Integrative and Comparative Biology

Integrative and Comparative Biology, volume 64, number 5, pp. 1309–1319
https://doi.org/10.1093/icb/icae026

Society for Integrative and Comparative Biology

COMPLIMENTARY/POSTER SESSION PAPER

Immune-pineal-ocular Axis in Amphibians: Unveiling A Novel Connection

Stefanny C. M. Titon[*,1], Patrício G. Garcia Neto[*], Braz Titon Jr.[*], Aymam C. de Figueiredo[*], Regina P. Markus[*], Fernando R. Gomes[*] and Vania R. Assis[*,†]

[*]Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, 101, São Paulo, SP 05508-090, Brasil; [†]College of Public Health, University of South Florida, Tampa, FL 33612, USA

From the symposium "Immunity in the 'omics age: what can 'omics approaches tell us about immunity in natural systems?" presented at the virtual annual meeting of the Society for Integrative and Comparative Biology, January 16–March 31, 2024.

[1]E-mail: stefannychristie@gmail.com

Synopsis Melatonin is a hormone known as an endogenous temporal marker signaling the dark phase of the day. Although the eyes seem to be the main site of melatonin production in amphibians, little information is available about the natural variation in ocular melatonin levels and its modulation following immune stimulation. We investigated the daily variation of plasma and ocular melatonin levels in bullfrogs (*Lithobates catesbeianus*) and their modulation following an immune stimulation with lipopolysaccharide (LPS) in yellow cururu toads (*Rhinella icterica*). For the daily variation, bullfrogs were bled and then euthanized for eye collection every 3 h over 24 h to determine plasma and ocular melatonin levels. We found a positive correlation between ocular and plasma melatonin levels, with maximum values at night (22 h) for both plasma and the eyes. For immune stimulation, yellow cururu toads received an intraperitoneal injection of LPS or saline solution during the day (10 h) or at night (22 h). Two hours after injection, toads were bled and euthanized for eye collection to obtain plasma and ocular melatonin levels. In addition, the liver and bone marrow were collected to investigate local melatonin modulation. Our results demonstrate that retinal light-controlled rhythmic melatonin production is suppressed while liver and bone marrow melatonin levels increase during the inflammatory assemblage in anurans. Interestingly, the LPS injection decreased only ocular melatonin levels, reinforcing the central role of the eyes (i.e., retina) as an essential organ of melatonin production, and a similar role to the pineal gland during the inflammatory response in amphibians. Together, these results point to a possible immune-pineal-ocular axis in amphibians, yet to be fully described in this group.

Introduction

The daily melatonin rhythm is the biological output of the photoperiodic information, with increased melatonin plasma levels signaling the darkness period of the day in all investigated vertebrates (Reiter 1993; Singh and Haldar 2007; Moore and Menaker 2011; Jessop et al. 2014; Saha et al. 2019). Melatonin is mainly produced in the pineal gland, with studies in mammals, fish, reptiles, and birds showing that pinealectomy suppresses plasma melatonin nocturnal peak and daily rhythm (Cogburn et al. 1987; Reiter 1993; Bubenik and Pang 1997; Tosini et al. 2001; Muñoz-Pérez et al. 2016). In addition, melatonin can be rhythmically produced by the eyes in birds, fish, reptiles, amphibians, and mammals, including humans (Tosini et al. 2001; Lundmark et al. 2006; Summers Rada and Wiechmann 2006; Wright et al. 2006; Iigo et al. 2007). In humans and chickens, the melatonin synthesized in the eyes is mostly a paracrine substance, modulating local physiology and pathophysiology (Lundmark et al. 2006; Summers Rada and Wiechmann 2006; Brennan et al. 2007). Meanwhile, the eye and pineal contribute to the resulting plasma melatonin levels in amphibians and some fish (Bayarri et al. 2003; Wright et al. 2006). Specifically in amphibians, the eyes produce high concentrations of melatonin, which seems to contribute to at least half of the circulating melatonin levels (Serino et al. 1993; Chiba et al. 2005; Wright et al. 2006). In addition, the

Advance Access publication April 24, 2024

circadian rhythm of melatonin in amphibians shows increased plasma melatonin and retinal levels at the dark phase (Wright et al. 2006; Titon et al. 2021a), with both plasma and eye melatonin being synchronized in some species (D'Istria et al. 1994; Chiba et al. 2005; Hu et al. 2022a,c).

Besides its function in signaling darkness, melatonin is an immunomodulatory hormone that plays an essential role in the inflammatory response (Summers Rada and Wiechmann 2006; Markus et al. 2018; Horodincu and Solcan 2023). This melatonin-induced immunomodulatory effect has been described in snakes (Tripathi et al. 2014), fish (Ángeles Esteban et al. 2013), chickens (Horodincu and Solcan 2023), and mammals (Markus et al. 2018). Regarding the inflammatory response, melatonin modulates the leukocyte migration and cellular activity, including proliferation and phagocytosis (Markowska et al. 2001; Pires-Lapa et al. 2013; Tripathi et al. 2014). Following immune stimuli (pathogen-associated molecular patterns and/or damage-associated molecular patterns), it has been described that increased melatonin attenuated the lipopolysaccharide (LPS)-induced inflammatory response and inhibited the phytohemagglutinin-induced lymphocyte proliferation in chickens (Markowska et al. 2001; Li et al. 2018). For mammals, the immune stimuli suppress the nocturnal melatonin by direct action on the pineal gland, which increases blood leukocyte migration to the site of infection. Later, in the inflammatory response, leukocytes in the site of infection increase melatonin production. This shift from pineal to immune cell melatonin production is termed the immune-pineal axis (Markus et al. 2018).

Well described in mammals, the first phase of the inflammatory response, called the alarm phase, is characterized by the activation of two main axes: (1) the hypothalamic-hypophyseal-adrenal axis, which culminates in increased glucocorticoid secretion; and (2) the activation of the immune-pineal axis, which culminates in decreased systemic melatonin synthesis and secretion, while immune cells increase the production of chemokines and pro-inflammatory interleukins (Cain and Cidlowski 2017; Markus et al. 2018). In accordance, studies with amphibians have shown that LPS induces increases in plasma corticosterone levels, in the expression of interleukin (IL)-1β, IL-6, and IL-10 mRNA levels, and bacterial killing ability in some amphibian species (Gardner et al. 2018; Titon Junior et al. 2021; Bastos et al. 2022; Floreste et al. 2022; Garcia Neto et al. 2022). In addition, we previously observed decreased plasma and ocular melatonin following LPS in *R. diptycha* and *R. icterica* toads (Ferreira et al. 2021; Titon Junior et al. 2021; Bastos et al. 2022), suggesting a direct melatonin suppression in the pineal and eyes of anurans.

