Patrício Getúlio Garcia Neto

Efeitos de um desafio imune sobre parâmetros imunológicos e endócrinos de sapos Cururus (*Rhinella icterica*) em seu habitat natural

Effects of immune challenge on immunological and endocrine parameters of Cururu toads (*Rhinella icterica*) in their natural habitat

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A tudo e a todos que tornaram isto possível

As horas de tolice são medidas pelo relógio, mas as de sabedoria nenhum relógio pode medir.

William Blake, *O Casamento do Céu e do Inferno*

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Primeiramente agradeço aos meus pais, pois sem seus esforços diários para que nada me faltasse durante toda minha vida eu não teria chegado onde estou hoje. Eles são meus maiores exemplos de vida, de superação e dedicação a tudo aquilo que fazem, sempre dando o melhor possível para atingirem seus objetivos. E seguindo o exemplo deles eu dei o meu máximo para realizar este trabalho. Também sou extremamente grato aos meus queridos irmãos, que são fontes de inspiração para que em momentos difíceis eu me mantenha firme e dê o meu melhor para alcançar meus objetivos, pois um dos deveres de um irmão mais velho é servir de exemplo para os mais novos.

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Resumo

Os hormônios glicocorticoides e melatonina são conhecidos por possuírem funções imunomodulatórias, podendo agir tanto como estimuladores quanto supressores da resposta imune, a depender do contexto. Enquanto suas propriedades imunológicas têm sido bem exploradas em mamíferos, em anfíbios ainda há poucos trabalhos que abordem essa interação imune-endócrina em um contexto inflamatório, e aqueles feitos recorreram ao uso do cativeiro para tal. Avaliar como esses animais reagem em campo frente a um desafio imune pode trazer informações relevantes acerca de como parâmetros imunofisiológicos são modulados em condições naturais. O objetivo deste trabalho foi investigar o efeito de um desafio imune, representado por uma injeção com lipopolissacarídeos (LPS), em distintos horários de atividade de anuros (*Rhinella icterica*) recém-capturados em seu ambiente natural na Floresta Atlântica. Foram avaliados os seguintes parâmetros: capacidade bactericida plasmática, níveis hormonais (corticosterona, melatonina e testosterona plasmática) e expressão gênica de citocinas. A injeção com LPS induziu o aumento de corticosterona e na expressão da interleucina-1β. O horário da injeção influenciou na resposta de algumas citocinas, indicando uma maior resposta inflamatória no começo da noite. Nossos resultados mostram que o LPS induziu uma resposta inflamatória, com horário de injeção influenciando alguns dos parâmetros avaliados, encontrando um padrão semelhante ao que foi visto em cativeiro em outros estudos. Enquanto este trabalho encontrou resultados mais associados à primeira etapa da resposta inflamatória, outros estudos devem ser feitos a fim de se avaliar etapas posteriores da montagem de uma resposta imune.

Palavras chave: Anfíbio; Citocina; Corticosterona; Imunidade; LPS; Melatonina.

Abstract

Glucocorticoids and melatonin show immunomodulatory functions, acting as both stimulators and suppressors of the immune response, depending on the context. While their immune properties are well explored in mammals, in amphibians there are still few studies on this immune-endocrine interaction in an inflammatory context, all of them under captivity conditions. Evaluating how these animals react in the field to an immune challenge can bring relevant information regarding how immune-physiological parameters are modulated in natural conditions. The aim of this study was to evaluate the effect of an immune challenge, represented by lipopolysaccharide (LPS) injection, in different activity times of toads (*Rhinella icterica*) recently captured in their natural habitat in Atlantic Forest. The following parameters were evaluated: bacterial killing ability, hormone levels (plasma corticosterone, melatonin and testosterone) and gene expression of cytokines. LPS injection induced an increase in corticosterone plasma levels and in the gene expression of interleukin-1β. Time of injection affected some of the cytokines response, indicating a higher inflammatory response at the beginning of the night. Our results show that LPS induced an inflammatory response, with time of injection affecting some of the evaluated parameters, showing a similar pattern found in other studies in captivity. While this work found results associated more with the first stage of the inflammatory response, other studies should evaluate further stages in the montage of an immune response.

Keywords: Amphibian; Cytokine; Corticosterone; Immunity; LPS; Melatonin

List of Abbreviations

ACTH	Adrenocorticotropic hormone
ANCOVA	Analysis of covariance
ANOVA	Analyses of variance
BKA	Bacterial killing ability
Bd	*Batrachochitrium dendrobatidis*
BI	Body index
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
DAMP	Damage associated molecular pattern
DNA	Deoxyribonucleic acid
GC	Glucocorticoid
GR	Glucocorticoid receptor
HPA/I	Hypothalamus – Pituitary – Adrenal/Interrrenal axis
HPG	Hypothalamus – Pituitary – Gonadal axis
IFN γ	Interferon γ
IL1β	Interleukin 1 beta
IL6	Interleukin 6
LPS	Lipopolysaccharides
MEL	Melatonin

NA	Noradrenaline
NF- κB	nuclear transcription factor κB
PAMP	Pathogen associated molecular patterns
PCA	Principal component analysis
PCR	Polymerase chain reaction
PKA	cAMP-dependent protein kinase
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerase chain reaction
SAL	Saline
SCG	Superior cervical ganglion
SCN	Suprachiasmatic nucleus
SVL	Snout-vent length
T	Testosterone
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor α

1. Introduction

1.1. Anurans and global epidemics

The Brazilian amphibian diversity is one of the greatest in the world, with 1136 species spread across the national territory, most of it composed of anurans, with 1093 species, divided into 20 families (Segalla *et al.*, 2019). In the family Bufonidae, the species *Rhinella icterica*, popularly known as Cururu toad, is composed by large animals with rough skin and cutaneous venom glands, called parotid glands, which protects them against predators (Maciel *et al.*, 2010). *R. icterica* individuals are nocturnal animals with a generalist lifestyle, and can be found not only in forest zones, but also in urban regions, capable of surviving despite anthropic perturbations (Maciel *et al.*, 2010). They reproduce seasonally, during winter within the region of the state of São Paulo, extending from July to August, exhibiting peak of activity in August (Jim, 2002). Since they have an indirect development, with aquatic stage larvae, the tadpoles, they depend on the presence of water bodies (such as temporary ponds created during the rain) for reproduction (Maciel *et al.*, 2010).

In the last decades amphibian populations in many regions of the globe have suffered with declines caused by diverse factors, such as habitat destruction and epidemics from different types of pathogens, including Ranavirus and the fungi *Batrachochitrium dendrobatidis* (*Bd*), responsible for causing iridoviral infections and chytridiomycosis, respectively (Daszak *et al*., 1999). Both pathogens are associated with dissemination through water bodies, affecting not only adult individuals but also tadpoles, presenting high mortality rates across populations (Daszak *et al.*, 1999). Responsible for the "red-leg disease", *Aeromonas hydrophila* is a gram-negative bacteria present in different types of environments, specially fresh and brackish water (Hazen *et al.*, 1978), and capable of infecting amphibians (Hill *et al.*, 2010). This pathogen has

been accounted for the high mortality of wild and captive amphibian populations during the 20th century (Rivas, 2016). However, it's known today that part of this mass report may be due to the infection of other pathogens with similar symptoms (such as the Ranavirus and *Bd*), suggesting that *A. hydrophyla* acts as an opportunistic pathogen when the hosts are immunocompromised (Rivas, 2016). Considering these threats, it's important to investigate how different species of amphibians react to immune challenges, how their organisms respond to an infection and how immune-physiological parameters involved with the responses are modulated.

1.2. Glucocorticoids: considerations in anurans' physiology

The Hypothalamus – Pituitary (Hypophysis) – Adrenal/Interrrenal axis (HPA/I) is an important endocrine axis involved in a vast array of functions in vertebrates, including metabolism, cardiovascular tone, neuroplasticity and immunity, among others (Sapolsky *et al.*, 2000; Norris, 2007). Following stimulation, the hypothalamus secretes the corticotropin-releasing hormone (CRH). CRH stimulates the release of adrenocorticotropic hormone (ACTH) in the hypophysis, which stimulates the production and secretion of glucocorticoids by the adrenocortical cells in the adrenal/interrenal glands (GCs; Whitnall, 1997). GCs, once in the blood, inhibits the production of CRH and ACTH in the hypothalamus and hypophysis, respectively, negatively regulating the activity of the HPA/I axis (Whitnall, 1997). In vertebrates, even though there is a ubiquitous presence of adrenocortical cells capable of producing GCs, they are arranged in different forms according to the taxon. In amphibians, specifically anurans, we can find adrenocortical cells arranged into a pair of interrenal glands located on the ventral surface of kidneys, whose main secretion is corticosterone (CORT; Norris, 2007).

One of the most well understood functions of GCs is their metabolic effects, mobilizing energetic resources for activity (Sapolsky *et al.,* 2000). It's known that a preparatory increase in GCs secretion happens close to the period of activity. In a nocturnal species, such as the anuran *R. icterica,* the peak of CORT secretion happens around sunset, just before the beginning of the night, preparing the organism physiology for the activity in the next hours (Bastos, 2017). Other known important function of GCs is immunomodulation. GCs secretion can be influenced by several stressful conditions (Sapolsky *et al.*, 2000) and, among them, those related to immunological challenges (Sapolsky *et al.*, 2000). In this sense, cytokines (regulatory components of the immune system), such as interleukins (IL)-1β, IL6 and tumor necrosis factor-α (TNFα), are capable of stimulating the production of CRH by the hypothalamus, ultimately leading to an increase in GCs levels (Cain & Cidlowski, 2017). Furthermore, adrenocortical cells present toll-like receptors (TLR2 and TLR4) capable of recognizing pathogen/damage associated molecular patterns (PAMPS and DAMPS) and to induce GC production directly (Bornstein *et al.*, 2006).

GCs may induce immune-stimulatory effects, such as increasing macrophage phagocytosis at moderate concentrations (Zen *et al.*, 2011) and modulating leukocytes distribution in the organism, reducing the amount of lymphocytes in the blood (Dhabhar, 2006), and leading to an increase in the proportion of circulating neutrophils. This change in the ratio of neutrophils and lymphocytes in the blood can be used as a parameter to evaluate stress response, with a higher ratio often being associated with the montage of a stress response (Davis *et al.*, 2008; Garcia-Neto *et al.*, 2020). Furthermore, GCs may also present immunosuppressive effects, inhibiting the expression of pro-inflammatory cytokines (IL1β, TNFα, IL6 and interferon-γ [IFNγ], for example), adjusting the inflammatory response (Cain & Cidlowski, 2017). Also, GCs reduce the

activation and proliferation of T and B cells and shift the immune response from Th1 (associated with pro-inflammatory cytokines) towards a more Th2/Treg (associated with the production of regulatory and anti-inflammatory cytokines such as IL10) activity pattern (Sapolsky *et al.*, 2000).

The HPA/I axis is well known to be associated with stress responses, since environmental stressors (infectious or not) can stimulate the activity of this axis resulting in increased production of GCs (Whitnall, 1997). A short-term stress response, lasting hours, can be beneficial to the organism, mobilizing energy resources to regions where they are most needed at that moment (Wingfield & Romero, 2001). However, when the stress reaches chronic conditions, lasting weeks, we can observe deleterious effects arising from the exacerbated GCs activity. We can highlight their immunosuppressive effects under this condition, including the apoptosis of T cells and atrophy of lymphoid organs, thus making the individual more vulnerable to pathogens (Sapolsky *et al.*, 2000; Córdoba-Moreno *et al.*, 2019). In anurans, for example, decreased bacterial killing ability (BKA) and phagocytosis have been detected after movement restriction challenge and/or maintenance in captivity during several days (Assis *et al.*, 2015; Titon *et al.*, 2017). Other important consequence of chronic elevated GC levels is the inhibition of the activity of the Hypothalamus – Pituitary (Hypophysis) – Gonadal axis (HPG), culminating in reduced secretion of androgens [such as testosterone (T)] and reproductive function. CORT and T may also interact in other contexts. In vocalizing anurans, high levels of CORT are correlated with high levels of T, probably because CORT mobilizes energetic resources towards the oblique muscles involved with calling rate and intensity (Emerson, 2001). Conversely, when the individuals are exposed to a stressor like starvation or predators, the increase in CORT production could lead to decreasing levels of T (Emerson, 2001).

The immunoregulatory properties of T are still a matter of discussion in vertebrates. Classically, T is considered an immunosuppressive hormone (Schroderus *et al.*, 2010; Roberts *et al.*, 2004), with high levels of it being associated with depletion of humoral and cellular mediated immune responses and thymic mass (Grossman, 1984; 1985). Accordingly, the immunocompetence handicap hypothesis predicts that males benefit from high levels of T in the development of secondary sex traits and reproductive behaviors relevant to its fitness; but these high levels of T also weights down on males suppressing immune response and elevating the parasitic load (Folstad & Karter, 1992). However, others studies reported that T may have some immunostimulatory effects, more specifically, increasing antibodies response and their production in male birds (Peters, 2000; Evans *et al.*, 2000). Evaluating how T interacts with other hormones during the inflammatory assemblage could help to elucidate more of its immune properties.

1.3. Melatonin: considerations in anurans' physiology

Melatonin (MEL), produced by the pineal gland of vertebrates, is popularly known as the "darkness hormone", transducing darkness signals from the environment to the organism and synchronizing the endogenous oscillatory system (Reiter *et al.*, 2010). In mammals, after receiving the light stimuli, captured by the photopigment melanopsin present at specialized ganglion cells (Provencio *et al*., 2002), the environmental lighting information is transmitted via the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN, entrained by the light-dark cycle (adjustment of clock genes), regulates the circadian rhythms of peripheral oscillators, including adrenals and the pineal gland (Sakamoto *et al.*, 1998). The environmental lightning information, firstly decoded by the SCN, is transmitted through the

paraventricular nucleus, hindbrain, spinal cord, superior cervical ganglion (SCG) and reaches the pineal (Arendt, 1998). Plasma MEL levels are high at night and low during the day in all vertebrates. However, the pineal gland has different structures depending on the group. In ectotherms, the pineal gland possesses special structures which allow light perception in the form of photoreceptor cells resembling a retina. This gland is also located below the skull in a thinner area in ectotherms, facilitating light entry, while in mammals it's more internalized, depending more on the signals transmitted by the SCN (Fálcon *et al.*, 2009). MEL is produced not only in the pineal gland, but also in other regions of the organism, including the retina (Hardeland, 2009; Fálcon *et al.*, 2009). The retina is the main source of MEL in amphibians, fluctuating according to daytime (Serino *et al.*, 1993; Baker *et al.*, 1965; Delgado & Vivien-Roels, 1989) and its biosynthesis pathway is the same as in the pineal (Fálcon *et al.*, 2009).

