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SYMPOSIUM

Day Versus Night Melatonin and Corticosterone Modulation by LPS in Distinct Tissues of Toads (*Rhinella Icterica*)

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Synopsis Pathogen-associated molecular patterns modulate melatonin (MEL) production in the pineal and extra-pineal sites and corticosterone (CORT) synthesis in the adrenal/interrenal and other tissues. Both MEL and CORT play essential and complex immunomodulatory roles, controlling the inflammatory response. Given that most of what we know about these interactions is derived from mammalian studies, discovering how MEL and CORT are modulated following an immune challenge in anurans would increase understanding of how conserved these immune-endocrine interactions are in vertebrates. Herein, we investigated the modulation of MEL and CORT in plasma *vs*. local tissues of toads (*Rhinella icterica*) in response to an immune challenge with lipopolysaccharide (LPS; 2 mg/kg) at day and night. Blood samples were taken 2 hours after injection (noon and midnight), and individuals were killed for tissue collection (bone marrow, lungs, liver, and intestine). MEL and CORT were determined in plasma and tissue homogenates. LPS treatment increased MEL concentration in bone marrow during the day. Intestine MEL levels were higher at night than during the day, particularly in LPS-injected toads. Bone marrow and lungs showed the highest MEL levels among tissues. Plasma MEL levels were not affected by either the treatment or the phase. Plasma CORT levels increased in LPS-treated individuals, with an accentuated increase at night. Otherwise, CORT concentration in the tissues was not affected by LPS exposure. Modulation of MEL levels in bone marrow suggests this tissue may participate in the toad's inflammatory response assembly. Moreover, MEL and CORT levels were different in tissues, pointing to an independent modulation of hormonal concentration. Our results suggest an important role of immune challenge in modulating MEL and CORT, bringing essential insights into the hormone-immune interactions during anuran's inflammatory response.

Introduction

Immune function activation in response to immune challenges is regulated by several neural and endocrine signals, including immunomodulatory hormones such as glucocorticoids and melatonin (MEL) (Falso et al. 2015; Paavonen 2016). MEL, for example, is a hormone produced in the pineal gland of vertebrates rhythmically (Carrillo-Vico et al. 2005; Bastos et al., 2022). Pineal MEL is synthesized from tryptophan and then is released into the blood, peaking during the night to signal darkness (Carrillo-Vico et al. 2005). Extra-pineal sources of MEL were also described in retina cells (Gern and Ralph 1979; Cahill and Besharse 1989), intestine (Bubenik 2002), skin (Grace et al. 1991; Slominski et al. 2005; Slominski et al. 2008), liver (Slominski et al. 2018), lungs (Martin and Frevert 2005), and immune cells (Markus et al. 2018) in different vertebrates, such as birds, amphibians, and mammals. As much as MEL has an essential role as an endogenous synchronizer of the light/dark cycle when produced by the pineal, the hormone also acts as a regulator of other physiological processes when produced by different cell types, playing an essential role in the complex neuroimmune-endocrine system (Acuña-Castroviejo et al. 2014; Markus et al. 2018).

MEL plays an immunomodulatory role in the assembly and resolution phases of the inflammatory response (Skwarlo-Sonta et al. 2003; Markus et al. 2018).

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Increased plasma MEL shows an anti-inflammatory role in healthy conditions, reducing adhesion proteins' production by endothelial cells, thus inhibiting immune cells' migration to peripheral tissues (Markus et al. 2018). During an infectious process, macrophages recognize pathogen-associated molecular patterns (PAMPs) and signal the synthesis of pro-inflammatory cytokines (Markus et al. 2007). PAMPs signalization combined with higher plasma glucocorticoid levels inhibits pineal MEL production, facilitating the transmigration of immune cells to the peripheral tissues, initiating the inflammatory response assembly (Markus et al. 2007, 2018). Meanwhile, at the site of infection, PAMPs induce MEL production, increasing phagocytosis activity and production of anti-inflammatory cytokines, leading to the resolution of the inflammation (Markus et al. 2018).

Glucocorticoids are another important immunomodulatory class of hormones (Dhabhar 2014; Cain and Cidlowski 2017). Produced by the activation of the hypothalamic-pituitary-adrenal axis, the glucocorticoids are produced rhythmically, signalizing the active phase, and are the main mediator of the stress response (Sapolsky et al. 2000). Such as MEL, corticosterone (CORT), the main glucocorticoid in amphibians, has been described to modulate and be modulated by the inflammatory response (Cain and Cidlowski 2017). CORT plasma levels increase following an immune challenge in different vertebrates (Salome et al. 2008; Gibb et al. 2008; Titon Jr. et al. 2021). Indeed, CORT modulates the inflammatory response by regulating several immune functions, such as cell influx to the inflammatory site, phagocytosis activity, and production of pro and anti-inflammatory cytokines, among others (Cain and Cidlowski 2017). In addition, increased glucocorticoids during the initial inflammatory response decrease the secretion of pineal MEL (Fernandes et al. 2009; Falso et al. 2015). Once the immune challenge is controlled, glucocorticoids participate in the resolution of the inflammatory response and inhibit the synthesis of cytokines and pro-inflammatory mediators, besides contributing to pineal MEL production restauration (Fernandes et al. 2009). In addition, extra-adrenal/interrenal CORT production has been already described in several tissues, such as lungs (Hostettler et al. 2012), lymphoid organs (Taves et al. 2016, 2017; Slominski et al. 2020), and skin (Taves et al. 2011; Slominski et al. 2013), in contexts of homeostasis and infection. This local production seems to be important to modulate the immune cell maturation (lymphoid organs), as well as local immune activation and inflammation (other tissues) (Taves et al. 2011).