Regarding the production of melatonin by extrapineal/ocular sources, it has been described that the lungs, liver, intestine, stomach, bone marrow, and immune cells can synthesize melatonin in vertebrates (Bubenik and Pang 1997; Tan et al. 1999; Martin and Frevert 2005; Markus et al. 2018; Slominski et al. 2018; de Figueiredo et al. 2021, 2022; Cyrino et al. 2022; Hu et al. 2022c). Moreover, evidence of local increases in melatonin following an immune challenge and with protective cellular actions has been observed in several tissues, such as bone marrow, spleen, gastrointestinal tract, and immune cells, among others (Bubenik 2001; Acuña-Castroviejo et al. 2014; Markus et al. 2018; Córdoba-Moreno et al. 2020). In particular, immune cells play a crucial role as sources of melatonin, and heightened melatonin production at the local level facilitates an enhanced immune response in the immediate vicinity (Markus et al. 2018). In amphibians, the intestine and stomach are important melatonin sources under physiological and pathophysiological conditions (Bubenik and Pang 1997; de Figueiredo et al. 2021, 2022). However, to date, melatonin production by immune cells has not been described in this taxon.

A better understanding of the daily variations in ocular and plasma melatonin will help to shed light on the role of the eyes in contributing to systemic melatonin in amphibians. Herein, we investigated the daily variation of concomitant plasma and ocular melatonin in bullfrogs (*Lithobates catesbeianus*). We predicted (1) increased plasma and ocular melatonin levels during the dark phase, with a peak within the middle of the dark phase. In addition, little is known about the melatonin production by the eyes and immune cells, especially following an immune stimulation along distinct day phases. Then, isolating the eyes, plasma, and immune tissues, such as the liver and bone marrow, can be the first step to shed light on the immune-endocrine relations in anurans during distinct periods of the day. Herein, we also investigated plasma, ocular, liver, and bone marrow melatonin modulation following an immune stimulation with LPS in the yellow cururu toad (*Rhinella icterica*). We predicted (2) decreased systemic melatonin levels (plasma and ocular) following LPS injection, with a more accentuated response being evident during the time in which systemic melatonin is maximum, ~22 h (Bastos et al. 2022); and (3) increased local melatonin levels (liver and bone marrow) following LPS injection at both 10–22 h.

Materials and methods

Experiment 1: daily variation

In experiment 1, we selected the *L. catesbeianus* species, an amphibian model used in numerous studies (De Abreu Manso et al. 2009; Becerra López et al. 2017; Blaustein et al. 2018; Lima et al. 2022; Madelaire et al. 2023; Ruiz and Robert 2023), making our results comparable with pre-existing physiological data. Moreover, this species is easy to manipulate and readily available to purchase from farms in the State of São Paulo. Adult males of *L. catesbeianus* ($N = 40$) were bought from a commercial facility at the end of April 2019 (Rãs World, São Paulo, Brazil). Frogs with similar sizes and weights were purchased from the farm to avoid differences in these variables. The mean body mass ± *SD* was 142.34 ± 5.46 g, and the mean snout–vent length ± *SD* was 288.33 ± 31.79 mm. Animals were randomly divided and kept individually in 20-L plastic containers with a meshed lid to allow air circulation, filled with 1.5-L aged tap water. Containers had a plastic pipe (75 mm) to provide a dry area, and the water was changed every 3 days, 1 h before the lights turned off. All the individuals were maintained in an environmental chamber (Eletrolab EL011, São Paulo/SP, Brazil) with a controlled temperature (20 ± 2°C) and photoperiod (12:12 LD cycle, lights on at 7 h and off at 19 h). Frogs were kept in conditions similar to those from the farm where they were bred; therefore, 7 days were allowed to adjust to the individual containers after transportation and relocation.

On the 8th day of captivity, animals were randomly sampled (independent samples) throughout 24-h day, within 3-h intervals (5 frogs every 3 h, at each specific hour: 1, 4, 7, 10, 13, 16, 19, and 22 h). Blood samples were collected (∼1 ml) by cardiac puncture using heparinized 1 ml syringes and 26 G×1/2″ needles within 1 min. Thereafter, animals were euthanized by decapitation. All procedures for using biological material were performed with the approval of the IB/USP Ethical Committee (CEUA, #325/2018).

Experiment 2: field collection and day and night LPS administration

For experiment 2, *R. ictherica* species was selected as a model considering the previous knowledge about endocrine-immune interactions, including LPS exposure on this species (Assis et al. 2015, 2017, 2022; Titon et al. 2018; Titon et al. 2021b; Bastos et al. 2022; Garcia Neto et al. 2022), allowing a better understanding of amphibian's physiology. Adult male toads of *R. ictherica* [$N = 24$, snout–vent-length ≥70 mm (Krakauer 1968)] were collected in Botucatu (22°46′55″ S and 48°28′29″ W), São Paulo/Brazil, in July 2019. Toads were kept in plastic containers (43.0 × 28.5 × 26.5 cm—5 toads *per* box), with free access to water for 72 h under natural photoperiod and temperature until they were taken to the laboratory. At the laboratory, the animals were individually accommodated in plastic containers (43.0 × 28.5 × 26.5 cm), whose lids had holes to allow air circulation placed in an environmental chamber (Eletrolab EL011, São Paulo/SP, Brazil), with the temperature kept at 20 ± 2°C and photoperiod at 12/12 LD lights turned on at 7 h and off at 19 h. Toads were kept under these conditions for 10 days.

Three days before the experiment, the animals were weighed (0.00 g) and had their snout–vent length measured (0.00 mm). Toads were randomly divided into four groups (LPS day, saline day, LPS night, and saline night). Mean body mass ± *SD* was 159.74 ± 56.04 g, and mean snout–vent length ± *SD* was 119.79 ± 13.22 mm. The toads were intraperitoneally injected with LPS (2 mg/kg, *Escherichia coli*, Serotype 0127: B8, L3129, Sigma, St. Louis, MO, USA) or saline, according to Titon Junior et al. (2021). Intraperitoneal injections of LPS or saline were performed at 10 or 22 h and 4 h after lights were on or off, respectively. These times were selected since captive anurans, including *R. ictherica* species, showed decreased melatonin during the day and increased melatonin at night (Jessop et al. 2014; Titon et al. 2021a; Bastos et al. 2022), then we wanted to detect possible differences in the hormonal profile at the selected times. The individuals were injected within 5 min intervals, LPS and saline, respectively. Two hours after the injections, the toads were sampled by cardiac puncture (∼500 ml) and were euthanized by decapitation.

Animal collection and all the procedures were performed under license from the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, #29,896-1) and the approval of the animal ethics committee of the Bioscience Institute of the University of São Paulo (#242/2016).

Blood processing and tissue removing

Immediately after collection, the blood was placed on ice for a maximum of 30 min. Blood samples were centrifuged at 604 g, 23°C, and 4 min. The plasma was isolated and stored in a −80°C freezer. After decapitation, the eyes, liver, and bone marrow were removed with sterile surgical material and stored in a −80°C freezer. All tissues were used for further melatonin quantification. In addition, for all the experimental procedures (intraperitoneal injections, blood sampling, and eye removal), red lights (15 W, 220 V, and 650 nm wavelength)

were used during the night to prevent animals' exposure to white light, which inhibits melatonin production (Trinder et al. 1996).