The immune effects associated with MEL had been recently reviewed by ours and others groups (Carrillo-Vico *et al.*, 2013; Markus *et al.*, 2018). During a healthy context of the organism, MEL produced by the pineal gland reduces the expression of adhesion molecules by endothelial cells (Lotufo *et al.*, 2001). This property diminishes the rolling and adhesion of phagocytic cells to the endothelium, negatively regulating the migration of leukocytes through the endothelium, which avoids an unnecessary immune response from the organism (Markus *et al.*, 2007). However, during acute inflammatory responses, MEL production in this gland is inhibited by the association of neuro (pattern of adrenoceptors activation; Fernandes *et al.*, 2017), immune (cytokines and PAMPS; Fernandes *et al.*, 2006; Domínguez-Rodriguez *et al.*, 2002) and endocrine (high levels of GCs; Zhao & Touitou, 1993) signals, allowing the migration of leukocytes through the endothelial layer to the site of lesion (Markus *et al.*, 2018). Once these leukocytes arrive, MEL produced by phagocytic cells acts in an autocrine/paracrine manner,

enhancing the expression of molecules responsible for potentiating phagocytosis, which will lead to the clearing of inflammatory debris, thus contributing to the resolution of the immune response (Yi & Kim, 2017). Also, during the resolution phase, the interaction between a decreased adrenergic stimulus, GCs in the pineal, and immune signals such as IFNγ (Barbosa-Lima *et al.*, 2019) normalize MEL production, restoring the inhibition of leukocyte migration through the endothelium (Fernandes *et al.*, 2017). This switch in the MEL action and bimodal interaction with the immune system is known as the Immune-Pineal Axis (Markus *et al.*, 2018).

Besides the communication between immune system and pineal MEL, there is also a crosstalk between the pineal and adrenal glands in mammals, as it was briefly exposed above. During healthy condition, MEL production is stimulated through sympathetic input represented by activation of β-adrenoceptors. At night, the observed increase in MEL production is represented by β and α-adrenoceptors activation in the pineal (Fernandes *et al.*, 2017). Sympathetic stimulation enhances the expression of *Aanat* (gene) and phosphorylation of AANAT (protein), enzymes involved in the MEL synthesis pathway. Both β and α-adrenoceptors activation will lead to an increase in the cellular content of cyclic adenosine monophosphate (cAMP), which will activate the cAMP-dependent protein kinase (PKA), responsible for inducing the expression of *Aanat* and phosphorylating AANAT, preventing its degradation by proteasomes (Simonneaux & Ribelayga, 2003). During an inflammatory response, GCs will bind with glucocorticoid receptors (GRs) in pineal cells. The resulting stimuli from both adrenergic receptors and GRs will inhibit MEL production, resulting in the scenery observed in the immune-pineal axis. And during the resolution phase of the immune response, when there is a decreased adrenergic stimulus represented by activation of only β-adrenoceptors, this along with the activation of GR will restore the MEL production by the pineal (Fernandes *et*

al., 2017). Therefore, in mammals, GCs have bimodal effects on pineal MEL production depending on the immune context.

1.4. Rhythmicity and the immune system

GCs and MEL, both immunoregulatory hormones, present circadian rhythm of production/inhibition, synchronized by environmental cues, allowing the individual to anticipate and respond effectively to daily challenges, such as foraging and exposition to pathogens (Reiter *et al.*, 2010; Pancak & Taylor, 1982). Similarly, other immune components also present circadian rhythms controlled by their endogen circadian clocks (Labrecque & Cermakian, 2013), tuning the appropriate response to any threats in the organism. Macrophages, for example, show a time-dependent response to PAMPs, even after being synchronized in vitro and then stimulated at different times for several days (Keller *et al.*, 2009). It's also known that neutrophils express clock genes (Haimovich *et al.*, 2010), and present a time-dependent response to inflammatory challenges in the lung of mice, being more present at the early rest period (Gibbs *et al.*, 2014). Furthermore, treating rats with PAMPs at the beginning of their period of activity evokes a stronger cytokine response than when administrated during resting phase (Gibbs *et al.*, 2012). Immune components present different timing of activity depending on their role in the organism, each own with their endogenous circadian machinery, and while this time-dependent activity is being actively explored in mammals, in amphibians it still needs to be more uncovered. Studying the effects of inflammatory challenges at different times of the day in amphibians could reveal more about how their immune system operates within a circadian rhythm.

1.5. Lipopolysaccharide challenge: effects and field perspectives

Lipopolysaccharides (LPS) are endotoxins derived from the cell wall of gram-negative bacteria, such as *Escherichia coli*. They have been often used in studies on innate immune response (Mekaouche *et al.*, 1994; Tamura *et al.*, 2009) and behavioral fever (Kluger, 1991; Bicego *et al.*, 2002) in several animal models. LPS binds to TLR4 and triggers the nuclear translocation of NF-κB dimers (nuclear transcription factor κB), which regulates the transcription of several genes, such as the inflammatory cytokines IL1β, IL6 and TNFα (Lu *et al.*, 2008).

The NF-κB signaling pathway is present in rat pineal cells and is associated with the regulation of MEL production during healthy conditions (Cecon *et al.*, 2010; Da Silveira Cruz-machado *et al.*, 2017) and innate immune responses (Da Silveira Cruz-Machado *et al.*, 2010). The activation of TLR4 receptors in the cells of the pineal gland increases the expression of cytokine TNFα, responsible for inhibiting the transcription of *Aanat*, the synthesis of N-acetylserotonin, and MEL (Fernandes *et al.*, 2006; Da Silveira Cruz-Machado *et al.*, 2010). Similar effects were verified in amphibians by Bastos (2017): *R. icterica* individuals presented high levels of plasma CORT and low levels of both plasma and ocular MEL two hours after injection with LPS. In toads from genus *Rhinella* there is also literature regarding LPS effects on gene expression of inflammatory cytokines (Gardner *et al.*, 2018), hormones and cytokines levels (Bastos, 2017) and, also, other studies evaluating its effects on the development of behavioral fever (Bicego *et al.*, 2002; Moretti *et al.*, 2018). However, there is still more to explore considering amphibians and how their physiological parameters change during an innate immune response, especially when in their natural habitat.

Studies involving LPS commonly use animals in captivity, with some few studies analyzing its effects on behavior and immune-endocrine parameters on birds in the field (Owen-Ashley *et al.*, 2006; Hegemann *et al.*, 2013). In amphibians, to the best of our knowledge, the

studies evaluating immune-endocrine interactions in the context of an inflammatory challenge were done in captivity. Studies in captivity are essential when we need to understand complex interactions such as between the immune and endocrine systems. However, captivity conditions can induce immune-endocrine changes on the individuals. In previous studies, it was demonstrated that, when exposed to captivity conditions, toads from genus *Rhinella* presented high plasma levels of CORT, but low plasma levels of T, associated with a reduction in BKA, in animals kept in captivity for short (seven days) and long (months) periods (Assis *et al.*, 2015; Titon *et al.*, 2017). Therefore, studies in the field could offer more relevant information about how immune-physiologic parameters can be modulated in animals in their natural habitat.

To evaluate how an inflammatory response varies in distinct periods may provide interesting information about how its components interact in different contexts. Concerning anurans, investigating how immunoregulatory hormones, such as GCs, MEL and T respond to an immune challenge in different moments during their period of activity will not only help us understand better about their roles during an immune response, which still needs more investigation in this group, but also how their effects can change depending on the temporal context.

2. Objective

The objective of the present study was to investigate LPS-induced effects on the immune-endocrine parameters in freshly captured *R. icterica* toads. We hypothesized that LPS induces a more intense inflammatory response in the onset of the evening when compared with late evening. We predicted (1) increased CORT, BKA and pro-inflammatory cytokines expression, associated with (2) decreased MEL and T following LPS exposure. Also, (3) increases in CORT and pro-inflammatory cytokines expression should be more pronounced at the onset of the evening, while, (4) decreased MEL should be more accentuated in the late evening, when plasma MEL levels are maximum.

3. Materials & Methods

3.1. Specimen sampling and experimental design

Animals were collected in the municipality of São Luís do Paraitinga (23º 13' 22'' S, 45º 18' 42'' W) in the State of São Paulo, Brazil, in September 2018. In order to assess the weather conditions, HOBO data loggers were used to register humidity and temperature at the days of capture (data were recorded within 5 min intervals). Additionally, toads' body temperature was measured using infrared thermometer (TR-300, Equitherm, São Paulo/SP, Brazil) at the moment of animal capture.

Adult males of the *Rhinella icterica* (n = 45) were manually collected and immediately divided into two treatment groups: saline (n = 22) and LPS (n = 23). Sampling started at 7 pm, after sunset (around 6:30 pm) and in total darkness. Toads were collected mainly in pairs, one for each treatment group, throughout the night, from 7 pm until 11:30 pm, to investigate variation in the physiological parameters associated with toads' activity period. Flashlights using red filter were used during animal collection, sampling and processing in the field, to prevent animals' exposure to white light, since MEL production can be inhibited by light (Trinder *et al.*, 1996).

Immediately after capture, the animals were identified, weighted and measured. Then, LPS (2 mg/kg diluted in saline solution; *Escherichia coli* O127:B8, L3129 Sigma Aldrich) or saline injections were administered via intraperitoneal (Gardner *et al.*, 2018). After injection, toads were allocated in individual plastic boxes (40 x 28,5 x 26,5 cm), with perforated lids which permitted the air flow. Blood sampling was done 2 h after injection (Figure 1), followed by euthanasia (decapitation) to remove the spleen used to measure the gene expression of cytokines. Animals injected from 7 pm to7:59 pm were euthanized from 9 pm to 9:59 pm. Toads in this timeframe were called the Early Night group. Otherwise, toads injected from 10 pm to 10:59 pm

were euthanized from 12 am to 12:59 am. Toads in this timeframe were called the Late Night group.

Toads were bled by cardiac puncture using previously heparinized 1 ml syringes and 26G x 1/2'' needles. Blood samples were collected within 3 min, given that CORT can be influenced by the stress of capture and handling after this time period (Romero & Reed, 2005). Blood samples were kept in ice for approximately 4 h, and then centrifuged (3000 rpm, 4 min). Plasma samples were kept in liquid nitrogen and then transferred to a -80 ºC freezer to measure the concentration of CORT, MEL, T and BKA assay. Spleens were immediately frozen (liquid nitrogen), and then transferred to the -80 ºC freezer.

Toads were collected under authorization from Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, 8132-1). The procedures were performed according to the ethical standards of the Brazilian National Council on Experimental Animal Control (CONCEA) and were approved by the local Ethics Committee (protocol nº 323/2018).

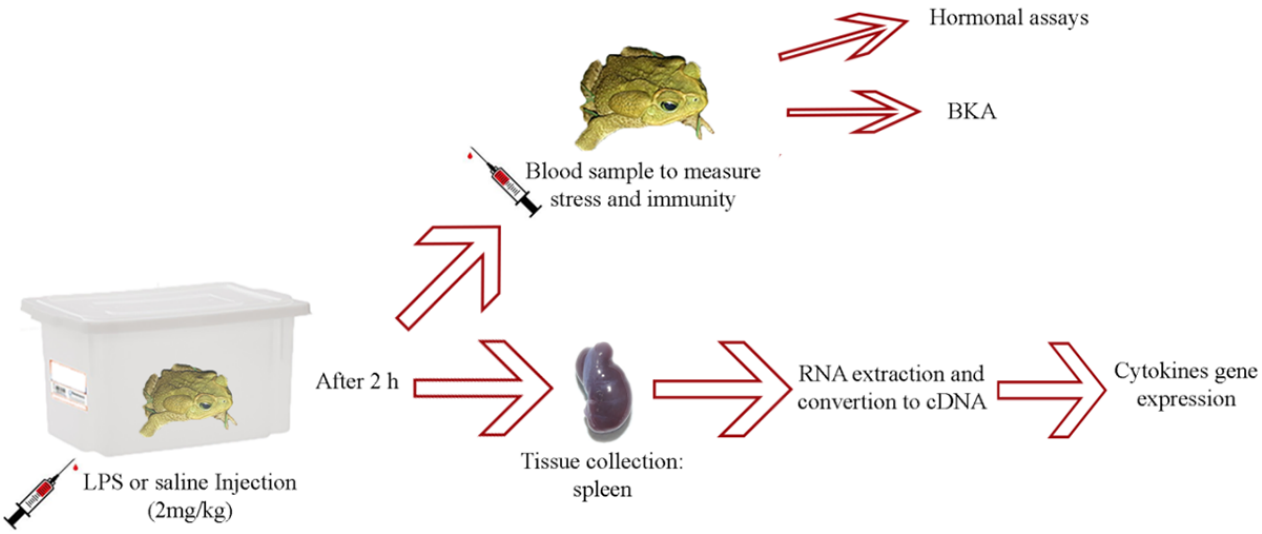


Figure 1. Experiment design. Animals were collected during their activity period at night. They were divided in two groups: SAL (injected with saline solution) and LPS (injected with LPS solution). Then, they were allocated in individual plastic boxes and after 2 h blood samples were collected, following euthanasia by decapitation to collect the spleen (*R. icterica* photography by Vania R. Assis).

3.2 Bacterial killing ability assay (BKA)

The BKA was determined according to Assis *et al.* (2013), with slight modifications. Plasma samples (10 µL) diluted in ringer for amphibians (190 µl) were incubated with *Aeromonas hydrophila* (2.5 x 10^5 microorganisms) (10 µl) for 1 h. The positive control was established with the same concentration of bacteria diluted in ringer (10 µl), and the negative control contained only ringer solution (210 µl). After incubation, 500 µl of TSB was added to each sample and then they were transferred in duplicate to a 96 wells microplate. After 1 h, the optical density of the samples was measured hourly in a plate spectrophotometer (595 nm), totaling 4 readings. BKA was calculated as: 1 – (optical density of sample / optical density of positive control), representing the proportion of killed microorganisms in the samples compared to the positive control. BKA was evaluated at the beginning of the bacterial exponential growth phase, since it is at this moment that their maximum growth becomes clear and, consequently, the highest BKA index for the samples.