Evidence of central and local MEL and CORT modulation following an immune challenge has been observed differently among vertebrate groups (Taves et al. 2011, 2017); Markus et al. 2018, with some pieces of the complete frame also observed in anurans. One type of PAMP commonly used as an immune challenge in different vertebrates is the main component of the outer membrane of gram-negative bacteria: the lipopolysaccharide or LPS (Leulier et al. 2003; Llewellyn et al. 2010; Gardner et al. 2018). The LPS is recognized by the toll-like receptor 4 (TLR4) present in mammalian immune cells like lymphocytes, neutrophils, and macrophages (Triantafilou and Triantafilou 2002). Amphibians possess receptors homologous to mammalian TLR4 receptors (Ishii et al. 2007). Once the recognition is made, signaling cascades that control the production of antimicrobial substances and chemokines are activated, assembling inflammatory response (Leulier and Lemaitre 2008). Recent studies showed increased plasma CORT (Gardner et al. 2020; Bastos et al., 2022; Titon Jr. et al. 2021; Titon et al. 2021a) and decreased plasma MEL (Bastos et al., 2022; Titon Jr. et al. 2021; Figueiredo et al. 2021a) levels following LPS exposure for three *Rhinella species* and *Lithobates catesbeianus*. Still, as much as changes in MEL and CORT have already been described in anurans in the context of stress and digestion (Bubenick and Pang 1997; Barsotti et al. 2017; Titon et al. 2018), local and systemic CORT and MEL modulation remains to be further explored in a context of an immune challenge, especially the association of these hormonal responses with the day cycle. It is known that plasma CORT and MEL levels show daily variation, as well as this fluctuation co-varies with immune function in anurans (Titon et al. 2021b). However, the CORT and MEL concentration in toad's organs is unknown. Then, understanding the responses to immune challenges are dependent on the day cycle helps to understand anuran chronobiology better and if the inflammatory response could be favored during a specific phase.

This study aimed to investigate the effect of an immune challenge on the MEL and CORT secretion by extra-pineal and extra-interrenal tissues, respectively, in different phases (day *vs.* night) in toads (*Rhinella icterica*). As during inflammatory response, CORT plasma levels increase and the MEL source switches from central to extra-pineal production, we tested the following hypothesis: (1) MEL and CORT concentration in the extra-pineal and extra-interrenal tissues (bone marrow, lungs, liver, and intestine) increase in response to an immune challenge with lipopolysaccharides (LPS), regardless the phase; (2) Plasma CORT levels increase, and MEL levels decrease in response to an immune challenge with LPS; (3) reduction in plasma MEL levels in

response to LPS is more evident in the night phase when MEL is higher.

Methods

Studied species and experimental design

Rhinella icterica is a large toad from the *Rhinella marina* group, which shows a wide geographic distribution in the Southern Brazilian Atlantic Rain Forest (Maciel et al. 2010). *R. icterica* was selected as a model for this study considering the previous knowledge about endocrine-immune interactions on this species (Gomes et al. 2012; Moretti et al. 2017; Assis et al. 2019, 2017, 2015; Titon et al. 2021, 2018; Bastos et al., 2022), allowing a better understanding of endocrine modulation in amphibians.

Individuals from the *R. icterica* species were collected in Botucatu (22° 53′ 11.8″S, 48° 29′ 23.2″W), Sao Paulo/Brazil (n = 24), in July/2019, with the authorization from the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (ICMBio n° 29896). The animals were transported to the laboratory and kept individually in plastic containers (43 × 28.5 × 26.5 cm) with free water access. The lids of the boxes had holes to allow air circulation. Toads were kept inside a climatic chamber (Fitotron), submitted to controlled photoperiod 12:12 LD (lights turn on at 6 am and turn off at 6 pm), and temperature (20 ± 1°C) for ten days based on previous studies with the same species and experimental design (Bastos et al., 2022). All the animals were fasting during this habituation.