Liver and bone marrow processing

The bone marrow and liver were processed according to de Figueiredo et al. (2021) and Cyrino et al. (2022). Briefly, the liver was weighed, and 60 mg was pulverized with liquid nitrogen in a porcelain mortar and homogenized in the vortex in 400 µL of tris-HCl. The mix was centrifuged (20,000 g, 5 min, and 4°C). The supernatant was isolated and frozen at −80°C for hormone quantification.

For the bone marrow, cells were obtained by centrifuging the femur in a 1.5-ml microtube (14,000 g, 5 min, and 4°C). Next, the opposite bone extremity was cut, then the bone was turned upside down in the same microtube and centrifuged again to collect more bone marrow cells (14,000 g, 5 min, and 4°C). Thereafter, the bone marrow was removed from the microtube, and the resulting cells were resuspended in 400 µL of tris-HCl, then homogenized in the vortex and centrifuged (14,000 g, 5 min, and 4°C). The supernatant was collected and frozen (−80°C) for hormone quantification.

Ocular tissue processing

For ocular melatonin determination, the eyes were defrosted, the crystalline was removed, and the remaining eye tissue was homogenized in 2 ml of saline solution in a homogenizer (Ultra Turrax, T25 Basic, IKA Labortechnik; 16,000 rpm) and centrifuged (1562 g, 20 min, and 4°C). The supernatant was collected for melatonin quantification.

Melatonin quantification

Plasma, ocular, liver, and bone marrow melatonin levels were determined by the ELISA commercial kit (IBL, RE54021) according to the manufacturer's instructions. The melatonin quantification with the IBL ELISA kit has been conducted in some anuran species, including *L. catesbeianus* and *R. icterica* (Barsotti et al. 2017; de Figueiredo et al. 2021; Titon Junior et al. 2021; Titon et al. 2021a; Bastos et al. 2022; Garcia Neto et al. 2022). The sensitivity of the assay was 3.0 pg/ml, and intra-assay variations for experiments 1 and 2 were 9.55 and 2.86%, respectively. Tissue proteins were quantified with 1 μl of tissue supernatant in a Nanodrop apparatus, and the final bone marrow and liver melatonin values in pg/mg of tissue were calculated as the melatonin value (pg/ml) from the ELISA kit divided by the protein values (mg/ml) obtained with the Nanodrop, according to de Figueiredo et al. (2021).

Statistical analysis

All variables were submitted to the Shapiro–Wilk normality test and Levene's homogeneity test.

Experiment 1: daily variation

The variables for experiment 1 did not fit the parametric test assumptions. Then, we performed a Kruskal–Wallis test, with plasma and ocular melatonin levels as the dependent variables and hour of the day as a factor (1, 4, 7, 10, 13, 16, 19, and 22 h).

Experiment 2: day and night LPS administration

Considering the LPS treatment in the day and night LPS exposure (experiment 2), we first performed sets of two-way ANCOVAs with plasma, ocular, liver, and bone marrow melatonin levels as dependent variables, treatment (saline and LPS), and period (day and night) as factors, and body mass as a covariate. For the variables not affected by body mass, we performed sets of two-way ANOVAs by excluding the covariate from the models.

When appropriate, pairwise comparisons with Bonferroni adjustment followed all analyses (experiments 1 and 2). Pearson or Spearman correlation tests were used to investigate the correlations among variables. All statistics were performed using IBM SPSS Statistics 26 for Windows.

Results

Experiment 1: daily variation

There was a daily variation (experiment 1) in the plasma ($H_{(7)} = 17.783$, $P = 0.013$, Fig. 1A) and ocular ($H_{(7)} = 20.626$, $P = 0.004$, Fig. 1B) melatonin levels in captive males of *L. catesbeianus*. In addition, there is a positive correlation between plasma and ocular melatonin levels over 24 h ($rs_{(39)} = 0.384$, $P = 0.019$, Fig. 1C).

Experiment 2: day and night LPS administration

Plasma and ocular melatonin levels were not affected by body mass ($P = 0.344$ and $P = 0.703$, respectively). The LPS injection showed no effect on plasma melatonin levels but decreased ocular melatonin levels independently of the period of the day ($F_{1,12} = 0.044$; $P = 0.837$; and $F_{1,19} = 5.551$; $P = 0.029$; Fig. 2A and B) in males of *R. icterica* toads from experiment 2. Regarding the immune tissues, liver melatonin levels were affected by body mass ($F_{1,17} = 8.924$ and $P = 0.008$), with the larger animals showing higher melatonin levels. The melatonin levels in the liver tended to be affected by LPS treatment ($F_{1,17} = 3.191$ and $P = 0.092$), with increased mean values in the LPS group than in the saline group

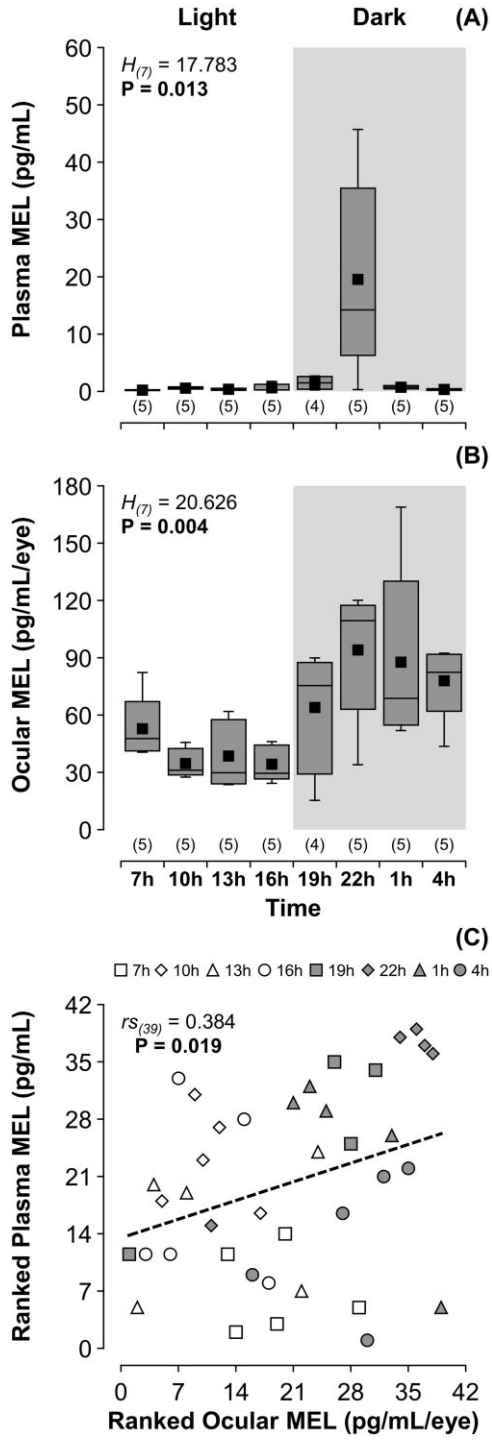

Fig. 1 Daily variation in melatonin levels in *Lithobates catesbeianus* (experiment 1). (A) Plasma melatonin levels; (B) ocular melatonin levels; and (C) correlation between plasma and ocular melatonin levels. Boxplots inside lines indicate medians; lower and upper borders represent first and third quartiles, respectively; black squares indicate means; whiskers represent upper and lower limits of 1.5 times interquartile range; and circles represent data out of this range. Ranked values are depicted as the Spearman correlation correction, in which measure values are considered in ordinal sequence for each variable. The *N* is indicated below bars in parentheses. In the statistics, *P* indicates a significance value.