3.3. Hormonal assays

Plasma concentrations of CORT, T and MEL were determined *via* ELISA kits (CORT, Caymam Chem. – 501320; T, Caymam Chem. – 582701; MEL, IBL – RE54021), according to the manufacturer's instructions and previous studies conducted with amphibians, including this same species (Assis *et al.*, 2015; Assis *et al.*, 2017; Bastos, 2017).

Steroids were extracted from 10 µl of plasma adding 3 mL of ethyl ether, according to Mendonça *et al.* (1996). For CORT and T quantification, samples were resuspended in ELISA buffer and assayed in the plate following the fabricant instructions. For MEL, samples were

extracted through silica columns (Waters Sep- Pak® Vac), using 150 µl of plasma, and assayed in the plate according to fabricant instructions.

3.4. Primers design

Primers previously described by Gardner *et al.* (2018) for the species *R. marina* were used to evaluate gene expression, since it is phylogenetically close to the species we used in this study. For target genes not analyzed in the aforementioned study, new primers were designed using the *R. marina* transcriptome as basis (Gardner *et al.*, 2018) and nucleotides data from other anuran species available in the National Center for Biotechnology Information (NCBI) (Table 1).

Table 1. Primers sequence used to evaluate gene expression

Gene	Forward (5') primer	Reverse (3') primer	Length (bp)	Design	Used
IL6	CAGTGATCTCCTGACGTTCC	AGCATTTGCCAAGGAGATGG	112	Gardner *et al.*, 2018	Yes
C1S	GCTGCCTGTACGACAGTCTT	GCTGCCTGTACGACAGTCTT	102	Gardner *et al.*, 2018	Yes
IL1β	GAGAACATTGCGCAAGAAGC	AAATAGAGTTGACGGCCTGC	110	Gardner *et al.*, 2018	Yes
IL8	GCCACAGCTCATACGAAAGG	GTCATTCCTTCTGAAAGCGC	110	Gardner *et al.*, 2018	No
TNFα	ACCAACGCCTTCAAAGATGG	ATCTTTGCCCAGTGAACACC	109	Gardner *et al.*, 2018	No
IL10	AGGACAAGCTCCTAGACCTGA	TCCAACTGCCTTGTACATCCC	140	Done in this study	Yes
IFNγ	TGTGAGCAGCCACAAGACAT	GCATGCGGCCTTGGATCTTA	84	Done in this study	Yes
Actin[a]	ATGACACAGATAATGTTTGAGAC	ATCACCAGAGTCCATCACAAT	117	Halliday *et al.*, 2008	Yes

Note. **IL6** = interleukin-6. **C1S** = complement component 1s. **IL1β** = interleukin-1β. **IL8** = interleukin-8. **TNFα** = tumor necrosis factor-α. **IL10** = interleukin-10. **IFNγ** = interferon-γ. [a] Housekeeping gene.

3.5. RNA extraction and qualitative polymerase chain reaction

Fifty milligrams of spleen were homogenized and transferred to microcentrifuge microtubes. RNA was isolated using TRIzol reagent (Invitrogen™, Cat. Nº 15596018), according to the manufacturer's instructions. RNA samples were treated with DNase I (ThermoScientific™, Cat. Nº EN0521), and the RNA concentration was determined using a spectrophotometer at A260 / A280 (Nanodrop ND1000, Thermo Scientific, USA). Reverse transcription was performed using 2 µg of total RNA as a template, reverse transcriptase and random primers (Revertaid H minus Reverse Transcriptase kit, Thermo-Scientific, Cat. Nº EP0451), according to the manufacturer's instructions.

After obtaining the cDNA (complementary DNA), several tests were done using electrophoresis method (agarose gel) to verify the functionality of the primers through amplification of the target genes. For this method a polymerase chain reaction (PCR) mix was prepared for each sample using a total volume of 15 µl with 7.5 µl of 2X DreamTaq Master Mix (ThermoScientific™, Cat. Nº K1081), 0.15 µl of our primer of interest (10µM; 0.075 µl forward primer + 0.075 µl reverse primer), 50 ng of cDNA and 2.35µl of water. The reaction was incubated in thermocycler according to the following steps: 1 cycle at 95 °C for 5 min, 40 cycles of 95 °C for 1 min, 60 °C for 30 s and 72 °C for 15 s, 1 cycle 72 °C for 5 min and hold at 8 °C. The obtained amplicon was analyzed in a 2 % agarose gel using a 50 bp ladder. Once observed the formation of bands in the correct length, the cytokines being amplified were used in the quantitative PCR (qPCR). A standard curve was generated from a ten-fold dilution of a quantified PCR product to confirm the reaction efficiency (Figure 2). The following cytokines were amplified and, therefore, used in this work: IL6, IL1β, IL10 and IFNγ, also the protein C1s.

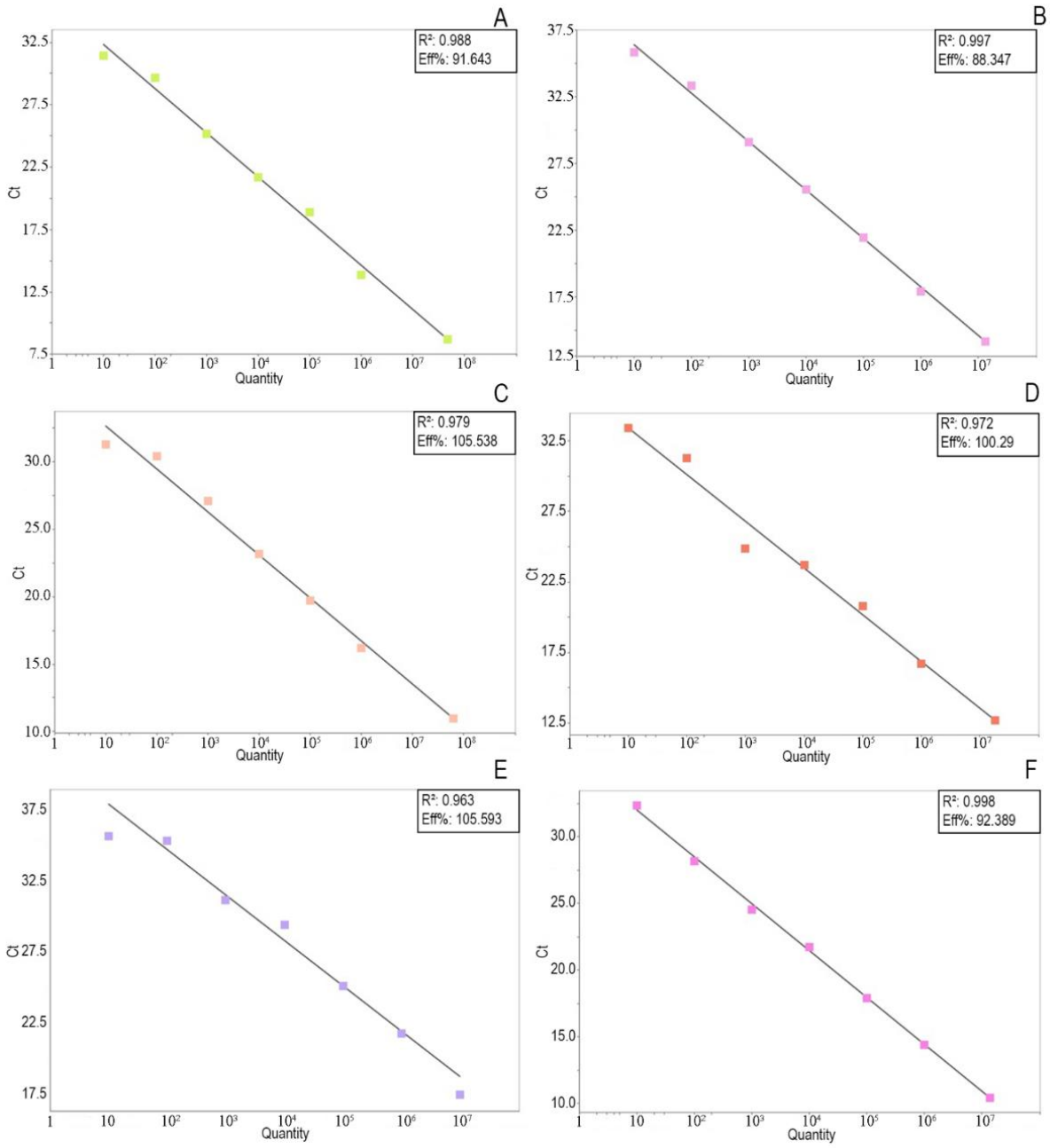


Figure 2. Primers efficiency curves generated from serial dilution of quantified PCR product. (A) Beta-actin primer efficiency curve. (B) IL1β primer efficiency curve. (C) IL6 primer efficiency curve. (D) IL10 primer efficiency curve. (E) IFNγ primer efficiency curve. (F) C1S primer efficiency curve.

3.6. Real time quantitative polymerase chain reaction (RT-qPCR)

For the quantitative analyses of gene expression, 2 µg of cDNA were assessed in a total volume of 15 µl containing Maxima SYBR Green qPCR Master Mix 2X (Thermo Scientific,

Lithuania, EU) and primers (10 µM). The mixture was incubated at 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s, 60 ºC for 60 s, and then after the cycles 95 ºC for 15 s, 60 ºC for 60 s and 95 ºC for 15 s. A negative control in the absence of cDNA was included in RT-qPCR assays to detect contamination. Reactions were carried out using StepOne™ realtime PCR System (Thermo Scientific, Finland). Reactions were performed in duplicate and analyses were performed using StepOne™ Software v2.3 (Thermo Scientific). The fold change was calculated by relative quantification using the ΔΔCt method (Livak & Schmittgen, 2001), normalized by *Beta-actin* expression.

3.7. Statistical analysis

Shapiro-Wilk normality tests and variance homogeneity tests were performed in order to investigate the prerequisites of parametric tests. All variables presented normal distribution and variance homogeneity. Additionally, body index (BI) was calculated as the residuals from the regression of body mass as a function of snout-vent length (SVL) and included in the analyses.

For hormonal and immune variables (plasma levels of CORT, T and MEL; BKA), analysis of covariance (ANCOVA) were performed, considering BI and body mass as covariates separately, and time (Early and Late Night) and treatment (Saline and LPS) as factors. If significant effects were not observed in the ANCOVA, analyses of variance (ANOVA) were performed in sequence, considering time and treatment as factors, along with multiple tests of comparison of the means with Bonferroni adjustments. If there was no effect of time, data was grouped, and only treatment was considered as factor using one-tailed Student's *t*-test. For cytokine expression, all data was first transformed into logarithmic scale (Log_{10}), and then statistical analysis was first based on ANCOVAs considering BI and body mass as separate

covariates, and time and treatment as factors, in the same manner as for the plasma variables. If significant effects were not observed in the ANCOVAs, ANOVAs considering time and treatment as factors were performed, also with tests of comparison of the means with Bonferroni adjustments. Similarly to the previous analyses, if there was no effect of time, data was grouped, and only treatment was considered as factor using one-tailed Student's *t*-test. For all tests, $p \leq 0.05$ was considered as statistically significant.

Principal component analysis (PCA) was performed for the environmental parameters: air and water temperature, and relative humidity. Component scores with eigenvalues higher than 1.0 were tested as covariates in ANCOVAs for all studied variables.

All analyses were performed using the program IBM SPSS Statistics 22.0. Graphs were done using the program GraphPad Prism 7.04.

4. Results

4.1. Effects of body mass, body index (BI) and environmental variables

BI and body mass didn't affect any of the studied hormonal and immune plasmatic variables ($p \geq 0.204$; Tables A1 and A2), and neither the profile of cytokines and complement protein expressions ($p \geq 0.105$; Tables A3 and A4).

Principal component analysis performed for environmental variables retained one component (Table 2). This component explained 71.23 % of total variance observed in the environmental variables data set and represents a direct association between air and water temperatures and an inverse association between each of these two parameters and relative humidity. Covariance analyses were performed using this component 1 as the covariate for all studied variables and no significant effects were observed ($p \geq 0.124$; Tables A5 and A6).

Table 2. Factors retained in the principal component analysis of environmental variables.

Environmental Variable	Component 1
Air Temperature	0.971
Water Temperature	0.960
Relative Humidity	-0.978
Eigenvalues	2.849
% of Variance	71.229

4.2. Hormonal and immune plasmatic variables

Time did not affect any of the variables ($p \geq 0.169$; Table A7 and Figure 3). After grouping data from different times, there was a difference between treatments in CORT plasma levels ($t_{20} = 2.89$; $p = 0.004$) with toads injected with LPS displaying higher CORT levels (Figure 4A). There was no effect of treatment on MEL and T plasma levels and on plasmatic BKA ($p \geq 0.139$; Figure 4B-D).