To investigate the MEL and CORT concentration in stimulated animals after an immune challenge with LPS day and night. The experiments were conducted in darkness using infra-red light, invisible to frogs due to being outside their eye's region of spectral sensitivity (Jaeger & Hailman 1973). The animals were divided into four groups: Vehicle day (n = 6), LPS day (n = 6), Vehicle night (n = 6), and LPS night (n = 6), taking into consideration their body mass and snout-vent-length, in a way that each group had similar sized animals. Toads were intraperitoneally injected with LPS (serotype O127: B8, 2 mg/kg) diluted in Anuran phosphate-buffered saline (APBS; NaCl 8 g, KCl 0.2 g, Na_2PO_4 1.44 g, KH_2PO_4 0.24 g, diluted in 1.3 L of distilled water), or only APBS at 10 am or 10 pm. Animals were killed by decapitation (Holmes 1993) two hours after the injection (noon or midnight). Both the LPS dose and the sampling point were chosen based on previous studies with species from the *Rhinella* group, including *R. icterica*, which facilitates comparisons (Bastos et al., 2022; Gardner et al. 2018, 2020; Moretti et al. 2018; Titon Jr. et al. 2021; Titon et al. 2021a).

All the experimental procedures were performed with the approval of the local animal ethics committee of the Bioscience Institute of the University of São Paulo (CEUA n° 242/2016).

Blood samples and tissue collection

Blood collection (700 μL) was performed two hours after the injection (LPS or APBS) through cardiac puncture. The blood sample was centrifuged (4 min, 604 g), and the plasma was isolated and frozen at a −80°C freezer for MEL and CORT plasma levels quantification. After the decapitation, the liver, lungs, intestine, and bone marrow were surgically and aseptically removed and immediately frozen (−80°C). Bone marrow cells were obtained from the femur by centrifuging the bone (14,000 g, 5 min, 4°C). Next, the opposite bone extremity was cut, then the bone was turned upside down and centrifuged again to collect the bone marrow cells (14,000 g, 5 min, 4°C). The centrifugation's resulting cells were collected in a microtube (1.5 mL) and immediately frozen (−80°C).

Tissue samples processing

Each tissue (intestine, liver, and lung) was weighed, and 60 mg was selected for processing. After MEL and CORT quantification, the hormone concentration was normalized by protein and wet tissue weight and expressed as pg/mg protein/g tissue. The tissues were pulverized with liquid nitrogen in a porcelain mortar, then the tissues were homogenized in 100 μL of tris-HCl (Trizma base, Sigma #T6066-500G; HCl, EDTA, and EGTA; pH 7), and more 300 μL of tris-HCl were added. After homogenization in the vortex, the mix was centrifuged (20,000 g, 5 min, 4°C). The supernatant was isolated and frozen at −80°C for hormone quantification. For the bone marrow, 400 μL of tris-HCl was added to the bone marrow cells, which were homogenized and centrifuged after (14,000 g, 5 min, 4°C). The supernatant was collected and frozen (−80°C) for hormone quantification (Cardinali et al. 1979; Figueiredo et al. 2021b).

Melatonin quantification

To quantify the plasma MEL levels and the MEL concentration in the tissues, the samples (plasma and supernatant from the homogenized tissues) were extracted through silica extraction columns (Waters Sep- Pak® Vac). Briefly, kit extraction columns were washed with 1 mL of pure methanol and ultrapure water and centrifuged (120 g, 1 min, 23°C). Then, samples were added to the columns and subsequentially washed with 500 μL ultrapure water and twice with 1 mL of methanol

(10%). Retained MEL in the column was recovered with pure methanol. Samples + pure methanol were allowed to evaporate for about 2 h at 45 °C, and pellets were resuspended in ultrapure water for the hormone assay. The MEL concentrations were determined by the ELISA commercial kit (IBL—RE54021) following the manufacturer's instructions and previous studies with amphibians (Barsotti et al. 2017; Bastos et al., 2022; Titon Jr. et al 2021; Figueiredo et al. 2021a; Titon et al. 2021b). Briefly, 50 µL of the resuspended sample was plated along with 50 µL of melatonin biotin and 50 µL of melatonin antiserum and incubated overnight at 4°C. The incubation solution was discarded the following day. The plate was washed three times with a buffer solution, and 150 µL of enzyme conjugate was added for the subsequent 2 h incubation at room temperature on an orbital shaker (500 rpm). The supernatant was discarded, and the plate was again washed three times. Then, 200 µL of the PNPP substrate solution was added, and the plate was again incubated at room temperature on an orbital shaker (500 rpm) for 40 min. After the last incubation, 50 µL of PNPP stop solution was added, and the plate was read in a spectrophotometer at 405 nm. The kit sensibility was 13.20 pg/mL, and the intra and inter-assay coefficients were 8.01% e 6.12%, respectively.

Corticosterone quantification

To quantify the plasma CORT levels and the CORT concentration in the tissues, the samples (plasma and supernatant from the homogenized tissues) were extracted with ether, according to the CORT parallelism for this same species (Assis et al. 2017). The CORT concentrations were determined by the ELISA commercial kit (Cayman Chemical CORT number 501320) following the manufacturer's instructions and previous studies with the same species, *R. icterica* (Assis et al. 2015, 2017; Titon et al. 2018, 2021a). The kit sensibility was 16.04 pg/ml, and the intra and inter-assay coefficients were 5.05% and 4.88%, respectively.