(Table 1; Fig. 1C). Bone marrow melatonin levels were affected by body mass ($F_{1,17} = 8.114$ and $P = 0.011$), where larger animals showed lower melatonin levels. Melatonin levels in the bone marrow were affected by the treatment × period interaction ($F_{1,17} = 7.430$ and $P = 0.014$). The melatonin levels in the bone marrow were higher in the LPS group than in the saline group during the day (Table 1; Fig. 2D). Bone marrow melatonin levels were also higher during the day than at night in the LPS group. There is no correlation among the studied variables.

Discussion

Experiment 1: daily variation

Our results demonstrated plasma and ocular melatonin variation over 24 h, with the dark phase duration better characterized in ocular melatonin than in plasma in *L. catesbeianus*. Interestingly, melatonin plasma levels showed mean increased values at only one point during the dark phase at 22 h. Plasma melatonin levels in amphibians show the pattern of nocturnal melatonin peaks varying with species (i.e., melatonin peaks may occur within the first hours of the night, late in the dark phase, in the middle of the dark phase, or throughout the dark phase) (Serino et al. 1993; Jessop et al. 2014; Titon et al. 2021a; Bastos et al. 2022; Hu et al. 2022c). According to Wright (2002), melatonin clearance is very fast when melatonin plasma levels are high. Hence, it is plausible that there was a transient surge in plasma melatonin levels (probably in the 19–22 h and 22–1 h intervals), with such rapid clearance that our study only captured a single data point (at 22 h). In the meantime, ocular melatonin showed increased mean values from the beginning of the dark phase, in which mean values were at least three to four times higher than plasma mean values. Indeed, ocular melatonin levels seem to be higher than in the plasma in amphibians (Delgado and Vivien-Roels 1989; Wright et al. 2006; Hu et al. 2022c) and sea bass (Bayarri et al. 2003). In addition, we found a positive correlation between plasma and ocular melatonin levels in *L. catesbeianus*, as previously observed in *Rana perezi* (Delgado and Vivien-Roels 1989; Hu et al. 2022a). These and other studies demonstrate the eyes express the melatonin rhythm and contribute to systemic melatonin levels in amphibians (Delgado and Vivien-Roels 1989; Serino et al. 1993; D'Istria et al. 1994; Hu et al. 2022a,c).

Experiment 2: day and night LPS administration

As expected, ocular melatonin decreased following LPS exposure; however, plasma melatonin was not modu-

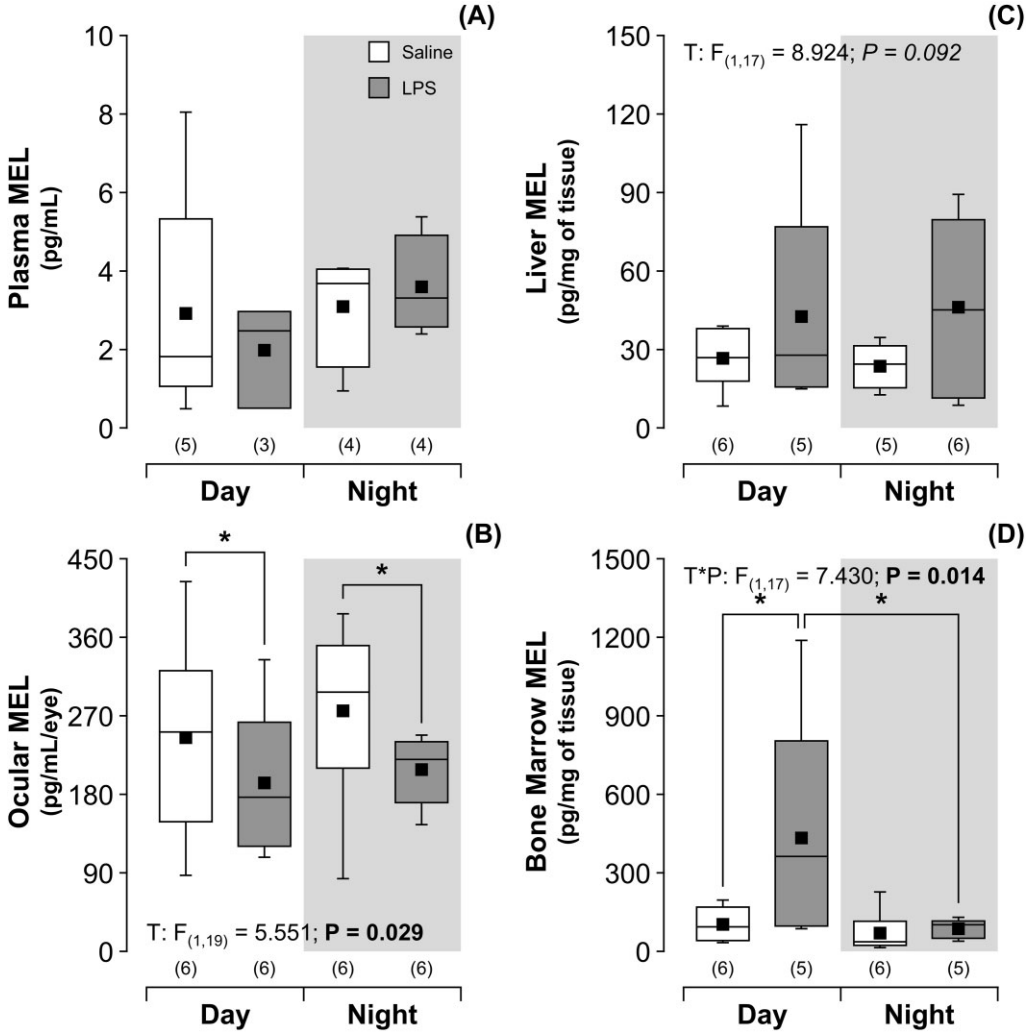

Fig. 2 Lipopolysaccharide (LPS) effects on melatonin (MEL) levels in *R. icterica* toads at day and night (experiment 2). (A) LPS effects on plasma melatonin levels; (B) LPS effects on plasma melatonin levels; (C) LPS effects on liver melatonin levels; and (D) LPS effects on bone marrow melatonin levels. Boxplots inside lines indicate medians; lower and upper borders represent first and third quartiles, respectively; black squares indicate means; whiskers represent upper and lower limits of 1.5 times interquartile range; and circles represent data out of this range. The asterisk (∗) denotes differences between treatments. Significance level at $P \leq 0.05$. The *N* is indicated below bars in parentheses. In the statistics, T = treatment factor; $T \times P$ = treatment × period interaction; and P indicates significance values.