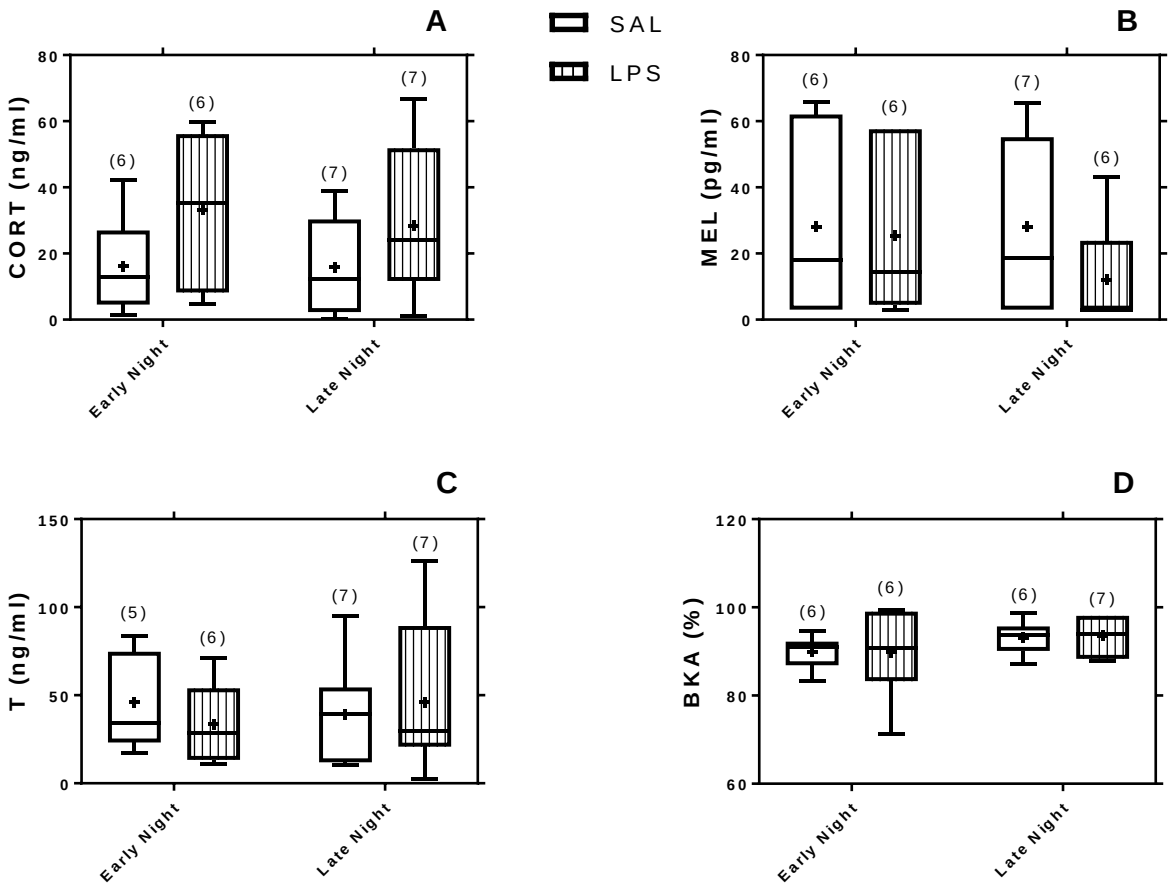


Figure 3. Parameters assessed in toads, comparing different times and treatments. (A) Corticosterone plasma levels. (B) Melatonin plasma levels. (C) Testosterone plasma levels. (D) Bacterial killing ability. Boxplots show the median, interquatile range (IQR), whiskers show min and max values, plus signals show the mean.

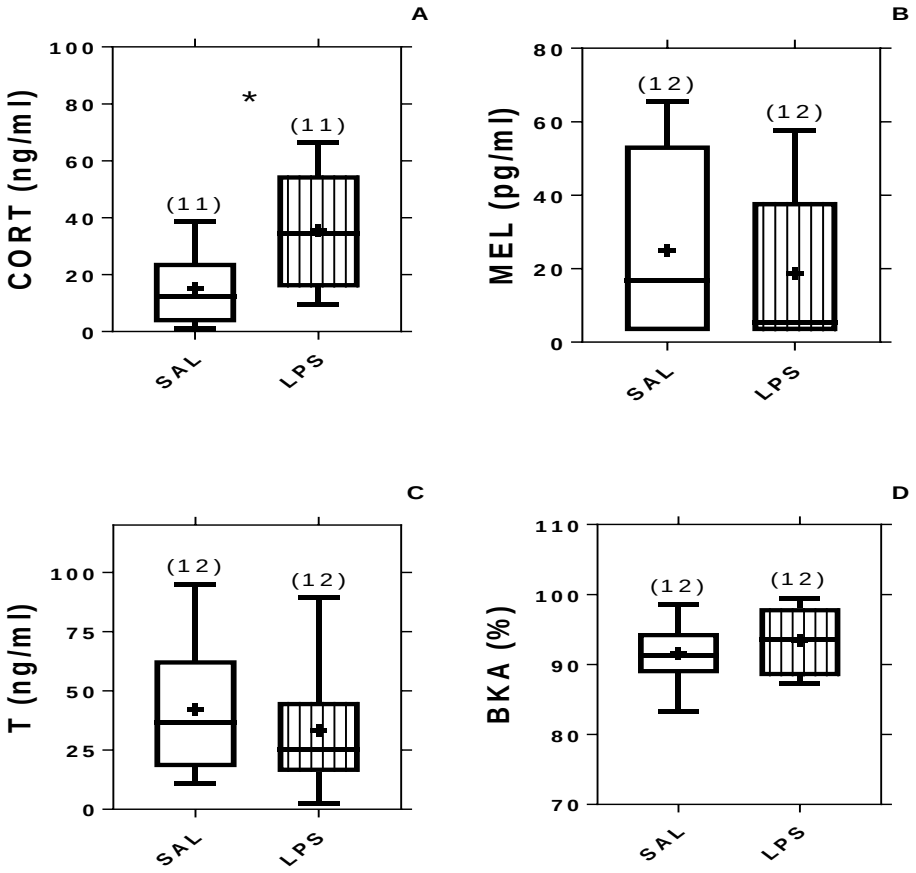


Figure 4. Hormonal and immune parameters in *R. icterica* after immunological challenge with LPS. (A) Plasma corticosterone levels. (B) Plasma melatonin levels. (C) Testosterone plasma levels. (D) Bacterial killing ability. Boxplots show the median, interquatile range (IQR), whiskers show min and max values, plus signals show the mean. Asterisk shows significant difference ($p \leq 0.05$) between groups.

4.3. Splenic profile of cytokines and complement protein expressions

Time did not affect the expression of any cytokines and complement protein ($p \geq 0.074$; Table A8 and Figure 5) but, for the Early Night group, the expression of IL1β in animals injected with LPS was significantly higher than in the ones injected with saline (p = 0.007; Figure 5A). For the Late Night group, the expression of IL10 in animals injected with LPS was significantly lower than in the ones injected with saline (p = 0.047; Figure 5C).

After grouping data from different times, there was an effect of treatment in the IL1β expression (t_{22} = -2.514; p = 0.01) with toads injected with LPS showing upregulation of IL1β

(Figure 6A). No differences were observed between the groups for IL6, IL10, IFNγ and C1S ($p \geq 0.06$; Figure 6B-E).

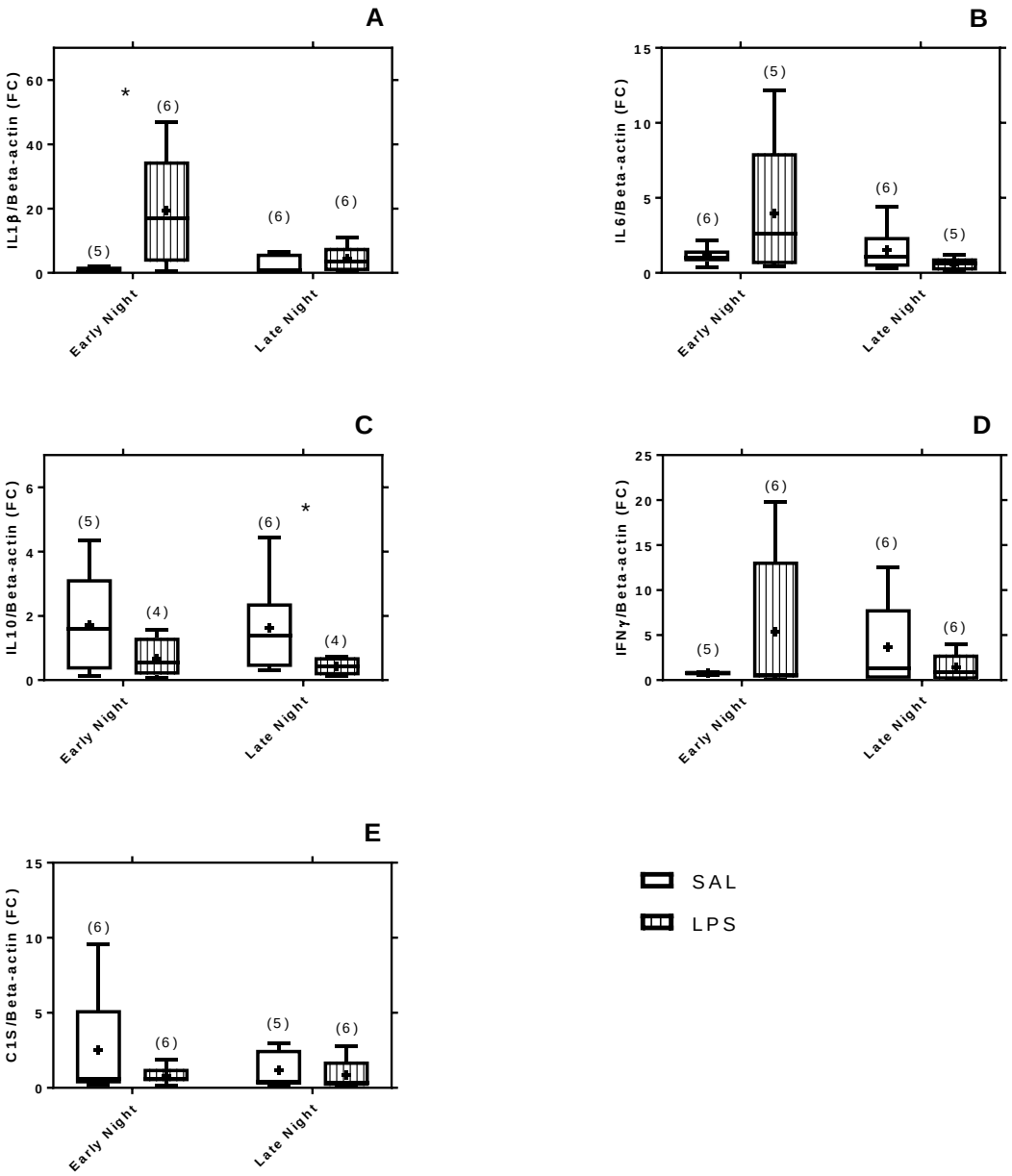


Figure 5. Cytokine expression in *R. icterica* after immunological challenge with LPS in different times**.** (A) Interleukin-β expression. (B) Interleukin-6 expression. (C) Interleukin-10 expression. (D) Interferon-γ expression. (E) Complement component 1S expression. FC: fold change. Boxplots show the median, interquatile range (IQR), whiskers show min and max values, plus signals show the mean. Asterisk shows significant difference ($p \leq 0.05$) between groups according to Bonferroni comparisons.

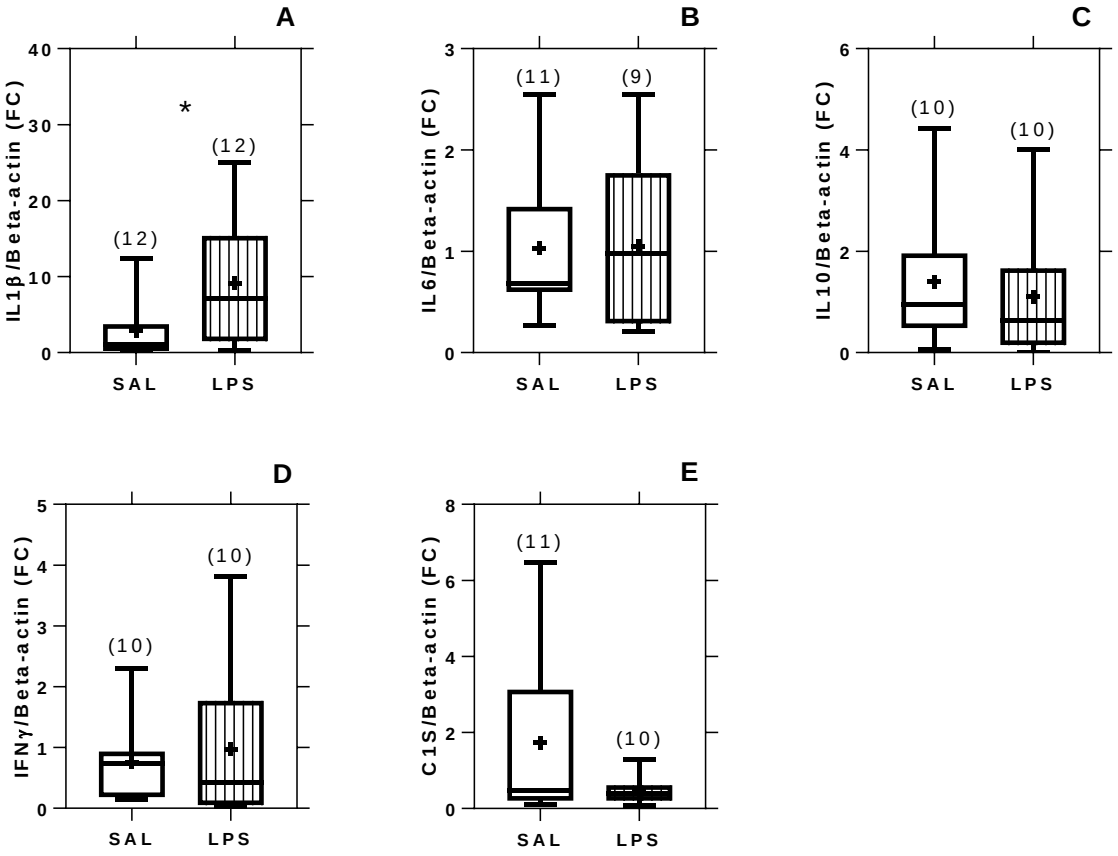


Figure 6. Cytokine expression in *R. icterica* after immunological challenge with LPS. (A) Interleukin-1β expression. (B) Interleukin-6 expression. (C) Interleukin-10 expression. (D) Interferon-γ expression. (E) Complement component 1S expression. FC: fold change. Boxplots show the median, interquartile range (IQR), whiskers show min and max values, plus signals show the mean. Asterisk shows significant difference ($p \leq 0.05$) between groups.

5. Discussion

In the present study, we evaluated the immune-endocrine response following LPS exposure in freshly captured toads in the field. Overall, the results showing that the LPS injection increased CORT plasma levels and IL1β spleen expression demonstrate that recently captured toads mount an immune response similarly to the pattern observed in others anurans (Bastos, 2017; Gardner *et al.*, 2018), mammals and other groups (Terrazzino *et al.*, 1997; Takemura *et al.*, 1997; Owen-Ashley *et al*., 2006). Importantly, we also disclosed that alterations in some of the parameters evaluated depend on the moment of the dark phase that the injection was performed, with IL1β expression responding to the treatment at Early Night and IL10 at Late Night.