Parallelism curve for melatonin and corticosterone in the lungs

A parallelism curve was performed with lung samples to validate the MEL and CORT kit sensibility in detecting the MEL and CORT in the *R. icterica* samples. Serial dilutions were made to the parallelism curve with a pool of lung samples (n = 6) and the standard kit curves (1:1, 1:2, 1:4, 1:8, and subsequently). The sample dilution used to quantify the MEL and CORT in the tissue was based on the pool samples from the parallelism that resulted in 50% binding (B/B0) (Fig. S1).

Protein quantification

To normalize the MEL and CORT concentration in the tissue, we performed the protein quantification in each sample in a nanodrop (Nagorny et al. 2011). First, we divided the MEL and CORT quantified in the tissues by the protein quantity in each sample. The values of MEL and CORT in the tissues were expressed as pg/mg protein/g tissue. We used the ND1000 V3 3.1 program in the "proteins A280–sample type BSA" configuration to perform the protein quantification. The procedure was performed by placing 1 µL of water in the NanoDrop device and pressing the "Blank" button, then placing 1 µL of Tris-HCl buffer and pressing "Re-blank," and then placing 1 µL of the sample and pressing "measure." Eight samples were the maximum performed in a row, and then the water and buffer were made again to recalibrate the machine (Figueiredo et al. 2021b).

Statistical analysis

Complete descriptive statistics were performed for each treatment. The data were tested for homogeneity of variance and normality, as prior requirements of parametric tests. Two data (1 CORT in the intestine from LPS day and 1 CORT in bone marrow from LPS night) were excluded according to the Z-score ($Z > 3$), in which the values were more than 20 times higher than the highest values. Other data were missing because the values were outside the maximum detection limit of the kit (4 CORT bone marrow, being 1 from the Vehicle day, 1 from LPS day, and 2 from the Vehicle night; 2 MEL bone marrow, being 1 from LPS day and 1 from LPS night; 1 CORT intestine LPS night; 1 CORT liver from LPS day; 2 MEL liver being 1 from LPS day and 1 from LPS night; and 1 CORT lung from LPS day). Since it was not possible to predict maximum values, we did not consider them. Also, for the MEL and CORT plasma levels, we did not have the necessary volume of plasma to run the assay for all the samples. Then we did not perform the MEL and CORT quantification for these samples: 7 MEL plasma levels (1 from vehicle day, 2 from LPS day, 2 from Vehicle night and 2 from LPS night) and 2 CORT plasma levels (1 from LPS day and 1 from LPS night). The N for each group can be observed below each corresponding treatment bar in the figures.

The effects of the treatment (LPS and Vehicle) and the phase (day and night) over the MEL and CORT concentration in the plasma and the tissues were verified through analyses of covariance (ANCOVA). We first conducted mixed ANCOVA models, with the MEL and CORT in the plasma and tissues used as dependent variables, body mass used as a co-variable, treatment (LPS and Vehicle) and phase (day and night) were used as independent factors, and tissue was used as within-subject

