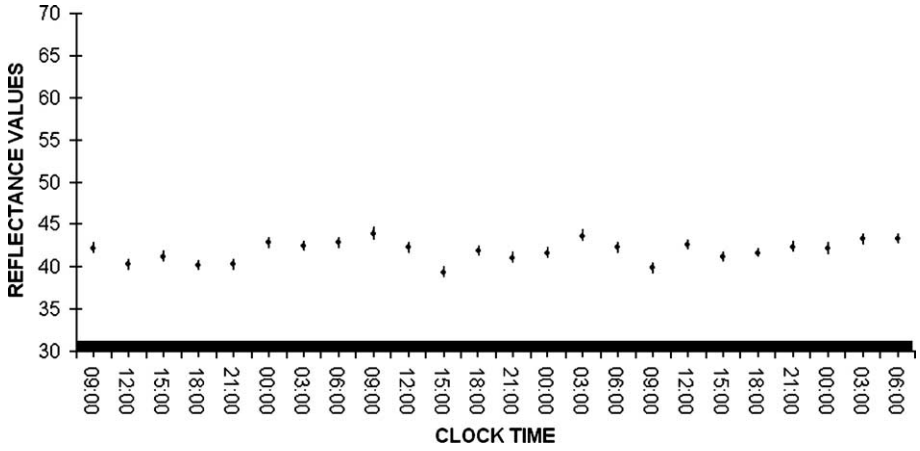

Fig. 3. Plexogram of the data of the experiment with toads maintained in DD. Changes of reflectance values are the mean of 12 toads, ±1 standard error. The dark phase is shaded.

has already been detected in other studies of circadian rhythms, such as that of the *N*-acetyltransferase (the main enzyme in the melatonin biosynthesis) rhythmic activity in pineal gland of chicks (Binkley, 1981), and in the lizard *A. carolinensis* color change rhythm (Binkley et al., 1987).

On the other hand, reflectance values did not exhibit a significant circadian rhythm in the LL regimen (Table 1, Fig. 4), showing that this is a not permissive condition for the expression of the color change rhythm. The absence of a significant circadian rhythm when the animals are exposed to the LL condition (Fig. 4, Table 1) was also verified in the lizard *A. carolinensis* (Binkley et al., 1987). The non-permissive constant light effect can also be related, for example, to either a light-induced arrhythmia on the pineal melatonin secretion (Binkley, 1988), or to a direct effect of light on the chromatophores (Provencio et al., 1998; Oshima, 2001). In addition, the mesors reflectance values in DD (41.792) and LD 12:12 (47.958) regimens were lower in comparison with the LD 14:10 (53.667) regimen (Table 1). This may indicate that higher reflectance values are in some way associated with a total higher previous time of light exposure.

In fact, direct effects of light on vertebrate chromatophores are conspicuous and characterize a kind of physiological color change that is called 'primary color response' (Bagnara, 1965; Binkley, 1988; Rollag, 1988). In this response, chromatophores react independently as receptors and effectors, transducing light energy into the intracellular process of pigment migration. In most invertebrates, light induces pigment dispersion, but mainly in fish and in amphibian chromatophores, it induces pigment aggregation (Oshima, 2001). For example, melanophores of the tail fin of later stages *X. laevis* tadpoles, in vivo and in vitro, exhibit a full pigment aggregation in response to normal conditions of illumination (Oshima, 2001), although the inverse response can be obtained with melanophores of younger *Xenopus* tadpoles (Provencio et al., 1998; Oshima, 2001). The light-induced melanosome response is probably mediated by melanopsin and/or rhodopsin photopigments; the first, for example, has already been detected in *X. laevis* melanophores (Provencio et al., 1998; Oshima, 2001).

Our results showed that the mesor values in LD 14:10 were higher than in LD 12:12 and DD (Table 1). It seems that the reflectance values exhibited a light increase in the regimen with a higher number of light hours. Since amphibian chromatophores show pigment aggregation when they are exposed to light (Oshima, 2001), the higher reflectance values in LD 14:10 could be related with a direct photopigment-mediated light action on the chromatophores. However, further studies are necessary to confirm this hypothesis.