The increase in CORT plasma levels in animals injected with LPS reflects the stimulation of the HPA/I axis (Rivier *et al.*, 1989), a result similar to the observations in animals of the same species under laboratory conditions after LPS treatment (Bastos, 2017). The LPS induction of CORT secretion might be explained by the increase of IL1β, since the injection of this cytokine induces a similar effect in rats (Bumiller *et al.*, 1999). In fact, rats injected with LPS present increased levels of inflammatory cytokines (including IL1β) in association with high CORT plasma levels (Terrazzino *et al.*, 1997; Takemura *et al.*, 1997). Additionally, these responses could also be explained by other mechanisms seen in mammals, which are the LPS-induced activation of TLR4 receptors present in adrenocortical cells (Bornstein *et al.*, 2006), and the increased production of others pro-inflammatory cytokines (IL6 and TNFα), which can induce the production of CRH in the hypothalamus (Dunn, 2000). These immune-endocrine interactions are more elucidated in mammals (Cain & Cidlowski, 2017) when compared to what is known in amphibians.

Decreased circulating MEL levels in response to LPS or others inflammatory stimuli has been observed in rats (Tamura *et al.*, 2010), hamsters (Laranjeira-Silva *et al.*, 2015), anurans (Bastos, 2017) and chickens (Piesiewicz *et al.*, 2012). In our study, although there is a trend of decreased MEL levels in toads injected with LPS, no significant differences between LPS and saline groups were observed. Considering that restrictive conditions can inhibit MEL production, as it was seen in doves (Barriga *et al.*, 2002) and rats (Couto-Moraes *et al.*, 2009), it is possible that the capture could have acted as a stressor in both groups inhibiting MEL production and, therefore, attenuating the inhibitory effect induced by LPS. Accordingly, the mean values of plasma MEL found in both groups of our study were below those reported by Bastos (2017) in *R. icterica* maintained in captivity for 31 days and posteriorly injected with saline. In fact, recently captured animals presented basal levels of MEL similar to those observed in animals injected with LPS after a month in captivity (Bastos, 2017), reinforcing the hypothesis that the experimental procedure *per se* is imposing an inhibition of circulation MEL levels.

The inhibition of MEL synthesis is a multi-mediated process regulated by different immune signals (Markus *et al.*, 2018). In mammals, LPS and TNF respectively activate toll-like and cytokine receptors in pinealocytes, and through the nuclear translocation of NF-κB dimers inhibit the MEL synthesis by decreasing adrenergic induced transcription of *Aanat*, the gene responsible for the expression of a key enzyme involved in the synthesis pathway of MEL (Markus *et al.*, 2013). Importantly, CORT exerts dual effects on melatonin production depending on the pattern of stimulation of adrenergic receptors (Fernandes *et al.*, 2017). In a context of increased adrenergic stimuli activating both α and β-adrenoceptors, the CORT-induced GRs nuclear translocation inhibits the *Aanat* transcription. On the other hand, in situations that only β-adrenoceptors are active, GR stimulation leads to an increase of MEL production by the pineal

gland (Fernandes *et al.*, 2017). Considering that stress increases the release of noradrenaline (NA) from the sympathetic system (Sabban *et al.*, 2004), it is possible that the change on adrenergic receptors pattern are also modulating CORT effect upon pineal MEL synthesis in toads, as observed in mammals. Although we don't know whether these mechanisms are operating in amphibians, there are some evidences for melatonin inhibition through inflammatory challenge and ACTH injection similar to what is observed in mammals (Bastos 2017; Barsotti *et al.*, 2017), possibly illustrating a conservation of the immune-pineal axis system through different vertebrate groups. Moreover, the better description of these mechanisms in amphibians might also open an important research field for other vertebrate groups.

We also observed no differences for T plasma levels between LPS and saline treated groups. We expected an accentuated decrease in testosterone levels in animals injected with LPS, since the increase in CORT plasma levels frequently promotes a decrease in T, at least in a context of stress exposure in toads (Assis *et al.*, 2017; Barsotti *et al.*, 2017; Titon *et al.*, 2017; Titon *et al.*, 2018). In *Rhinella* species, including *R. icterica*, decreased levels of T were described following several types of stressors, such as 24 h of restraint and captivity (Titon *et al.*, 2017; Titon *et al.*, 2018; Assis *et al.*, 2019). Moreover, *Boana faber* kept in captive conditions, the treatment with ACTH led to a decrease in T levels 1 h after treatment at nighttime (Barsotti *et al.*, 2017). The activation of GR receptors suppresses T biosynthesis at the Leydig cells of rats (Dong *et al.*, 2004), and it has been reported that GCs inhibit steroidogenesis via GR-mediated mechanism in toads (Czuchlej *et al.*, 2019). It is possible that the evaluation after a longer period of treatment with LPS in our toads would result in a more pronounced and evident effect. Although T is commonly associated with immune inhibitory effects, there are evidences in the literature pointing to enhanced immunocompetence correlated with increased androgen levels in

male birds (Peters, 2000; Evans *et al.*, 2000). In this sense, considering that this is also a relevant point of investigation for different groups of vertebrates, further researches, should consider T, as well as CORT and MEL, as an immunomodulatory hormone, and not only as suppressors of the immune response. To better understand the mechanisms behind the CORT modulation on T and MEL production, one interesting way would be exploring how GRs are modulating the biosynthetic pathway of both hormones in amphibians. Clarifying how GRs operate *per se* and/or in connection with others signals could help to elucidate the role of these endocrine interactions in amphibians. Therefore, the usage of GR antagonists in future evaluations might be an interesting way to evaluate how the activation of these receptors are interfering with T and MEL levels in both, saline and LPS injected animals.

Considering immune-related parameters of the circulation, it has been demonstrated in previous studies that plasma BKA is sensitive to inflammatory challenges and confinement stress (Assis *et al.*, 2015; Miller *et al.*, 2007). Our protocol was adapted from a study by Millet *et al*. (2007), which showed an increase in the bactericidal activity after LPS challenge in chicken blood 16 h after the treatment. Assis *et al.* (2015) observed a decrease in BKA 24 h after a stressor (restraint with movement restriction) in *R. icterica*. Gardner *et al.* (2020) compared the changes in the BKA of different *R. marina* populations by LPS stimulus at 2 and 20 h following injection and noticed a significant increase in one of the populations after 20 h of treatment. Considering that BKA measures the ability of constitutive components of the innate immune system (represented by antimicrobial peptides and proteins) in the plasma to deal with pathogens, we expected an increase in BKA in LPS-injected toads, as observed in those studies. However, we found no differences between groups, which could be associated with the short time interval between injection and blood sampling in our study compared to the time intervals used in other

works. Accordingly, Titon *et al.* (2019) described that BKA stress related-changes are more evident after long-stress exposure. There is also evidence suggesting immune responsiveness in anurans is higher during the reproductive season, being positively correlated with high levels of androgens and corticosterone (Madelaire & Gomes, 2016; Madelaire *et al.*, 2017). Since we sampled toads during their reproductive season, we could be dealing with high BKA in both groups; therefore, it would be difficult to notice immunostimulatory effects on this parameter. Alternatively, other interesting point we should be aware is that this is the only work assessing plasma BKA after inflammatory challenge in recently captured anurans. Considering that most probably the animals were previously and continuously exposed to pathogens in the environment, when assessing constitutive elements of the immune system, it is possible that both saline and LPS groups already have a defense system with higher vigilance to deal with PAMPs as LPS. When transferring toads from their natural habitat to captivity conditions, even if they were also previously exposed to pathogens, they spend time acclimating to these new conditions, with no or at least much less pathogens exposition, a condition that might influence the immune system readiness to deal with PAMPs exposure. In fact, when we look at the mean values of plasma BKA for both groups they range from 90 to close to 100, which can be considered high in comparison to the mean value (40.17) found in toads of the same species after long-term captivity (three months; Assis *et al.*, 2015). Then, we could expect that, since we are dealing with toads which were already exposed to pathogens in their natural habitat, LPS injection could not have affected them as much as if they were in captivity, in a clean environment, where constant investment in this line of defense would be too expensive to maintain in a prolonged absence of threats. Following this line of thought, high values for plasma BKA is an expected condition.

Complementarily, we did not find any effect of LPS injection on the expression of the gene C1s, a protein part of the complement system that is related to the montage of the innate immune response (Müller-Eberhard, 1988). Considering that there were no observable changes in the BKA, which evaluates the action of proteins deriving from the complement system, the absence of changes in the expression of C1S reinforces this result. There are no studies evaluating the expression of C1S gene in amphibians, but in mammals it is highly expressed in different types of situations, such as tissue damage and carcinoma (Yasojima *et al.*, 1998; Chang *et al.*, 2016). Fishes *Oplegnathus fasciatus* increased five- and four-fold the expression of C1S in the liver 6 h after injection with the bacteria *Edwardsiella tarda* and *Streptococus iniae*, respectively (Godahewa *et al.*, 2015). Exploring the gene expression in the liver should be a good addition in order to evaluate the activity of C1s.

In mammals, MEL and GCs are known to modulate immune responses, however little is known about their reaction to inflammatory stimuli and immunomodulatory effects during the different phases of inflammatory contexts in amphibians. We found significant effects of LPS treatment at specific times for IL1β and IL10 expression, possibly illustrating how the moment of injection can differently modulates certain aspects of the immune response. At Early Night we observed a significant increase in the IL1β expression in LPS-injected toads, accompanied by a trend of increased expression of other pro-inflammatory cytokines (IL6 and IFNγ). Otherwise, the Late Night group showed decreased expression of IL10 after the LPS treatment. These results suggest a more evident montage of inflammatory response at the beginning of the nighttime, when *R. icterica* toads begin their activity period. The significant decrease in IL10 expression in the Late Night group accompanied by the lack of higher IL1β, IL6 and IFNγ expression suggests a compensatory mechanism, avoiding a response too weak to deal with the inflammatory

challenge. Similar pattern is also observed in mammals, with increased inflammatory-related activity (such as the expression of cytokines) after LPS treatment occurring at the beginning of their period of activity (Gibbs *et al.*, 2012). Overall, LPS treatment induced the upregulation of the cytokine IL1β in the spleen, reinforcing its role in the mounting of an inflammatory response, probably through the activation of phagocytic cells, such as macrophages, which will act in the clearance of pathogens, as observed in mammals (reviewed by Mebius & Kraal, 2005). The same pattern of increased expression of IL1β after LPS injection was previously observed in the spleens of *R. marina* (Gardner *et al.*, 2018) and *Xenopus laevis* (Zou *et al.*, 2000), and in the plasma of *R. icterica* (Bastos, 2017). This cytokine is known for its pro-inflammatory effects and association with innate immune response, stimulating phagocytosis of macrophages and inducing sickness behavior (Zimmerman, 2014). In mammals, IL1β is also known for its role in the activity of the HPA/I axis, associated with the release of ACTH (Gądek-Michalska *et al.*, 2011) and the transcription of other important pro-inflammatory cytokines, such as TNFα and IL6 (Zimmerman *et al.*, 2014). In amphibians, individuals of *X. laevis* (adults and tadpoles) showed upregulation of IL1β, along with the pro-inflammatory cytokines TNFα and IFNγ, starting at one day after infection with ranavirus Frog Virus 3 (Morales *et al.*, 2010; Andino *et al.*, 2012) and after one day exposition to environmental contaminants (Martini *et al.*, 2012).

In our study, we found a trend in increased expression of IL6 and IFNγ (cytokines associated with the inflammatory response assemblage) following LPS treatment. IFNγ is a cytokine strongly associated with the pro-inflammatory response, being produced by natural killer, CD4 Th1, and CD8 cytotoxic T cells, mediating the adaptive response (Samuel, 2001). Moreover, IFNγ is highly conserved across the vertebrate taxa (Savan *et al.*, 2009). Humans treated with LPS showed a peak of INFγ serum levels 1.5 h after treatment (Kemna *et al.*, 2005).

Considering the metabolic differences between endotherms and ectotherms, with thermoconforming ectotherms having a metabolism positively correlated to environmental temperature (Pough, 1980), we might expect that IFNγ in the evaluated toads could be upregulated later after LPs challenge. IL6, in mammals, is also known for its pro-inflammatory profile, promoting the differentiation of Th17 cells, which inhibits immune suppression (Dienz & Rincon, 2009), and is also believed to be highly conserved (Zimmerman *et al.*, 2014). In anurans, IL1β and IFNγ were responsive to distinct challenges, being up-regulated by ranavirus (Andino *et al.*, 2012), heavy metals exposure (Jayawardena *et al.*, 2015), and LPS injection (Gardner *et al.*, 2018). In the meantime, besides showing increased values of IL1β, IL6 and IFNγ, the timeframe to detect increased values may differ, being 2h, 24h and even 28 days after treatments (Gardner *et al.*, 2018; Andino *et al.*, 2012; Jayawardena *et al.*, 2015; respectively). In our study, *R. icterica* toads were euthanized at the predicted beginning of the pro-inflammatory response, as evidenced by the increase in CORT levels and IL1β, but other cytokines didn't present evident alterations at this time point. IL10 is a cytokine associated with regulatory/anti-inflammatory effects (Zimmerman *et al.*, 2014; Jayawardena *et al.*, 2015). Takiguchi (2018) observed higher circulating levels of IL10 in the beginning of an inflammatory response in rats (2 h after LPS injection), limiting the risk of sepsis. In the same study it was also observed even higher circulating levels of this cytokine 6 h after the LPS treatment, probably associated with the resolution phase of the immune response. Therefore, the observed up-regulation only for the IL1β in *R. icterica* may represent differences in the time frame. Exploring other times post-injections would be pivotal for a better understanding of the inflammatory cytokines dynamic in this group.

Timing is an important aspect to be analyzed when studying the montage of an inflammatory response, since some processes consist of different steps. This can be observed in the Immune-Pineal Axis in mammals when evaluating the modulation of CORT upon MEL production in the pineal gland, composed of a switch in inhibition and stimuli depending of the sympathetic context. Takiguchi (2018) also illustrates this by observing different temporal patterns in the production of cytokines in rats depending of the LPS dosage, with lethal doses leading to a more intense response and perceivable earlier than in rats injected with non-lethal doses. Perhaps an inflammatory challenge happening at the Late Night could take longer than the Early Night to be perceivable, needing more than 2 hours to be noticeable in the organism. This could be associated with the endocrine context at the beginning of their period of activity, represented by high levels of CORT and T, which are related to a higher energetic expenditure for locomotion and vocalization (Emerson, 2001). While at late night this endocrine context shift, with lower levels of these hormones accompanied by less activity, probably leading to less immunostimulatory effects, reducing the magnitude of the inflammatory response. Immune cells also present their own endogenous clock responsible for different activity patterns according to time (Labrecque & Cermakian, 2013), which can be influenced by the endocrine context. New predictions and hypothesis regarding the timing of immune response in amphibians can be constructed from the results observed in this study.