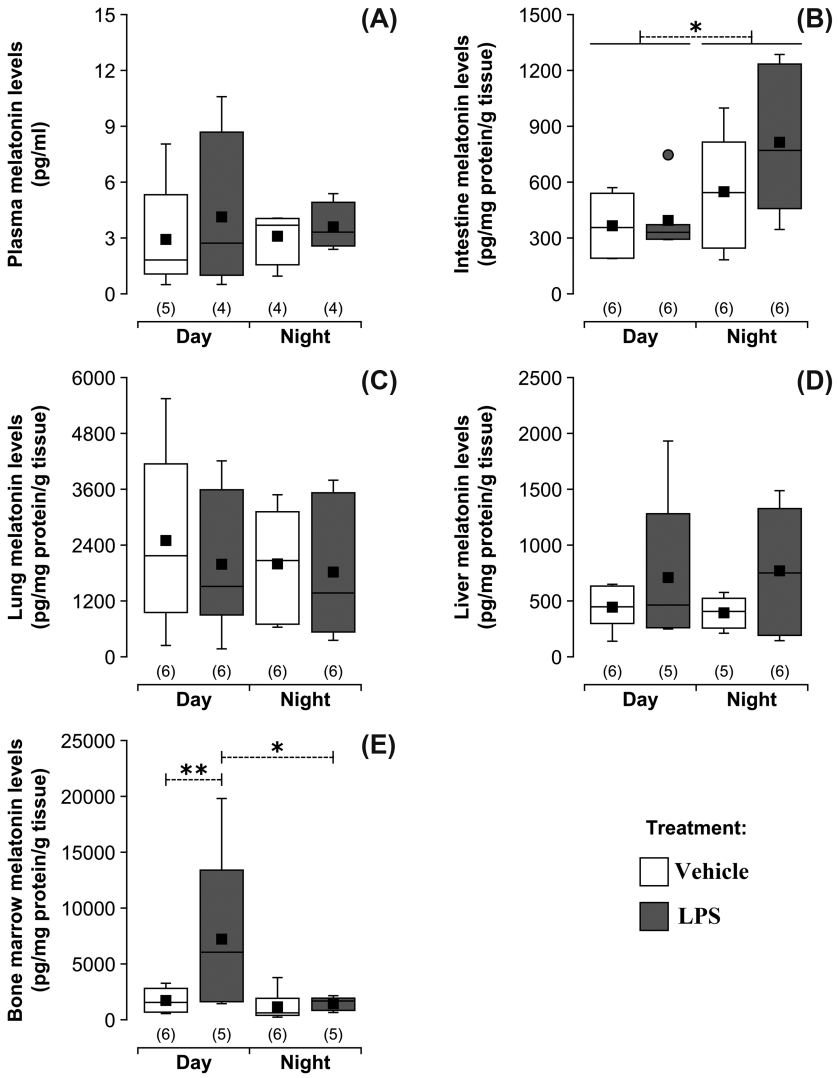

Fig. 1 Melatonin levels in plasma and organs of *Rhinella icterica* treated with LPS and vehicle during the day and night phases. **(A)** Plasma, **(B)** Intestine, **(C)** Lungs, **(D)** Liver, and **(E)** Bone Marrow. Boxplot inside lines indicate medians, lower and upper borders represent 1st and 3rd quartiles, respectively, black squares indicate means, whiskers represent upper and lower limits of 1.5 times interquartile range, and the circle represents values outside this range. The asterisk (∗) represents significant statistical differences ($P < 0.05$) by mean pars comparison with Bonferroni adjustment. One asterisk (∗) denotes the difference between phases and two (∗∗) between treatments.

factor. However, the co-variable (body mass) presented a significant interaction with tissue (For CORT: body mass∗tissue: $F_{3,33} = 8.981$, $P < 0.001$; For MEL: body mass∗tissue: $F_{3,45} = 9.299$, $P < 0.001$). Since this fact breaks the analysis assumption (Field 2013), we performed independent sets of ANCOVAs for each dependent variable.

We additionaly performed a mixed ANOVA model with treatment and phase as between-subject factors and tissue as a within-subject factor only to compare MEL and CORT levels among the tissues.

For all tests, we used a significance level of $P \leq 0.05$. We also considered a trend when significance levels were between $P > 0.05$ and $P \leq 0.100$ since there is a conventional use of 1-tail as significant too when tests apply to directional predictions. The analysis was followed by Bonferroni adjustment for multiple pairwise comparisons when applicable. The analyses were made through SPSS version 26.

Results

Melatonin

Plasma MEL and MEL levels in the lungs and intestine were not affected by body mass ($P \geq 0.259$). Plasma MEL levels were not affected by treatment ($F_{1,12} = 0.453$; $P = 0.514$) or phase ($F_{1,12} < 0.001$; $P > 0.999$; Fig. 1A). The MEL levels in the intestine were affected by phase ($F_{1,19} = 6.522$, $P = 0.019$), and toads showed higher MEL levels in intestines during the

night (Fig. 1B). The MEL levels in the lungs were not affected by treatment ($F_{1,19} = 0.284$, $P = 0.600$) or phase ($F_{1,19} = 0.298$; $P = 0.592$; Fig. 1C).

Liver MEL levels were affected by body mass ($F_{1,17} = 8.924$; $P = 0.008$), where larger animals showed higher MEL levels. The MEL levels in the liver tended to be affected by LPS treatment ($F_{1,17} = 3.191$, $P = 0.092$), with increased mean values in the LPS than in the Vehicle group (Fig. 1D). Bone marrow MEL levels were affected by body mass ($F_{1,17} = 8.114$; $P = 0.011$), where larger animals showed lower MEL levels. MEL levels in the bone marrow were affected by the treatment∗phase interaction ($F_{1,17} = 7.430$; $P = 0.014$). The MEL levels in the bone marrow were higher in the LPS than in the Vehicle group during the day (Fig. 1E). Bone marrow MEL levels were also higher during the day than at night in the LPS group (Fig. 1E).

MEL levels were different among the tissues depending on the phase (tissue∗phase) ($F_{3,48} = 3.598$; $P = 0.020$) and independently of treatment. During the day, MEL levels in the bone marrow and lungs were higher than in the intestine and liver ($P \leq 0.014$). During the night, only the MEL in the lungs was higher than in the intestine ($P = 0.014$).

Complete MEL analysis details can be found in the supplementary materials (ANCOVAs in Table S1 and mixed ANOVA in Table S2).