lated by LPS. Plasma melatonin levels have not been modulated after the first hours following LPS stimulation in some anurans (Figueiredo et al. 2021; Garcia Neto et al. 2022). In the meantime, other studies demonstrated decreased plasma melatonin levels after LPS stimulation (Ferreira et al. 2021; Figueiredo et al. 2021; Titon Junior et al. 2021). The absence of response in plasma melatonin levels to LPS in our study could be due to the fact we measured the hormone before the melatonin peak, when its values were still low in *R. icterica* (Bastos et al. 2022). Meanwhile, corroborating the findings of Bastos et al. (2022), we observed decreased ocular melatonin following LPS during the night. In addition, we observed the same pattern during the day, demonstrating the *R. icterica* eyes are responsive to LPS at any phase of the day. Decreased central melatonin levels are observed through the direct action of pathogen-associated molecular patterns, such as LPS, and increased glucocorticoid levels in mammals and amphibians (Barsotti et al. 2017; Markus et al. 2018; Titon Junior et al. 2021). In accordance, we previously observed decreased melatonin associated with increased corticosterone plasma levels in amphibians (Titon et al. 2022), demonstrating the eye is responsive to immune stimulation and suggesting increased corticosterone can participate in this ocular melatonin regulation.

Regarding the bone marrow and liver, we observed increased melatonin levels in the bone marrow during the day and a trend to increase melatonin levels in the

Table 1 Results of the sets of two-way ANOVAs showing the lipopolysaccharide effects (treatment: LPS or saline) on the plasma, ocular, liver, and bone marrow melatonin levels at different periods (day or night) in *R. icterica* (experiment 2).

Variable	Source	Type III SS	DF	MS	*F*	*P*
Plasma melatonin	Intercept	130.006	1	130.006	31.265	**<0.001**
	Treatment	0.184	1	0.184	0.044	0.837
	Period	3.116	1	3.116	0.749	0.404
	Treatment × Period	2.008	1	2.008	0.483	0.500
	Error	49.898	12	4.158		
Ocular melatonin	Intercept	1,319,791.681	1	1,319,791.681	205.460	**<0.001**
	Treatment	35,655.334	1	35,655.334	5.551	**0.029**
	Period	10,158.552	1	10,158.552	1.581	0.224
	Treatment × Period	4177.551	1	4177.551	0.650	0.430
	Error	122,048.512	19	6423.606		
Liver melatonin	Intercept	224.904	1	224.904	0.419	0.526
	Body mass	4785.593	1	4785.593	8.924	**0.008**
	Treatment	1711.287	1	1711.287	3.191	*0.092*
	Period	114.454	1	114.454	0.213	0.650
	Treatment × Period	596.656	1	596.656	1.113	0.306
	Error	9116.047	17	536.238		
Bone marrow melatonin	Intercept	561,344.007	1	561,344.007	16.374	**0.001**
	Body mass	278,176.205	1	278,176.205	8.114	**0.011**
	Treatment	127,232.780	1	127,232.780	3.711	*0.071*
	Period	312,071.663	1	312,071.663	9.103	**0.008**
	Treatment × Period	254,718.550	1	254,718.550	7.430	**0.014**
	Error	582,799.147	17	34,282.303		

Abbreviations are as follows: **Type III SS:** type III sum of squares; **DF:** degrees of freedom; **MS:** mean square. Variables with *P* significant ≤0.05 are highlighted in bold.

liver, independently of the day phase. In amphibians, the bone marrow is an organ that accumulates immune cells during the day (Yaparla et al. 2020; Ruiz and Robert 2023). Meanwhile, these immune cells are released from the bone marrow to the bloodstream at night (Yaparla et al. 2020; Ruiz and Robert 2023). Therefore, we hypothesized the increase in melatonin levels following LPS stimulation during the day is probably attributable to local melatonin synthesis by the immune cells within the bone marrow, as described for rats (Tan et al. 1999). Conversely, during the night, when these immune cells are released into the bloodstream, we can no longer find discernible alterations in local melatonin levels. This hypothesis remains to be tested. On the other hand, the liver is a hematopoietic organ in amphibians, showing resident hematopoietic stem and progenitor cells, which produce and replenish the blood with new mature immune cells (Yaparla et al. 2020; Ruiz and Robert 2023). The immune cells within the liver may contribute to melatonin production, as suggested by the observed trend for elevated melatonin levels in response to LPS stimulation both during the day and night in our results. Isolating the immune cells from the bone marrow and liver and identifying melatonin production following LPS administration would corroborate this hypothesis.

Under healthy physiological conditions in mammals, leukocytes circulate within the bloodstream, with heightened melatonin levels preventing their translocation across the endothelial barrier. However, following immune stimuli, the activation of the innate immune response induces a reduction in melatonin levels, triggering leukocyte migration to the injury site (Markus et al. 2018). The immune cells, mainly macrophages, in the inflammatory site produce melatonin during the resolution phase to increase the phagocytic activity of local cells (Markus et al. 2018). This bidirectional communication between the pineal gland and the immune system is termed the immune-pineal axis and is known only for mammals to date (Markus et al. 2018). We propose an immune-pineal-ocular axis in amphibians (see the scheme in Fig. 3) based on the following information: ocular and plasma melatonin daily cycles with increased values at night have been described under homeostatic conditions in several amphibian species (D'Istria et al. 1994; Chiba et al. 2005; Wright et al. 2006; Bastos et al. 2022; Hu et al. 2022a,c). In addition, studies with anurans have shown that melatonin can be