Free-living freshly captured *R. icterica* toads showed hormonal and immune changes following an immune challenge with LPS. The increase in CORT plasma levels and expression of IL1β indicate the initial pro-inflammatory response, demonstrating that freshly captured toads are responsive to an immune challenge. This pattern is similar to what was found in other studies involving different anuran species, including the same species, under captivity conditions. The

response as a whole was not time dependent, however we found significances in some parameters pointing to a more evident pro-inflammatory response when the LPS challenge was performed at the Early Night. Also, we present gene data to further evaluate expression for molecules related to the immune system in these animals both on field and captivity. As the first study examining the effects of an inflammatory challenge happening at different moments in free-living freshly captured toads, we could report findings that reinforce the results found in studies at captivity, but at the same time prospects for new questions to be answered in amphibian immuno-endocrinology.

6. Conclusions

→Recently captured *R. icterica* toads responded to LPS challenge on field, showing a pro-inflammatory immune profile after 2 h, with increased CORT plasma levels and IL1β expression, both variables associated with mobilization of leukocytes in the innate immune response.

→IL1β responded to LPS treatment at Early Night, along with a trend of increased expression of IL6 and IFNγ.

→ IL10 expression decreased at Late Night.

→The cytokines expression seems to indicate a higher response from *R. icterica* toads to an inflammatory challenge at the beginning of the period of activity.

7. References

ANDINO, F. J.; CHEN, G.; LI, Z.; GRAYFER, L.; ROBERT, J. Susceptibility of *Xenopus laevis* tadpoles to infection by the ranavirus Frog-Virus 3 correlates with a reduced and delayed innate immune response in comparison with adult frogs. **Virology**, vol. 432, p. 435-443. 2012.

ARENDT, J. Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. **Rev Reprod**, vol. 3, p. 13-22. 1998.

ASSIS, V. R.; TITON, S. C. M.; BARSOTTI, A. M. G.; SPIRA, B.; GOMES, F. R. Antimicrobial capacity of plasma from anurans of the Atlantic Forest. **S Am J Herpetol**, vol. 8, p. 155-160. 2013.

ASSIS, V. R.; TITON, S. C. M.; BARSOTTI, A. M. G.; TITON JR., B.; GOMES, F. R. 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**, vol. 10:e0121005. 2015.

ASSIS, V. R.; TITON, S. C. M.; QUEIROZ-HAZARBASSANOV, N. G. T.; MASSOCO, C. O.; GOMES, F. R. Corticosterone transdermal application in toads (*Rhinella icterica*): Effects on cellular and humoral immunity and steroid plasma levels. **J Exp Zool**, vol. 327, p. 200-213. 2017.

ASSIS, V. R.; TITON, S. C. M.; GOMES, F. R. Acute stress, steroid plasma levels, and innate immunity in Brazilian toads. **Gen Comp Endocrinol**, vol. 273, p. 86-97. 2019.

BAKER, P. C.; QUAY, W. B.; AXELROD, J. Development of hydroxyindole-O-methyltransferase activity in eye and brain of the amphibian, *Xenopus laevis*. **Life Sci**, vol. 4, p. 1981-1987. 1965.

BARBOSA-LIMA, L. E.; MUXEL, S. M.; KINKER, G. S.; CARVALHO-SOUSA, C. E.; CRUZ-MACHADO, S. S.; MARKUS, R. P.; FERNANDES, P. A. C. M. STAT1-NFκB crosstalk triggered by interferon gamma regulates noradrenaline-induced pineal hormonal production**. J Pineal Res**, vol. 67: e12599. 2019.

BARRIGA, C.; MARCHENA, J. M.; LEA, R. W.; HARVEY, S.; RODRÍGUEZ, A. B. Effect of stress and dexamethasone treatment on circadian rhythms of melatonin and corticosterone in ring dove (*Streptopelia risoria*). **Mol Cel Biochem**, vol. 232, p. 27-31. 2002.

BARSOTTI, A. M. G.; ASSIS, V. R.; TITON, S. C. M.; TITON JR., B., FERREIRA, Z. F. S.; GOMES, F. R. ACTH modulation on corticosterone, melatonin, testosterone and innate immune response in the tree frog *Hypsiboas faber*. **Comp Biochem Phys A**, vol. 204,p. 177-184. 2017.

BASTOS, P. R. O. **Inflamação sistêmica induzida por LPS em anuros da espécie *Rhinella icterica*: efeito sobre os mediadores inflamatórios citocinas, corticosterona e melatonina**. 2017. 75 p. Dissertation (Masters in Science) – Instituto de Biociências, Universidade de São Paulo, São Paulo. 2017.

BICEGO, K. C.; STEINER, A. A.; ANTUNES-RODRIGUES, J.; BRANCO, L. G. S. Indomethacin impairs LPS-induced behavioral fever in toads. **J of Appl Physiol**, vol. 93, p. 512-516. 2002.

BORNSTEIN, S. R.; ZIEGLER, C. G.; KRUG, A. W.; KANCZKOWSKI, W.; RETTORI, V.; MCCANN, S. M.; WIRTH, M.; ZACHAROWSKI, K. The role of toll-like receptors in the immune-adrenal crosstalk. **Ann N Y Acad Sci**, vol. 1088, p. 307–318. 2006.

BUMILLER, A.; GÖTZ, F.; ROHDE, W.; DÖRNER, G. Effects of repeated injections of interleukin 1β or lipopolysaccharide on the HPA axis in the newborn rat. **Cytokine**, vol. 11, p. 225-230. 1999.

CAIN, D. W.; CIDLOWSKI, J. A. Immune regulation by glucocorticoids**. Nat Rev Immunol**, vol. 17, p.233-247. 2017.

CARRILLO-VICO, A.; LARDONE, P. J.; ÁLVAREZ-SÁNCHEZ, N.; RODRÍGUEZ-RODRÍGUEZ, A.; GUERRERO, J. M. Melatonin: Buffering the Immune System. **Int J Mol Sci**, vol. 14, p. 8638-8683. 2013.

CECON E.; FERNANDES, P. A.; PINATO, L.; FERREIRA, Z. S.; MARKUS, R. P. Daily variation of constitutively activated nuclear factor kappa b (nfkb) in rat pineal gland. **Chronobiol Int**, vol. 27, p. 52-67. 2010.

CHANG, I. W.; LIN, V. C.; WU, W. J.; LIANG, P. I.; LI, W. M.; YEH, B. W.; HE, H. L.; LIAO, A. C.; CHAN, T. C.; LI, C. F. Complement Component 1, s Subcomponent overexpression is an independent poor prognostic indicator in patients with urothelial carcinomas of the upper urinary tract and urinary bladder. **J Cancer**, vol. 7, p. 1396-1405. 2016.

CÓRDOBA-MORENO, M. O.; TODERO, M. F.; FONTANALS, A.; PINEDA, G.; DANIELA, M.; YOKOBORI, N.; RAMOS, M. V.; BARRIENTOS, G.; TOBLLI, J. E.; ISTURIZ, M. A.; REARTE, B. Consequences of the lack of IL-10 in different endotoxin effects and its relationship with glucocorticoids. **Shock**, vol. 52, p. 264-273. 2019.

COUTO-MORAES, R.; PALERMO-NETO, J.; MARKUS, R. P. The Immune–Pineal Axis. **Ann N Y Acad Sci**, vol. 1153, p. 193-202. 2009.

CZUCHLEJ, S. C.; VOLONTERI, M. C.; REGUEIRA, E.; CEBALLOS, N. R. Effect of glucocorticoids on androgen biosynthesis in the testes of the toad *Rhinella arenarum* (Amphibia, Anura). **J Exp Zool**, vol. 331, p. 17– 26. 2019.

DA SILVEIRA CRUZ-MACHADO, S. S.; CARVALHO-SOUSA, C. E. ; TAMURA, E. K.; PINATO, L.; CECON, E.; FERNANDES, P. A. C. M.; DE AVELLAR, M. C.; FERREIRA, Z. S.; MARKUS, R. P. TLR4 and CD14 receptors expressed in rat pineal gland trigger NFKB pathway. **J Pineal Res**, vol. 49, p. 183-192. 2010.

DA SILVEIRA CRUZ-MACHADO, S.; TAMURA, E. K.; CARVALHO-SOUSA, C. E.; ROCHA, V. A.; PINATO, L.; FERNANDES, P. A. C. M.; MARKUS, R. P. Daily corticosterone rhythm modulates pineal function through NFκB-related gene transcriptional program. **Sci Rep**, vol. 7. 2017.

DÄNICKE, S.; BROSIG, B.; KERSTEN, S.; KLUESS, J.; KAHLERT, S.; PANTHER, P.; DIESING, A. K.; ROTHKÖTTER, H. J. The *Fusarium* toxin deoxynivalenol (DON) modulates the LPS induced acute phase reaction in pigs. **Toxicol Lett**, vol. 220, p. 172-180. 2013.

DASZAK, P.; BERGER, L.; CUNNINGHAM, A. A.; HYATT, A. D.; GREEN, D. E.; SPEARE, R. Emerging infectious diseases and amphibian population declines. **Emerg Infect Dis**, vol. 5, p. 735-748. 1999.

DAVIS, A. K.; MANEY, D. L.; MAERZ, J. C. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. **Funct Ecol**, vol. 22, p. 760-772. 2008.

DELGADO, M. J.; VIVIEN-ROELS, B. 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**, vol. 75, p. 46-53. 1989.

DHABHAR, F. S. Stress-induced Changes in Immune Cell Distribution and Trafficking: Implications for Imunoprotection versus Immunopathology. In WELSH, C. J.; MEAGHER, M.; STERNBERG, E. (Eds.). **Neural and Neuroendocrine Mechanisms in Host Defense and Autoimmunity**. New York: Springer, 2006. P. 7-25

DIENZ, O.; RINCON, M. The effects of IL-6 on CD4 T cell responses. **Cl Immunol**, vol. 130, p. 27-33. 2009.

DOMÍNGUEZ-RODRIGUEZ, A.; ABREU-GONZÁLEZ, P.; GARCÍA, M. J.; SANCHEZ, J.; MARRERO, F.; ARMAS-TRUJILLO, D. Decreased nocturnal melatonin levels during acute myocardial infarction. **J Pineal Res**, vol. 33, p.248-252. 2002.

DONG, Q.; SALVA, A.; SOTTAS, C. M.; NIU, E.; HOLMES, M.; HARDY, M. P. Rapid Glucocorticoid Mediation of Suppressed Testosterone Biosynthesis in Male Mice Subjected to Immobilization Stress. **J Androl**, vol. 25, p. 973-981. 2004.

DUNN, A. J. Cytokine activation of the HPA axis. **Ann N Y Acad Sci**, vol. 917, p. 608–617. 2000.

EMERSON, S. B. Male advertisement calls: behavioral variation and physiological processes. In RYAN, M. J. (Ed.). **Anuran Communication**. Washington, DC: Smithsonian Institution Press, 1º Ed., 2001. P. 36-44.

EVANS, M. R.; GOLDSMITH, A. R.; NORRIS, S. R. A. The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). **Behav Ecol Sociobol**, vol. 47, p. 156-163. 2000.

FÁLCON, J.; BESSEAU, L.; FUENTÈS, M.; SAUZET, S.; MAGNANOU, E.; BOEUF, G. Structural and functional evolution of the pineal melatonin system in vertebrates. **Ann NY Acad Sci**, vol. 1163, p. 101-111. 2009.

FERNANDES, P. A. C. M.; CECON, E.; MARKUS, R. P.; FERREIRA, Z. S. Effect of TNF-α on the melatonin synthetic pathway in the rat pineal gland: basis for a "feedback" of the immune response on circadian timing. **J Pineal Res**, vol. 41, p. 344-350. 2006.

FERNANDES, P. A. C. M.; TAMURA, E. K.; D'ARGENIO-GARCIA, L.; MUXEL, S. M.; CRUZ-MACHADO, S. S.; MARÇOLA, M.; CARVALHO-SOUSA, C. E.; CECON, E.; FERREIRA, Z. S.; MARKUS, R. P. Dual Effect of Catecholamines and Corticosterone Crosstalk on Pineal Gland Melatonin Synthesis. **Neuroendrocrinology**, vol. 104, p. 126-134. 2017.

FOLSTAD, A.; KARTER, A. J. Parasites bright males and the immunocompetence handicap. **Am Nat**, vol. 139, p. 603-622. 1992.

GĄDEK-MICHALSKA. A.; TADEUSZ, J.; RACHWALSKA, P.; SPYRKA, J.; BUGAJSKI, J. Effect of prior stress on interleukin-1β and HPA axis response to acute stress. **Pharmacol Rep**, vol. 63, p. 1393-1403. 2011.

GARCIA-NETO, P. G.; NOWAKOWSKI, A. J.; DA SILVA, A. F. C.; OLIVEIRA, O. C. C.; GUERRA, R. N. M.; ANDRADE, G. V. Leukocyte profiles of two neotropical anuran species affected by anthropogenic habitat alteration. **Anim Conserv**. doi:10.1111/acv.12564. 2020.

GARDNER, S.; ASSIS, V. R.; ZHAO, H.; GOMES, F. R.; PEATMAN, E.; MENDONÇA, M. T. Differential gene expression to an LPS challenge in relation to exogenous corticosterone in the invasive cane toad (*Rhinella marina*). **Dev Comp Immunol**, vol. 88, p. 114-123. 2018.

GARDNER, S.; ASSIS, V. R.; SMITH, K. M.; APPEL, A. G.; MENDONÇA, M. T. Innate immunity of Florida cane toads: how dispersal has affected physiological responses to LPS. **J Comp Physiol B**. doi:10.1007/s00360-020-01272-7. 2020.

GIBBS, J. E.; BLAIKLEY, J.; BEESLEY, S.; MATTHEWS, L.; SIMPSON, K. D.; BOYCE, S. H.; FARROW, S. N.; ELSE, K. J.; SINGH, D.; RAY, D. W.; LOUDON, A. S. The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. **Proc Natl Acad Sci USA**, vol. 109, p. 582–587. 2012.