Corticosterone

Plasma CORT and CORT levels in the lungs, intestine, and liver were not affected by body mass (body mass: $P \geq 0.196$). Plasma CORT levels were affected by the LPS treatment ($F_{1,17} = 4.464$; $P = 0.049$), with LPS treated toads showing higher values than those from the Vehicle group (Fig. 2A). CORT levels in the intestine were not affected by treatment ($F_{1,17} = 0.309$; $P = 0.268$) or phase ($F_{1,17} = 2.230$; $P = 0.154$; Fig. 2B). CORT levels in the lungs were also not affected by treatment ($F_{1,18} = 1.063$; $P = 0.316$) or phase ($F_{1,18} = 0.822$; $P = 0.376$; Fig. 2C). CORT levels in the liver were not affected by treatment ($F_{1,18} = 1.532$; $P = 0.232$), but tended to be affected by phase ($F_{1,18} = 3.503$; $P = 0.078$; Fig. 2D). CORT levels in bone marrow were affected by body mass ($F_{1,14} = 4.546$, $P = 0.051$), where larger animals showed lower CORT. CORT in the bone marrow was not affected by treatment ($F_{1,14} = 1.895$; $P = 0.190$; Fig. 2E), but tended to be affected by phase ($F_{1,14} = 3.152$; $P = 0.098$).

CORT levels were different among the tissues independently of treatment and phase ($F_{3,36} = 36.658$; $P < 0.001$), with higher CORT levels in the bone marrow and lungs than in the intestines and liver ($P \leq 0.018$).

Complete CORT analysis details can be found in the supplementary materials (ANCOVAs in Table S3 and mixed ANOVA in Table S4).

Discussion

Melatonin

The present study shows that plasma and lung MEL levels are not modulated by the immune challenge (LPS injection) or phase (day *vs.* night) in *Rhinella icterica* toads. Otherwise, toads showed higher MEL levels in the intestines during the night. Moreover, MEL levels in the bone marrow were higher in LPS treated toads during the day but not at night. Also, we were able to detect MEL in all the tissues sampled (bone marrow, liver, intestine, and lungs) in *R. icterica* toads. These results are in accordance with previous studies investigating tissue MEL production in bone marrow, spleen, intestine, and lungs of mammals (Bubenik 2002; Martin and Frevert 2005; Córdoba-Moreno et al. 2020). Furthermore, MEL presence in tissues in different concentrations than plasma was also previously described for *Lithobates catesbeianus* (Bubenik and Pang 1997; Figueiredo et al. 2021b). These results point to a discussion about the function of local MEL production in distinct tissues, which probably is tightly connected with immunity, displaying an essential role in the combat against pathogens (Tan et al. 1999; Acuña-Castroviejo et al. 2014; Kurhalul et al. 2018; Markus et al. 2018; Córdoba-Moreno et al. 2020). Besides being associated with immune functions, local basal MEL production is relevant to other physiological processes in tissues. For example, in the intestines, MEL influences the peristalsis and contributes to the digestive process synchronization (Bubenik et al. 1996; Bubenik 2001; Mukherjee and Maitra 2015).

LPS treatment, mimicking an immune challenge, led to increased MEL concentrations in the bone marrow and liver of *R. icterica* toads. The increase in MEL following LPS exposure indicates these tissues also participate in the immune response assembly and its systemic regulation when facing a pathogenic agent in amphibians, as previously demonstrated for mammal's immune cells (e.g., bone marrow and liver cells, macrophages, and microglia) (Tan et al. 1999; Kurhalul et al. 2018; Córdoba-Moreno et al. 2020; *reviewed in*: Carrillo-Vico et al. 2005; Markus et al. 2018). Otherwise, MEL plasma levels did not decrease in response to LPS treatment in *R. icterica*, contrary to expected. These results might be explained by an absence of a MEL peak in animals decapitated at midnight, precluding the identification of LPS effects on MEL plasma levels. Accordingly, *Lithobates temporaria* showed a MEL peak 6h after the lights were turned off (Serino et al. 1993; D'Istria