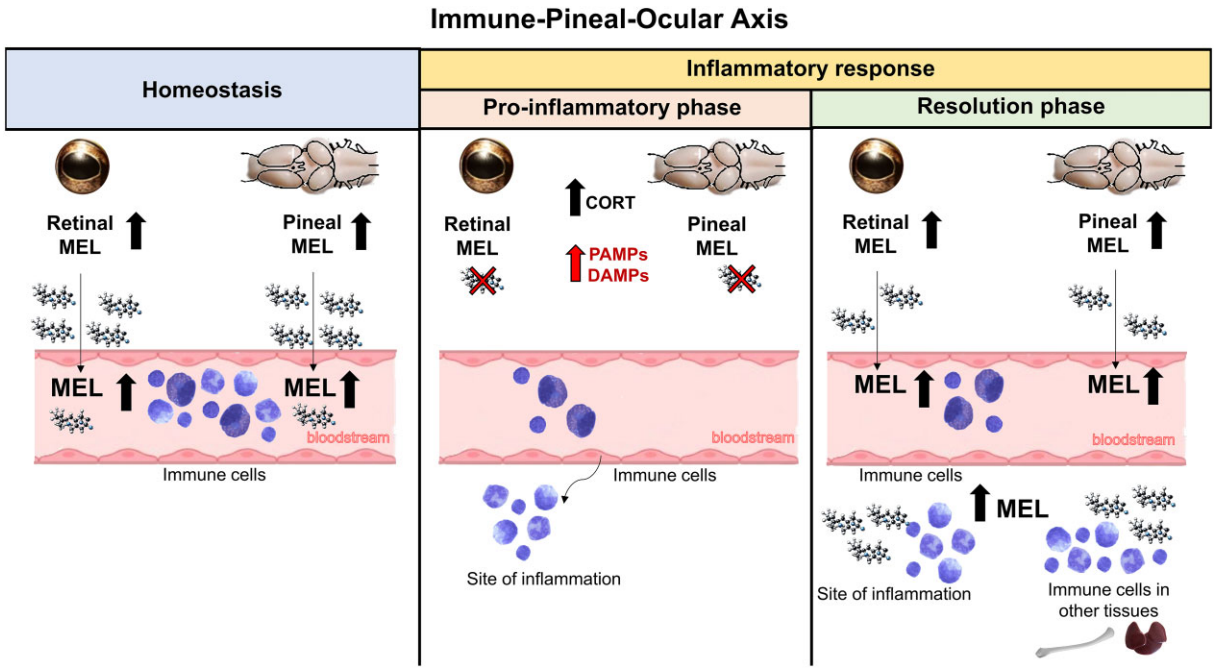

Fig. 3 Possible scheme of the immune-pineal-ocular axis in amphibians—backward and forward switch of melatonin synthesis from the pineal and eyes to immune cells. (A) *Homeostasis*—the eyes and the pineal gland produce melatonin (MEL) and release it into the bloodstream. (B) *Inflammatory response/Pro-inflammatory phase*—during the pro-inflammatory phase of the inflammatory response, pathogenic or damage-associated molecular patterns (PAMPs/DAMPs) set off a signaling cascade that inhibits MEL production in the eyes and the pineal gland. Additionally, the surge in corticosterone (CORT) levels further hampers MEL synthesis in these tissues. Concurrently, circulating immune cells transmigrate from the endothelium to the site of infection or inflammation. (C) *Inflammatory response/Resolution phase*—in the absence of PAMPs/DAMPs, immune cells at the site of infection or inflammation, as well as in other immune tissues, initiate MEL production. This serves as an autocrine/paracrine signal, enhancing the local immune response. Simultaneously, the eyes and pineal gland undergo recovery to resume MEL production, contributing to the restoration of elevated circulating MEL levels. The eyes and melatonin molecules were generated using AI (DALL-E).

produced by extra pineal and ocular tissues, such as the brain, skin, stomach, and intestine, by enterochromaffin cells acting as a paracrine hormone in other segments of the gastrointestinal tract (Bubenik and Pang 1997; Bubenik 2001; de Figueiredo et al. 2021; Cyrino et al. 2022; Hu et al. 2022b,c). In the meantime, melatonin production by immune cells has not been described in amphibians. However, our results demonstrate immune tissues such as bone marrow and the liver of amphibians have cells that can produce melatonin following LPS stimulation. In accordance, an immune enzyme, lysozyme, in *Rana dybowskii*'s serum and liver showed maximum activity during the darkness, suggesting a melatonin-induced immune augmentation in this function (Hu et al. 2022b). Besides, our results and other studies demonstrate that LPS suppresses ocular and plasma melatonin levels (Ferreira et al. 2021; Titon Junior et al. 2021; Bastos et al. 2022). Therefore, in light of the evidence presented here and in previous studies, we propose a model illustrating the function of the immune-pineal-ocular axis in amphibians (Fig. 3). However, further research is needed to explore the resolution phase and to comprehensively assess melatonin production and its enzymatic pathways in the eyes, pineal gland, and immune cells to substantiate the existence of the immune-pineal-ocular axis in this taxonomic group.

Conclusion

Our results showed a 24-h variation in ocular melatonin levels positively correlated with plasma melatonin levels. Interestingly, ocular melatonin levels were higher than in the plasma, indicating that the eyes might contribute to the resulting circulating melatonin levels in these toads. Our results also demonstrated that immune stimulation might impair ocular melatonin production. We observed decreased ocular melatonin levels following LPS exposure, with a concomitant increase in the bone marrow and an increased trend in the liver melatonin levels, possibly due to melatonin production by immune cells in these tissues. These results point to a possible immune-pineal-ocular axis in amphibians, which must be better characterized for understanding the role of melatonin during surveillance, as well as in the inflammatory response in amphibians.

Author contributions

SCMT: Conceptualization, Project administration, Methodology, Investigation, Formal analysis, Writing-Original draft preparation. BTJ: Methodology, Formal analysis, Writing- Reviewing, and Editing. ACF: Methodology, Writing- Reviewing, and Editing. PGGN: Methodology, Writing- Reviewing, and Editing. RPM: Investigation, Funding acquisition, Writing- Reviewing, and Editing. FRG: Conceptualization, Project administration, Writing-Original draft preparation, Funding acquisition, Writing-Reviewing, and Editing. VRA: Supervision, Conceptualization, Methodology, Investigation, Formal analysis, Writing-Original draft preparation.

Acknowledgments

We want to thank our teammates for their help with some lab procedures and the Managing Editor of ICB, Suzanne Miller, for selecting our work from the abstract list from SICB 2024 to be published.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP through the temático processes to RPM (2013/13,691–1) and to FRG (2014/16,320–7 and 2019/24,950–4) and through fellowships to BTJ (2016/01,782–0), and VRA (2015/23, 801–4). This work was also supported by the Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior—CAPES through a post-doc fellowship (88,887.473369/2020–00) to SCMT.

Data availability

The data supporting this article are available from the Mendeley Data repository at doi: https://data.mendeley.com/datasets/5xbhkm8w85/1

References

Acuña-Castroviejo D, Escames G, Venegas C, Díaz-Casado ME, Lima-Cabello E, López LC, Rosales-Corral S, Tan D-X, Reiter RJ. 2014. Extrapineal melatonin: sources, regulation, and potential functions. Cell Mol Life Sci 71:2997–3025.

Assis VR, Monteiro Titon SC, Teixeira Queiroz-Hazarbassanov NG, De Oliveira Massoco C, Ribeiro Gomes F. 2017. Corticosterone transdermal application in toads (*Rhinella icterica*): effects on cellular and humoral immunity and steroid plasma levels. J Exp Zool Pt A 327:200–213.

Assis VR, Titon SCM, Titon Jr. B, Gomes FR. 2022. The impacts of transdermal application of corticosterone on toad (*Rhinella icterica*) immunity. Integr Comp Biol 62:1640–1653.

Barsotti AMG, De Assis VR, Titon SCM, Titon B, Da Silva Ferreira ZF, Gomes FR. 2017. ACTH modulation on corticosterone, melatonin, testosterone and innate immune response in the tree frog *Hypsiboas faber*. Comp Biochem Physiol A Mol Integr Physiol 204:177–184.

Bastos PRO, Titon SCM, Titon Junior B, Gomes FR, Markus RP, Ferreira ZS. 2022. Daily and LPS-induced variation of endocrine mediators in cururu toads (*Rhinella icterica*). Chronobiol Int 39:89–96.