GIBBS, J.; INCE, L.; MATTHEWS, L.; MEI, J.; BELL, T.; YANG, N.; SAER, B.; BEGLEY, N.; POOLMAN, T.; PARIOLLAUD, M.; FARROW, S.; DEMAYO, F.; HUSSELL, T.; WORTHEN, G. S.; RAY, D.; LOUDON, A. An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action. **Nat Med**, vol. 20, p. 919–926. 2014

GODAHEWA, G. I.; BATHIGE, S. D. N. K.; HERATH, H. M. L. P. B.; NOH, J. K.; LEE, J. Characterization of rock bream (*Oplegnathus fasciatus*) complement components C1r and C1s in terms of molecular aspects, genomic modulation, and immune responsive transcriptional profiles following bacterial and viral pathogen exposure. **Fish Shellfish Immun**, vol. 46, p. 656–668. 2015.

GROSSMAN, C. J. Regulation of the immune system by sex steroids. **Endocr Rev**, vol. 5, p. 435-455. 1984.

GROSSMAN, C. J. Interactions between the gonadal steroids and the immune system. **Science**, vol. 227, p. 257-261. 1985.

HAIMOVICH, B.; CALVANO, J.; HAIMOVICH, A. D.; CALVANO, S. E.; COYLE, S. M.; LOWRY, S. F. In vivo endotoxin synchronizes and suppresses clock gene expression in human peripheral blood leukocytes. **Crit Care Med**, vol. 38, p. 751-758. 2010.

HARDELAND, R. Melatonin signaling mechanisms of a pleiotropic agent. **Biofactors**, vol. 35, p. 183–192. 2009.

HAZEN, T. C.; FLIERMANS, C. B.; HIRSH, R. P.; ESCH, G. W. Prevalence and distribution of *Aeromonas hydrophila* in the United States. **App Environ Microbiol**, vol. 36, p. 731-738. 1978.

HEGEMANN, A.; MATSON, K. D.; VERSTEEGH, M. A.; VILLEGAS, A.; TIELEMAN, B. I. Immune response to an endotoxin challenge involves multiple immune parameters and is consistent among the annual-cycle stages of a free-living temperate zone bird. **J Exp Biol**, vol. 216, p. 2573-2580. 2013.

HILL, W. A.; NEWMAN, S. J.; CRAIG, L.; CARTER, C.; CZARRA, J.; BROWN, J. P. Diagnosis of *Aeromonas hydrophila*, *Mycobacterium species*, and *Batrachochytrium dendrobatidis* in an African clawed frog (*Xenopus laevis*). **J Am Assoc Lab Anim Sci**, vol. 49, p. 215-220. 2010.

JAYAWARDENA, U. A.; RATNASOORIYA, W. D.; WICKRAMASINGHE, D. D.; UDAGAMA, P. V. Heavy metal mediated innate immune responses of the Indian green frog, *Euphlyctis hexadactylus* (Anura: Ranidae): Cellular profiles and associated Th1 skewed cytokine response. **Sci Total Environ**, vol. 566-567, p. 1194-1204. 2015.

JIM, J. **Distribuição altitudinal e estudo de longa duração de anfíbios na região de Botucatu, Estado de São Paulo**. 2002. 343 p. Thesis (Livre Docência) - Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Botucatu, São Paulo. 2002.

KELLER, M.; MAZUCH, J.; ABRAHAM, U.; EOM, G. D.; HERZOG, E. D.; VOLK, H. D.; KRAMER, A.; MAIER, B. A circadian clock in macrophages controls inflammatory immune responses. **Proc Natl Acad Sci USA**, vol. 106, p. 21407-21412. 2009.

LABRECQUE, N.; CERMAKIAN, N. Circadian Clocks in the Immune System. **J Biol Rhythms**, vol. 30, p. 277-290. 2015.

LARANJEIRA-SILVA, M. F.; ZAMPIERI, R. A.; MUXEL, S. M.; FLOETER-WINTER, L. M.; MARKUS, R. P. Melatonin attenuates *Leishmania* (*L.*) *amazonensis* infection by modulating arginine metabolism. **J Pineal Res**, vol. 59, p. 478-487. 2015.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. **Methods**, vol. 25, p. 402-408. 2001.

LOTUFO, C. M. C.; LOPES, C.; DUBOCOVICH, M. L.; FARSKY, S. H. P.; MARKUS, R. P. Melatonin and N-acetylserotonin inhibit leukocyte rolling and adhesion to rat microcirculation. **Eur J Pharmacol**, vol. 430, p. 351-357. 2001.

LU, Y.; YEH, W.; OHASHI, P. S. LPS/TLR4 signal transduction pathway. **Cytokine**, vol. 42, p. 145-151. 2008.

KEMNA, E.; PICKKERS, P.; NEMETH, E.; HOEVEN, H.; SWINKELS, D. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. **Blood**, vol. 106, p. 1864-1866. 2005.

KLUGER. M. J. Fever: role of pyrogens and cryogens. **Physiol Rev**, vol. 71, p. 93-127. 1991.

MACIEL, N. M.; COLLEVATTI, R. G.; COLLI, G. R.; SCHWARTZ, E. F. Late Miocene diversification and phylogenetic relationships of the huge toads in the *Rhinella marina* (Linnaeus, 1758) species group (Anura: Bufonidae). **Mol Phylogenet Evol**, vol. 57, p. 787-797. 2010.

MADELAIRE, C. B.; GOMES, F. R. Breeding under unpredictable conditions: Annual variation in gonadal maturation, energetic reserves and plasma levels of androgens and corticosterone in anurans from the Brazilian semi-arid. **Gen Comp Endocrinol**, vol. 228, p. 9-16. 2016.

MADELAIRE, C. B.; SOKOLOVA, I.; GOMES, F. R. Seasonal patterns of variation in steroid plasma levels and immune parameters in anurans from Brazilian semiarid area. **Physiol Biochem Zool**, vol. 90, p. 415-433. 2017.

MARKUS, R. P.; CECON, E.; PIRES-LAPA, M. A. Immune-Pineal Axis: Nuclear Factor κB (NF-κB) Mediates the shift in the melatonin source from pinealocytes to immune competent cells. **Int J Mol Sci**, vol. 14, p. 10979-10997. 2013.

MARKUS, R. P.; FERREIRA, Z. S.; FERNANDES, P. A. C. M.; CECON, E. The Immune-Pineal Axis: A Shuttle between Endocrine and Paracrine Melatonin Sources. **Neuroimmunomodulat**, vol. 14, p. 126-133. 2007.

MARKUS, R. P.; FERNANDES, P. A. C. M.; KINKER, G. S.; CRUZ-MACHADO, S. S.; MARÇOLA, M. Immune-pineal axis – acute inflammatory responses coordinate melatonin synthesis by pinealocytes and phagocytes. **Brit J Pharmacol**. doi:10.1111/bph.14083. 2018.

MARTINI, F.; FERNÁNDEZ, C.; TARAZONA, J. V.; PABLOS, M. V. Gene expression of heat shock protein 70, interleukin-1β and tumor necrosis factor α as tools to identify immunotoxic effects on *Xenopus laevis*: a dose-response study with benzo[a]pyrene and its degradation products. **Environ Pollut**, vol. 160, p. 28-33. 2012.

MEBIUS, R. E.; KRAAL, G. Structure and function of the spleen. **Nat Rev Immunol**, vol. 5, p. 606-616. 2005.

MEKAOUCHE, M.; GIVALOIS, L.; BARBANEL, G.; SIAUD, P.; MAUREL, D.; MALAVAL, F.; BRISTOW, A. F.; BOISSIN, J.; ASSENMACHER, I.; IXART, G. Chronic Restraint Enhances Interleukin-1-Beta Release in the Basal State and after an Endotoxin Challenge,

Independently of Adrenocorticotropin and Corticosterone Release. **Neuroimmunomodulat**, vol. 1, p. 292-299. 1994.

MENDONÇA, M. T.; CHERNETSBY, S. D.; NESTER, K. E.; GARDNER, G. L. Effects of sex steroids on sexual behavior in the big brown bat, *Eptesicus fuscus.* **Horm Behav**, vol. 30, p. 153–161. 1996.

MILLET, S.; BENNETT, J.; LEE, K. A.; HAU, M.; KLASING, K. C. Quantifying and comparing constitutive immunity across avian species. **Dev Comp Immunol**, vol. 31, p. 188-201. 2007.

MORALES, H. D.; ABRAMOWITZ, L.; GERTZ, J.; SOWA, J.; VOGEL, A.; ROBERT, J. Innate immune responses and permisiveness to Ranavirus infection of peritoneal leukocytes in the frog *Xenopus laevis*. **J Virol**, vol. 84, p. 4912-4922. 2010.

MORETTI, E. H.; CHINCHILLA, J. E. O.; MARQUES, F. S.; FERNANDES, P. A. C. M.; GOMES, F. R. Behavioral fever decreases metabolic response to lipopolysaccharide in yellow Cururu toads (*Rhinella icterica*). **Physiol Behav**, vol. 191, p. 73-81. 2018.

MÜLLER-EBERHARD, H. J. Molecular organization and function of the complement system. **Ann Rev Biochem**, vol. 57, p. 321-347. 1988.

NORRIS, D. O. **Vertebrate endocrinology**. New York: Elsevier Academic Press. 4th ed. 2007.

OWEN-ASHELY, N. T.; TURNER, M.; HAHN, T. P.; WINGFIELD, J. C. Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (*Zonotrichia leucophrys gambelii*). **Horm Behav**, vol. 49, p. 15-29. 2006.

PANCAK, M. K.; TAYLOR, D. H. Seasonal and Daily Corticosterone Rhythms in American Toads, *Bufo americanus*. **Gen and Comp Endocr**, vol. 50, p. 490-497. 1982

PETERS, A. Testosterone treatment is immunosuppressive in superb fairy-wrens, yet free-living males with high testosterone are more immunocompetent. **Proc R Soc Lond B**, vol. 267, p. 883-889. 2000.

PIESIEWICZ, A.; KEDZIERSKA, U.; ADAMSKA, I.; USAREK, M.; ZEMAN, M.; SKWARLO-SONTA, K.; MAJEWSKI, P. M. Pineal arylalkylamine *N-acetyltransferase* (*Aanat*) gene expression as a target of inflammatory mediators in the chicken. **Gen Comp Endocrinol**, vol. 179, p. 143–151. 2012.

POUGH, F. H. Advantages of ectothermy for tetrapods. **Am Nat**, vol. 115, p. 92-112. 1980.

PROVENCIO, I.; ROLLAG, M.; CASTRUCCI, A. Photoreceptive net in the mammalian retina. **Nature**, vol. 493. 2002. Available at: <https://doi.org/10.1038/415493a>. Access at: 02 July 2020.

REITER, R. J.; TAN, D. X.; FUENTES-BROTO, L. Melatonin: a multitasking molecule. **Prog Brain Res**, vol. 181, p. 127-151. 2010.

RIVAS, Z. P. ***Aeromonas hydrophila* in amphibians: harmless bystander or opportunistic pathogen**. 2016. 46 p. Honors Undergraduate Theses. University of Central Florida, 2016.

RIVIER, C.; CHIZZONITE, R.; VALE, W. In the Mouse, the Activation of the Hypothalamic-Pituitary-Adrenal Axis by a Lipopolysaccharide (Endotoxin) is Mediated through Interleukin-1*. **Endocrinology**, vol. 125, p. 2800-2805. 1989.

ROBERTS, M. L.; BUCHANAN, K. L.; EVANS, M. R. Testing the immunocompetence handicap hypothesis: a review of the evidence. **Anim Behav**, vol. 68, p. 227-239. 2004.

ROMERO, L. M.; REED, J. M. Collecting baseline corticosterone samples in the field: is under 3 min good enough? **Comp Biochem Phys A**, vol. 140, p. 73-79. 2005.

SABBAN, E. L.; NANKOVA, B. B.; SEROVA, L. I.; KVETNANSKY, R.; LIU, X. Molecular regulation of gene expression of catecholamine biosynthetic enzymes by stress: sympathetic ganglia versus adrenal medulla. **Ann N Y Acad Sci**, vol. 1018, p. 370-377. 2004.

SAKAMOTO, K.; NAGASE, T.; FUKUI, H.; HORIKAWA, K.; OKADA, T.; TANAKA, H.; SATO, K.; MIYAKE, Y.; OHARA, O.; KAKO, K.; ISHIDA, N. Multitissue circadian expression of rat period homolog (rPer2) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. **J Biol Chem**, vol. 273, p. 27039-27042. 1998.

SAPOLSKY, R. M.; ROMERO, M.; MUNCK, A. U. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. **Endocr Rev**, vol. 21, p. 55-89. 2000.

SAMUEL, C. E. Antiviral Actions of Interferons. **Clin Microbiol Rev**, vol. 14, p. 778-809. 2001.

SAVAN, R.; RAVICHANDRAN, S.; COLLINS, J. R.; SAKAI, M.; YOUNG, H. A. Structural conservation of interferon gamma among vertebrates. **Cytokine Growth F R**, vol. 20, p. 115-124. 2009.

SCHRODERUS, E.; JOKINEN, I.; KOIVULA, M.; KOSKELA, E.; MAPPES, T.; MILLS, S. C.; OKSANEN, T. A.; POIKONEN, T. Intra- and intersexual trade-offs between testosterone and immune system: Implications for sexual and sexually antagonistic selection. **Am Nat**, vol. 176, p. 90-97. 2010.

SEGALLA, M. V.; CARAMASCHI, U.; CRUZ, C. A. G.; GARCIA, P. C. A.; GRANT, T.; HADDAD, C. F. B.; SANTANA, D. J.; TOLEDO, L. F.; LANGONE; J. A. Brazilian Amphibians: List of Species. **Herpetologia Brasileira**, vol. 8, p. 65-96. 2019.

SERINO, I.; D'ISTRIA, M.; MONTELEONE, P. A comparative study of melatonin production in the retina, pineal gland and harderian gland of *Bufo viridis* and *Rana esculenta*. **Comp Biochem Physiol**, vol. 106C, p. 189-193. 1993.

SIMONNEAUX, V.; RIBELAYGA, C. Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. **Pharmacol Rev**, vol. 55, p. 325-395. 2003.