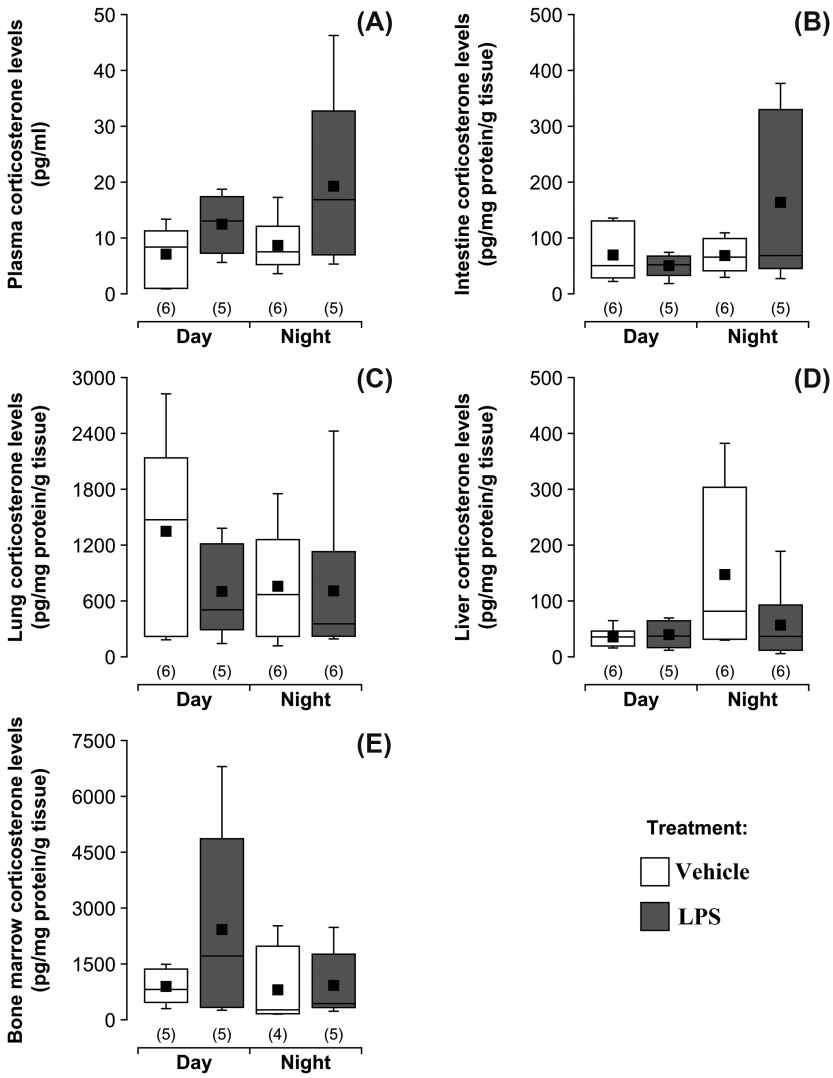

Fig. 2 Corticosterone levels in plasma and organs of *Rhinella icterica* treated with LPS and vehicle during the day and night phases. **(A)** Plasma, **(B)** Intestine, **(C)** Lungs, **(D)** Liver, and **(E)** Bone Marrow. Boxplot inside lines indicate medians, lower and upper borders represent 1st and 3rd quartiles, respectively, black squares indicate means, and whiskers represent upper and lower limits of 1.5 times inter-quartile range.

et al. 1994), and *Lithobates catesbeianus* 3h after the lights were turned off (Titon et al. 2021b), demonstrating that plasma MEL peak can be variable. We must also consider that impaired MEL secretion was previously observed after Vehicle treatment in rats (Tamura et al. 2010). Therefore, the injection itself could have promoted a decrease in pineal MEL secretion even in the Vehicle group, thus precluding the identification of LPS effects on central MEL production (Fernandes et al. 2017).

Another critical factor to be considered is the phase of the treatment. The LPS treatment increased bone marrow MEL concentration compared to the Vehicle group during the day. LPS induces an increase in MEL levels associated with the increased expressions of AANAT (Aralkylamine N-Acetyltransferase, enzyme related to the biosynthesis of melatonin and an essential regulator of the circadian rhythm) and ASMT (N-Acetylserotonin O-methyltransferase, enzyme that catalyzes the final reaction in the biosynthesis of melatonin) in rats (Córdoba-Moreno et al. 2020), a pattern that remains to be explored in amphibians. Besides, bone marrow regulates the efflux and the influx of the immune cells (mainly neutrophils and monocytes) into the bloodstream during a 24h day (Furze and Rankin 2008). Once bone marrow has higher neutrophil and macrophage concentrations during the light phase (Furze and Rankin 2008), this could be associated with a higher immune responsivity in this organ during this phase. It is interesting to observe that nocturnal rats present a higher global gene expression in the liver during the night, a daily pattern opposite to the one

seen in humans (Almon et al. 2008). These results suggest that the daily gene expression peak in some organs might be associated with the daily species phase of activity (Stebelová et al. 2007; Almon et al. 2008; Córdoba-Moreno et al. 2020). Previous studies have also evidenced increased MEL in the intestine plays a protective function during digestion (Huether 1994; Ayles et al. 1996; Bubenik 2001). In this way, it is interesting to observe that intestine MEL concentration was affected by phase, and LPS treated toads showed higher MEL concentration during the night, the phase these animals are more active and thus feeding (Pough 2007), than the ones that get their LPS injection during the day.

MEL concentration in the lungs was high at all points, although it was not affected by either phase or treatment. Besides the respiratory function, the lungs present an essential immune role in rats (Kang et al. 2011). The lungs are the interface between the individual and the environment; thus, lung immune cells act as the host defense against possible pathogens (Zaas and Schartz 2005). Since MEL production by immune cells plays a central role during infection (Markus et al. 2018), increased MEL following LPS in the lungs would be expected. However, the lack of response of lungs to LPS treatment in *R. icterica* might be explained by the higher responsiveness of this organ to local than systemic stimuli (Kaltreider 1976). Otherwise, rats show pulmonary proteins that bind to LPS sites, blocking LPS recognition by immune cells and consequently blocking its inflammatory effects (Knapp et al. 2006). Such proteins in toads might explain the observed lack of the LPS-induced effect lungs of *R. icterica*, which remains to be explored.