Bayarri Marı´a, Rol De Lama Marı´a, Madrid JA, Sánchez-Vázquez FJ. 2003. Both pineal and lateral eyes are needed to sustain daily circulating melatonin rhythms in sea bass. Brain Res 969:175–182.

Becerra López JL, Esparza Estrada CE, Romero Méndez U, Sigala Rodríguez JJ, Mayer Goyenechea IG, Castillo Cerón JM. 2017. Evidence of niche shift and invasion potential of *Lithobates catesbeianus* in the habitat of Mexican endemic frogs. PLoS One 12:1–15.

Blaustein A, Urbina J, Snyder P, Reynolds E, Dang T, Hoverman J, Han B, Olson D, Searle C, Hambalek N. 2018. Effects of emerging infectious diseases on amphibians: a review of experimental studies. Diversity 10:81.

Brennan R, Jan JE, Lyons CJ. 2007. Light, dark, and melatonin: emerging evidence for the importance of melatonin in ocular physiology. Eye 21:901–908.

Bubenik GA. 2001. Localization, physiological significance and possible clinical implication of gastrointestinal melatonin. Neurosignals 10:350–366.

Bubenik GA, Pang SF. 1997. Melatonin levels in the gastrointestinal tissues of fish, amphibians, and a reptile. Gen Comp Endocrinol 106:415–429.

Cain DW, Cidlowski JA. 2017. Immune regulation by glucocorticoids. Nat Rev Immunol 17:233–247.

Chiba A, Hattori A, Iigo M. 2005. Daily and circadian variations of the pineal and ocular melatonin contents and their contributions to the circulating melatonin in the Japanese newt, *Cynops pyrrhogaster*. Zoolog Sci 22:65–70.

Cogburn LA, Wilson-Placentra S, Letcher LR. 1987. Influence of pinealectomy on plasma and extrapineal melatonin rhythms in young chickens (*Gallus domesticus*). Gen Comp Endocrinol 68:343–356.

Córdoba-Moreno MO, De Souza EDS, Quiles CL, Dos Santos-Silva D, Kinker GS, Muxel SM, Markus RP, Fernandes PA. 2020. Rhythmic expression of the melatonergic biosynthetic pathway and its differential modulation *in vitro* by LPS and IL10 in bone marrow and spleen. Sci Rep 10:4799.

Cyrino JC, De Figueiredo AC, Córdoba-Moreno MO, Gomes FR, Titon SCM. 2022. Day versus night melatonin and corticosterone modulation by LPS in distinct tissues of toads (*Rhinella Icterica*). Integr Comp Biol 62:1606–1617.

De Abreu Manso PP, De Brito-Gitirana L, Pelajo-Machado M. 2009. Localization of hematopoietic cells in the bullfrog (*Lithobates catesbeianus*). Cell Tissue Res 337:301–312.

De Assis VR, Titon SCM, Barsotti AMG, Titon B Jr., Gomes FR. 2015. Effects of acute restraint stress, prolonged captivity stress and transdermal corticosterone application on immunocompetence and plasma levels of corticosterone on the cururu toad (*Rhinella icterica*). PLoS One 10:1–21.

De Figueiredo AC, Nogueira LAK, Titon SCM, Gomes FR, Carvalho JE de. 2022. Immune and hormonal regulation of the boa constrictor (Serpentes; Boidae) in response to feeding. Comp Biochem Physiol A Mol Integr Physiol 264:111119.

De Figueiredo AC, Titon SCM, Cyrino JC, Nogueira LAK, Gomes FR. 2021. Immune and hormonal modulation in the