Besides the primary color response, there is another type of physiological color change that is called the 'secondary color response'. This secondary response is mediated by the eyes, and is controlled by neural and/or endocrine systems (Fujji and Oshima, 1986). One of the secondary response subtypes, the rhythmic color change, has been already cited, and depends on pineal gland and melatonin (Binkley, 1988). The other secondary response subtype that is called 'background adaptation' depends on the interaction of the lateral eyes with the hypothalamus–pituitary axis leading to MSH release (Hadley, 1996). When amphibians are in a dark or light background, there is an increase or decrease in MSH release, and the animals get darker or lighter, respectively (Hadley, 1996). The stimulus induced by the background color can be considered more potent than the light-induced one, since in *A. carolinensis* maintained in LL, the resultant animal color is strongly dependent upon background color (Binkley et al., 1987).

In this study, a similar result was obtained, since *B. ictericus* submitted to LL, but maintained in dark or white background for three days, showed low and high reflectance values, respectively (data not shown). In fact it seems

Table 1
Results described by the cosinor parameters: amplitude, mesor and acrophase

Photoperiodic regimen	*n*	Acrophase	Amplitude	Mesor	*p*
LD 12:12	12	10:48	2.500	47.958	<0.05*
LD 14:10	12	05:06	2.833	53.667	<0.001*
DD	12	04:12	1.833	41.792	<0.005*
LL	6	02:24	5.667	53.333	>0.05

* Values indicate significant differences.

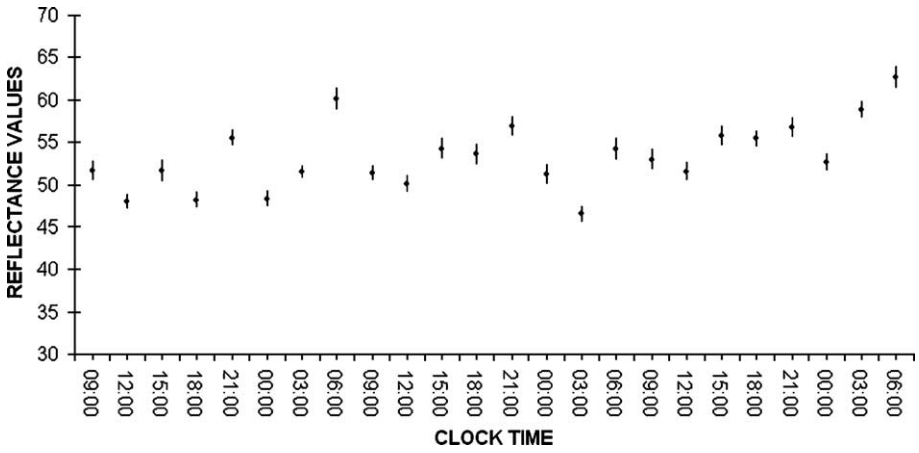

Fig. 4. Plexogram of the data of the experiment with toads maintained in LL. Changes of reflectance values are the mean of 6 toads, ±1 standard error.

appropriate that during the light phase of the day, animals can be adapted to the background color in natural conditions, despite the moment of their circadian skin color variation. On the other hand, during the dark phase of the day, animals could not be seeing the background color, therefore they would show a color resulting from the dark-time-release of melatonin. In addition, since serotonin (a melatonin biosynthesis substrate) stimulates the release of MSH in the pituitary, and its serum levels are reduced during the dark-time, it is possible that this dark-decreased serotonin availability (due to its conversion to melatonin) contributes to the reduction of MSH secretion in this phase (Binkley, 1988).

In conclusion, this study demonstrates the occurrence of a circadian color change rhythm in the toad *B. ictericus* that is maintained in free-running conditions. The circadian rhythms provide the animals adaptability to cyclic environmental factors, such as light–dark or temperature cycles. Then, probably, the process which are results from the color change in amphibians, such as mimetism, thermoregulation, protection of the skin from radiation and expression of behavioral signs (Bagnara and Hadley, 1973), can be in agreement with the environmental factors thorough the expression of this rhythm.

As perspectives for further studies, we can suggest the realization of: (1) phase-response curves, with the aim to elucidate the color responses to different ratios of light/dark; (2) measurements of color variation in animals maintained in their natural environment; (3) melatonin and MSH serum determinations in different moments of the circadian cycle; (4) dose-response curves to these hormones, in different moments of the circadian cycle, with the aim to investigate possible differences of sensitivity in the hormonal responses.

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