Corticosterone

Our results showed plasma CORT levels were affected by the LPS injection. Still, no LPS-induced effect on CORT levels was observed in the other tissues (bone marrow, lungs, intestine, and liver) in *R. icterica* toads in any phase (day vs. night). Increased plasma CORT levels following LPS are expected (Titon Jr et al. 2021; Ferreira et al. 2021) since LPS triggers the CORT synthesis and release by stimulating the hypothalamic hypophyseal adrenal axis and direct adrenal stimulation in mammals (Bornstein et al. 2006). Besides, LPS-induced indirect CORT production can be observed via interleukin 1β (IL1β) in the adrenal cells (Elenkov and Chrousos 2002). In accordance, studies conducted with anurans also found increased plasma CORT for *R. marina* (Gardner et al. 2020), *R. diptycha* (Titon Jr. et al. 2021), and *R. icterica* (Bastos et al., 2022; Titon et al. 2021a) in response to LPS. Augmented CORT during an infection mediates several immune functions, including reduced gene expression of pro-inflammatory cytokines and increased cellular phagocytosis (Cain and Cidlowski 2017). Indeed, increased circulating CORT presents important anti-inflammatory actions by suppressing pro-inflammatory cytokine expression and regulating inflammatory resolution (Cain and Cidlowski 2017). Therefore, increased CORT seems essential for modulating the inflammatory response (Cain and Cidlowski 2017).

Contrary to our expectation, CORT levels in the liver, lungs and intestine were not responsive to LPS in *R. icterica*. However, CORT levels in the bone marrow tend to be higher in the LPS group. Besides, CORT differed among tissues, pointing to local control of CORT concentrations. Immune tissues, such as the thymus and intestine, can synthesize glucocorticoids (CORT and cortisol), which act in a paracrine or autocrine manner playing physiological roles in local homeostasis, cell development, and immune cell activation in mammals (reviewed in: Talabér et al. 2013). Although glucocorticoids activate and modulate immune function in local tissues under healthy conditions (Lechner et al. 2001; Taves et al. 2011; Talabér et al. 2013), it is unclear if a systemic stimulus can influence local CORT production. The absence of LPS modulation in most tissues from *R. icterica* could be related to the fact that the LPS was intraperitoneally injected, promoting systemic inflammation. Noti et al. (2010a, 2010b) treated rat intestine cells with LPS showing an LPS-induced increase in intestinal glucocorticoid synthesis during an immune challenge. Thus, a local stimulus might be necessary to induce extra-adrenal glucocorticoid synthesis. At the same time, CORT in the bone marrow tended to be higher in the LPS group during the day, suggesting a local production of CORT following an immune challenge during the day. Additionally, local CORT in bone marrow, liver, lungs, and intestine do not reflect plasma CORT variation, suggesting the absence of modulation of CORT tissues following the systemic immune stimulus. More studies are necessary to determine the origin of CORT in these tissues, and if CORT is locally produced, the required stimuli to modulate its secretion in distinct tissues from *R. icterica* toads.

Conclusions

Our results showed that the concentration of MEL in extra-pineal tissues, including intestines and bone marrow, is modulated by an immune challenge and by phase (day *vs.* night) in *Rhinella icterica* toads. Additionally, the phase of increased MEL following LPS differs between tissues, evidencing the complexity of individual defense against pathogens. Moreover, MEL levels in the bone marrow and lungs were the

highest among tissues, suggesting bone marrow and lung cells are associated with increased MEL synthesis. In the meantime, plasma CORT was modulated by LPS, indicating hypothalamic-hypophyseal-interrenal activation following an immune challenge in *R. icterica*. Still, little evidence of modulation of CORT concentration in tissues by systemic LPS stimulus was found. These results bring new insights into the hormone-immune interactions in vertebrates and open new avenues of investigation for future studies on the functional relevance and the mechanisms of MEL and CORT production and its modulation in different tissues of amphibians. Including different doses of LPS and other times post-injection to induce MEL and CORT in different tissues may provide a better understanding of the local endocrine modulation in response to immune challenges in amphibians. We also would like to consider that larger sample sizes could give us a better picture of the reported changes. We understand that small sample size could have a meaningful impact on our results, preventing us from observing a possible response in LPS treated animals if it exists. Therefore, including larger samples must be considered in future studies since the individual variation is wide in wild-caught toads.

Author contributions

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

Competing interest statement

The authors have no competing interests to declare.

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Supplementary data

Supplementary Data available at *ICB* online.

Data availability statement

The original data used in this manuscript is available at the Mendeley Data, through the DOI: 10.17632/25jx9t827v.1

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