postprandial period of bullfrogs (*Lithobates catesbeianus*). J Exp Biol 224:1–9.
Delgado MJ, Vivien-Roels B. 1989. Effect of environmental temperature and photoperiod on the melatonin levels in the pineal, lateral eye, and plasma of the frog, Rana perezi: importance of ocular melatonin. Gen Comp Endocrinol 75:46–53.
D'istria M, Monteleone P, Serino I, Chieffi G. 1994. Seasonal variations in the daily rhythm of melatonin and NAT activity in the harderian gland, retina, pineal gland, and serum of the green frog, *Rana esculenta*. Gen Comp Endocrinol 96:6–11,
Esteban MÁ, Cuesta A, Chaves-Pozo E, Meseguer J. 2013. Influence of melatonin on the immune system of fish: a review. Int J Mol Sci 14:7979–7999.
Ferreira LF, Garcia Neto PG, Titon SCM, Titon B, Muxel SM, Gomes FR, Assis VR. 2021. Lipopolysaccharide regulates pro- and anti-inflammatory cytokines, corticosterone, and melatonin in toads. Integr Org Biol 3:obab025.
Figueiredo ACD, Titon SCM, Titon Jr. B, Vasconcelos-Teixeira R, Barsotti AMG, Gomes FR. 2021. Systemic hormonal and immune regulation induced by intraperitoneal LPS injection in bullfrogs (*Lithobates catesbeianus*). Comp Biochem Physiol A Mol Integr Physiol 253:110872.
Floreste FR, Titon B, Titon SCM, Muxel SM, Gomes FR, Assis VR. 2022. Time course of splenic cytokine mRNA and hormones during a lipopolysaccharide-induced inflammation in toads. Integr Comp Biol 62:1618–1628.
Garcia Neto PG, Titon SCM, Assis VR, Muxel SM, Titon Jr B, Ferreira LF, Markus RP, Gomes FR, Fernandes PACM. 2022. Immune and endocrine responses of Cururu toads (*Rhinella icterica*) in their natural habitat after LPS stimulation. Comp Biochem Physiol A Mol Integr Physiol 269:111213.
Gardner S, Assis VR, Zhao H, Gomes FR, Peatman E, Mendonça MT. 2018. Differential gene expression to an LPS challenge in relation to exogenous corticosterone in the invasive cane toad (*Rhinella marina*). Develop Comp Immunol 88: 114–123.
Horodincu L, Solcan C. 2023. Influence of different light spectra on melatonin synthesis by the pineal gland and influence on the immune system in chickens. Animals 13:2095.
Hu N, Wang J, Zhao Y, Wei H, Li X, Li Y. 2022a. Daily cycle of melatonin in different tissues of dybowski's frog (*Rana dybowskii*). Biol Rhythm Res 53:1364–1372.
Hu N, Yu C, Jin J, Zhao X, Zhao Y, Wei H, Li Y. 2022b. Impact of photoperiods on the specific activities of immune and antioxidant enzymes in different tissues of Dybowski's frog (*Rana dybowskii*). Biol Rhythm Res 53:1790–1799.
Hu N, Zhao X, Jin J, Zhao Y, Wei H, Li X, Li Y. 2022c. Effects of photoperiod on the melatonin cycle of Dybowski's frog (*Rana dybowskii*). Biol Rhythm Res 53:1539–1549.
Iigo M, Furukawa K, Nishi G, Tabata M, Aida K. 2007. Ocular melatonin rhythms in teleost fish. Brain Behav Evol 69:114–121.
Jessop TS, Dempster T, Letnic M, Webb JK. 2014. Interplay among nocturnal activity, melatonin, corticosterone and performance in the invasive cane toad (*Rhinella marinus*). Gen Comp Endocrinol 206:43–50.
Krakauer T. 1968. The ecology of the neotropical toad, bufo marinus, in south Florida. Herpetologica 24:214–221.
Li RX, Li J, Zhang SY, Mi YL, Zhang CQ. 2018. Attenuating effect of melatonin on lipopolysaccharide-induced chicken small intestine inflammation. Poult Sci 97:2295–2302.
Lima AS, De Figueredo AC, Floreste FR, Garcia Neto PG, Gomes FR, Titon SCM. 2022. Temperature extreme events decrease endocrine and immune reactive scope in bullfrogs (*Lithobates catesbeianus*). Integr Comp Biol 62:1671–1682.
Lundmark PO, Pandi-Perumal SR, Srinivasan V, Cardinali DP. 2006. Role of melatonin in the eye and ocular dysfunctions. Vis Neurosci 23:853–862.
Madelaire CB, Silva DP, Titon SCM, Lamadrid-Feris F, Floreste FR, Titon B, Jr, Gomes FR. 2023. Contrasting effects of transdermal and implant corticosterone treatments in the American bullfrog wound healing. Philos Trans R Soc B Biol Sci 378:20220119.
Markowska M, Waloch M, Skwarlo-Sońta K. 2001. Melatonin inhibits PHA-stimulated chicken lymphocyte proliferation *in vitro*. J Pineal Res 30:220–226.
Markus RP, Fernandes PA, Kinker GS, Da Silveira Cruz-Machado S, Marçola M. 2018. Immune-pineal axis—acute inflammatory responses coordinate melatonin synthesis by pinealocytes and phagocytes. Br J Pharmacol 175:3239–3250.
Martin TR. 2005. Innate immunity in the lungs. Proc Am Thorac Soc 2:403–411.
Moore AF, Menaker M. 2011. The effect of light on melatonin secretion in the cultured pineal glands of Anolis lizards. Comp Biochem Physiol A Mol Integr Physiol 160:301–308.
Muñoz-Pérez JL, López-Patiño MA, Álvarez-Otero R, Gesto M, Soengas JL, Míguez JM. 2016. Characterization of melatonin synthesis in the gastrointestinal tract of rainbow trout (*Oncorhynchus mykiss*): distribution, relation with serotonin, daily rhythms and photoperiod regulation. J Comp Physiol B 186:471–484.
Pires-Lapa MA, Tamura EK, Salustiano EMA, Markus RP. 2013. Melatonin synthesis in human colostrum mononuclear cells enhances dectin-1-mediated phagocytosis by mononuclear cells. J Pineal Res 55:240–246.
Rada JAS, Wiechmann AF. 2006. Melatonin receptors in chick ocular tissues: implications for a role of melatonin in ocular growth regulation. Invest Ophthalmol Vis Sci 47:25–33.
Reiter RJ. 1993. The melatonin rhythm: both a clock and a calendar. Experientia 49:654–664.
Ruiz VL, Robert J. 2023. The amphibian immune system. Philos Trans R Soc Lond B Biol Sci 378:20220123.
Saha S, Singh KM, Gupta BBP. 2019. Melatonin synthesis and clock gene regulation in the pineal organ of teleost fish compared to mammals: similarities and differences. Gen Comp Endocrinol 279:27–34.
Serino I, D'Istria M, Monteleone P. 1993. A comparative study of melatonin production in the retina, pineal gland and harderian gland of *Bufo viridis* and *Rana esculenta*. Comp Biochem Physiol Part C Comp 106:189–193.
Singh SS, Haldar C. 2007. Biological significance of daily variation in immunity of *Perdicula asiatica*: role of melatonin and testosterone. Biol Rhythm Res 38:95–106.
Slominski AT, Hardeland R, Zmijewski MA, Slominski RM, Reiter RJ, Paus R. 2018. Melatonin: a cutaneous perspective on its production, metabolism, and functions. J Invest Dermatol 138:490–499.
Tan D-X, Manchester LC, Reiter RJ, Qi W-B, Zhang M, Weintraub ST, Cabrera J, Sainz RM, Mayo JC. 1999. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochimica Et Biophysica Acta (BBA)—Gen Sub 1472:206–214.

Titon SCM, Titon B, Muxel SM, De Figueiredo AC, Floreste FR, Lima AS, Gomes FR, Assis VR. 2022. Day vs. night variation in the LPS effects on toad's immunity and endocrine mediators. Comp Biochem Physiol A Mol Integr Physiol 267:111184.

Titon SCM, Titon Junior B, Assis VR, Kinker GS, Fernandes PACM, Gomes FR. 2018. Interplay among steroids, body condition and immunity in response to long-term captivity in toads. Sci Rep 8:1–13.

Titon SCM, Titon Junior B, Assis VR, Vasconcelos-Teixeira R, Garcia Neto PG, Lima AS, Ferreira LDF, Fernandes PA, Gomes FR, Markus RP. 2021a. Hormonal daily variation co-varies with immunity in captive male bullfrogs (*Lithobates catesbeianus*). Gen Comp Endocrinol 303:113702.

Titon SCM, Titon Junior B, Gomes FR, Assis VR. 2021b. Short-term stressors and corticosterone effects on immunity in male toads (*Rhinella icterica*): a neuroimmune-endocrine approach. Brain Behav Immun—Health 13:100230.

Titon Junior B, Titon SCM, Assis VR, Barsotti AMG, Vasconcelos-Teixeira R, Fernandes PACM, Gomes FR. 2021. LPS-induced immunomodulation and hormonal variation over time in toads. J Exp Zool Pt A 335:541–551.

Tosini G, Bertolucci C, Foà A. 2001. The circadian system of reptiles: a multioscillatory and multiphotoreceptive system. Physiol Behav 72:461–471.

Trinder J, Armstrong S, O≫Brien C, Luke D, Martin M. 1996. Inhibition of melatonin secretion onset by low levels of illumination. J Sleep Res 5:77–82.

Tripathi MK, Singh R, Tripathi MK. 2014. Melatonin modulates splenocyte immune response in the freshwater snake, natrix piscator. Herpetol Conserv Biol 9:257–266.

Wright ML. 2002. Melatonin, diel rhythms, and metamorphosis in anuran amphibians. Gen Comp Endocrinol 126:251–254.

Wright ML, Francisco LL, Scott JL, Richardson SE, Carr JA, King AB, Noyes AG, Visconti RF. 2006. Effects of bilateral and unilateral ophthalmectomy on plasma melatonin in Rana tadpoles and froglets under various experimental conditions. Gen Comp Endocrinol 147:158–166.

Yaparla A, Reeves P, Grayfer L. 2020. Myelopoiesis of the amphibian *Xenopus laevis* is segregated to the bone marrow, away from their hematopoietic peripheral liver. Front Immunol 10:3015.