TAKIGUCHI, R. S. **Efeitos da modulação do sistema melatonérgico sobre o perfil inflamatório sistêmico induzido por endotoxemia letal em ratos**. 2018. 94 f. Dissertation (Masters in Science) – Instituto de Biociências, Universidade de São Paulo, São Paulo. 2018.

TAMURA, E. K.; CECON, E.; MONTEIRO, A. W. A.; SILVA, C. L. M.; MARKUS, R. P. Melatonin inhibits LPS-induced NO production in rat endothelial cells. **J Pineal Res**, vol. 46, p. 268-274. 2009.

TAMURA, E. K.; FERNANDES, P. A. C. M.; MARÇOLA, M.; CRUZ-MACHADO, S. D.; MARKUS, R. P. Long-Lasting Priming of Endothelial Cells by Plasma Melatonin Levels. **Plos One**, vol. 5:e13958. 2010.

TAKEMURA, T.; MAKINO, S.; TAKAO, T.; ASABA, K.; SUEMARU, S.; HASHIMOTO, K. Hypothalamic-pituitary-adrenocortical responses to single vs. repeated endotoxin lipopolysaccharide administration in the rat. **Brain Res**, vol. 167, p. 181-191. 1997.

TERRAZZINO, S.; PEREGO, C.; DE LUIGI, A.; DE SIMONI, M. G. Interleukin-6, tumor necrosis fator and corticosterone induction by central lipopolysaccharide in aged rats. **Life Sci**, vol. 61, p. 695-701. 1997.

TITON S. C. M.; ASSIS, V. R.; TITON JR., B.; CASSETTARI, B. O.; FERNANDES, P. A. C .M.; GOMES, F. R. Captivity effects on immune response and steroid plasma levels of a Brazilian toad (*Rhinella schneideri*). **J Exp Zool**, vol. 327, p. 127-138. 2017.

TITON, S. C. M.; TITON JR., B.; ASSIS, V. R.; KINKER, G. S.; FERNANDES, P. A. C. M.; GOMES, F. R. Interplay among steroids, body condition and immunity in response to long-term captivity in toads. **Sci Rep-Uk**, vol. 8, p. 1-13. 2018.

TRINDER, J.; ARMSTRONG, S. M.; O'BRIEN, C.; LUKE, D.; MARTIN, M. J. Inhibition of melatonin secretion onset by low levels of illumination. **J Sleep Res**, vol. 5, p. 77-82. 1996.

WHITNALL, M. H. Corticotropin-releasing hormone neurosecretory cells: regulation of peptide expression and release. **Mol Cell Endocrinol**, vol. 10A, p. 101-117. 1997.

WINGFIELD, J. C.; ROMERO, L. M. Adrenocortical responses to stress and their modulation in free-living vertebrates. In MCEWEN, B. S.; GOODMAN, H. M. (Eds.). **Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms**. New York: Oxford Univ. Press, 2001. P. 211-234

YASOJIMA, K.; SCHWAB, C.; MCGEER, E. G.; MCGEER, P. L. Human heart generates complement proteins that are upregulated and activated after myocardial infarction. **Circ Res**, vol. 83, p. 860-869. 1988.

YI, W.J.; KIM T.S. Melatonin protects mice against stress-induced inflammation through enhancement of M2 macrophage polarization. **Int Immuno Pharmacol**, vol. 48, p. 146–158. 2017.

ZEN, M.; CANOVA, M.; CAMPANA, C.; BETTIO, S.; NALOTTO, L.; RAMPUDDA, M.; RAMONDA, R.; IACCARINO, L.; DORIA, A. The kaleidoscope of glucocorticoid effects on immune system. **Autoimmun Rev**, vol. 10, p. 305-310. 2011.

ZHAO, Z. Y.; TOUITOU, Y. Kinetic changes of melatonin release in rat pineal perfusion at different circadian stages. Effect of corticosteroids. **Acta Endocrinol Openh**, vol. 129, p. 81–88. 1993.

ZIMMERMAN, L. M.; BOWDEN, R. M.; VOGEL, L. A. A vertebrate cytokine primer for eco-immunologists. **Funct Ecol**, vol. 28, p. 1061-1073. 2014.

ZOU, J.; BIRD, S.; MINTER, R.; HORTON, J. Molecular cloning of the gene for interleukin-1β from *Xenopus laevis* and analysis of expression in vivo and in vitro. **Immunogenetics**, vol. 51, p. 332-338. 2000.

8. Appendix

Table A1. Hormonal and immune variables for individuals of *R. icterica* analyzed by a set of ANCOVAs, with plasma corticosterone, testosterone and melatonin levels, and bacterial killing ability as dependent variables, body index as co-variable and treatment (saline and LPS) and time (early or late night) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
CORT	Model	3081.035	7	440.148	1.190	0.357
	Intercept	14364.199	1	14364.199	38.820	0.000
	Treat	1283.033	1	1283.033	3.467	0.079
	Time	58.306	1	58.306	0.158	0.696
	BI	0.835	1	0.835	0.002	0.963
	Treat*Time	26.166	1	26.166	0.071	0.793
	Treat*BI	115.703	1	115.703	0.313	0.583
	Time*BI	1231.700	1	1231.700	3.329	0.085
	Treat*Time*BI	236.011	1	236.011	0.638	0.435
	Error	6660.393	18	370.022		
	Total	23906.941	26			
T	Model	2981.024	7	425.861	0.307	0.941
	Intercept	49309.156	1	49309.156	35.543	0.000
	Treat	449.184	1	449.184	0.324	0.576
	Time	16.696	1	16.696	0.012	0.914
	BI	647.005	1	647.005	0.466	0.503
	Treat*Time	1295.091	1	1295.091	0.934	0.347
	Treat*BI	1.286	1	1.286	0.001	0.976
	Time*BI	15.077	1	15.077	0.011	0.918
	Treat*Time*BI	305.716	1	305.716	0.220	0.644
	Error	24971.752	18	1387.320		
	Total	77626.569	26			
MEL	Model	3917.855	7	559.694	0.844	0.567
	Intercept	13665.780	1	13665.780	20.619	0.000
	Treat	739.453	1	739.453	1.116	0.306
	Time	0.249	1	0.249	0.000	0.985
	BI	388.007	1	388.007	0.585	0.455
	Treat*Time	96.708	1	96.708	0.146	0.707
	Treat*BI	2456.039	1	2456.039	3.706	0.071
	Time*BI	42.741	1	42.741	0.064	0.803
	Treat*Time*BI	1162.325	1	1162.325	1.754	0.203
	Error	11267.071	17	662.769		
	Total	29629.601	25			
BKA	Model	123.223	7	17.603	0.402	0.888
	Intercept	207082.993	1	207082.993	4732.107	0.000
	Treat	0.067	1	0.067	0.002	0.969
	Time	73.295	1	73.295	1.675	0.213
	BI	10.672	1	10.672	0.244	0.628
	Treat*Time	0.553	1	0.553	0.013	0.912
	Treat*BI	1.615	1	1.615	0.037	0.850
	Time*BI	29.088	1	29.088	0.665	0.426
	Treat*Time*BI	1.123	1	1.123	0.026	0.875
	Error	743.942	17	43.761		
	Total	210933.554	25			

Note. **Type III SS** = Type III Sum of Squares; **DF** = Degrees of Freedom; **MS** = Mean Square. **CORT** = corticosterone; **TEST** = testosterone; **MEL** = melatonin; **BKA** = bacterial killing ability. **Treat** = treatment. **BI** = body index.

T**able A2.** Hormonal and immune variables for individuals of *R. icterica* analyzed by a set of ANCOVAs, with plasma corticosterone, testosterone and melatonin levels, and bacterial killing ability as dependent variables, body mass as co-variable and treatment (saline and LPS) and time (early or late night) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
CORT	Model	2007.693	7	286.813	0.668	0.697
	Intercept	767.309	1	767.309	1.786	0.198
	Treat	307.278	1	307.278	0.715	0.409
	Time	318.244	1	318.244	0.741	0.401
	Mas	45.798	1	45.798	0.107	0.748
	Treat*Time	184.424	1	184.424	0.429	0.521
	Treat*Mas	56.287	1	56.287	0.131	0.722
	Time*Mas	254.014	1	254.014	0.591	0.452
	Treat*Time*Mas	139.475	1	139.475	0.325	0.576
	Error	7733.736	18	429.652		
	Total	23906.941	26			
T	Model	5703.293	7	814.756	0.659	0.703
	Intercept	8254.062	1	8254.062	6.678	0.019
	Treat	351.284	1	351.284	0.284	0.600
	Time	1018.206	1	1018.206	0.824	0.376
	Mas	803.449	1	803.449	0.650	0.431
	Treat*Time	2717.671	1	2717.671	2.199	0.155
	Treat*Mas	238.577	1	238.577	0.193	0.666
	Time*Mas	1054.897	1	1054.897	0.853	0.368
	Treat*Time*Mas	1743.865	1	1743.865	1.411	0.250
	Error	22249.484	18	1236.082		
	Total	77626.569	26			
MEL	Model	3797.014	7	542.431	0.810	0.591
	Intercept	5.133	1	5.133	0.008	0.931
	Treat	50.085	1	50.085	0.075	0.788
	Time	2389.760	1	2389.760	3.567	0.076
	Mas	1168.460	1	1168.460	1.744	0.204
	Treat*Time	56.238	1	56.238	0.084	0.776
	Treat*Mas	1.833	1	1.833	0.003	0.959
	Time*Mas	2640.508	1	2640.508	3.942	0.063
	Treat*Time*Mas	125.977	1	125.977	0.188	0.670
	Error	11387.912	17	669.877		
	Total	29629.601	25			
BKA	Model	134.852	7	19.265	0.447	0.859
	Intercept	14331.732	1	14331.732	332.698	0.000
	Treat	21.532	1	21.532	0.500	0.489
	Time	1.593	1	1.593	0.037	0.850
	Mas	5.935	1	5.935	0.138	0.715
	Treat*Time	20.685	1	20.685	0.480	0.498
	Treat*Mas	25.547	1	25.547	0.593	0.452
	Time*Mas	1.550	1	1.550	0.036	0.852
	Treat*Time*Mas	22.031	1	22.031	0.511	0.484
	Error	732.313	17	43.077		
	Total	210933.554	25			

Note. **Type III SS** = Type III Sum of Squares; **DF** = Degrees of Freedom; **MS** = Mean Square. **CORT** = corticosterone; **T** = testosterone; **MEL** = melatonin; **BKA** = bacterial killing ability. **Treat** = treatment. **Mas** = Body Mass.

Table A3. Cytokines and complement protein expression for individuals of *R. icterica* analyzed by a set of ANCOVAs, with IL1β, IL6, IL10, IFNγ and C1S expression as dependent variables, body index as co-variable and treatment (saline and LPS) and time (early or late night) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
IL1β	Model	5.946	7	0.849	1.994	0.124
	Intercept	1.710	1	1.710	4.014	0.064
	Treat	3.255	1	3.255	7.639	**0.014***
	Time	0.185	1	0.185	0.434	0.520
	BI	0.000	1	0.000	0.001	0.979
	Treat*Time	1.228	1	1,228	2.883	0.110
	Treat*BI	0.012	1	0.012	0.028	0.869
	Time*BI	0.020	1	0.020	0.048	0.830
	Treat*Time*BI	1.289	1	1.289	3.026	0.102
	Error	6.391	15	0.426		
	Total	14.633	23			
IL6	Model	2.098	7	0.300	1.862	0.153
	Intercept	0.006	1	0.006	0.036	0.852
	Treat	0.015	1	0.015	0.091	0.767
	Time	0.714	1	0.714	4.437	0.054
	BI	0.000	1	0.000	0.002	0.965
	Treat*Time	0.955	1	0.955	5.936	0.029
	Treat*BI	0.087	1	0.087	0.541	0.474
	Time*BI	0.005	1	0.005	0.030	0.865
	Treat*Time*BI	0.858	1	0.858	5.333	0.037
	Error	2.253	14	0.161		
	Total	4.354	22			
IL10	Model	5.946	7	0.849	1.994	.124
	Intercept	1.710	1	1.710	4.014	0.064
	Treat	3.255	1	3.255	7.639	**0.014***
	Time	0.185	1	0.185	0.434	0.520
	BI	0.000	1	0.000	0.001	0.979
	Treat*Time	1.228	1	1.228	2.883	0.110
	Treat*BI	0.012	1	0.012	0.028	0.869
	Time*BI	0.020	1	0.020	0.048	0.830
	Treat*Time*BI	1.289	1	1.289	3.026	0.102
	Error	6.391	15	0.426		
	Total	14.633	23			
IFNγ	Model	2.479	7	0.354	0.478	0.836
	Intercept	0.179	1	0.179	0.242	0.630
	Treat	0.082	1	0.082	0.110	0.744
	Time	0.009	1	0.009	0.013	0.912
	BI	1.431	1	1.431	1.933	0.185
	Treat*Time	0.251	1	0.251	0.340	0.569
	Treat*BI	0.092	1	0.092	0.124	0.730
	Time*BI	0.242	1	0.242	0.327	0.576
	Treat*Time*BI	0.126	1	0.126	0.170	0.686
	Error	11.108	15	0.741		
	Total	13.941	23			
C1S	Model	1.218	7	0.174	0.458	0.850
	Intercept	0.863	1	0.863	2.270	0.153
	Treat	0.294	1	0.294	0.774	0.393
	Time	0.201	1	0.201	0.529	0.478
	BI	0.192	1	0.192	0.506	0.488
	Treat*Time	0.012	1	0.012	0.032	0.860
	Treat*BI	0.017	1	0.017	0.045	0.834
	Time*BI	0.047	1	0.047	0.124	0.729
	Treat*Time*BI	0.427	1	0.427	1.124	0.306
	Error	5.701	15	0.380		
	Total	7.958	23			

Note. **Type III SS** = Type III Sum of Squares; **DF** = Degrees of Freedom; **MS** = Mean Square. **Treat** = Treatment. **BI =** Body Index. **Asterisk (*)** = $p \leq 0.05$.

Table A4. Cytokines and complement protein expression for individuals of *R. icterica* analyzed by a set of ANCOVAs, with IL1β, IL6, IL10, IFNγ and C1S expression as dependent variables, body mass as co-variable and treatment (saline and LPS) and time (early or late night) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
IL1β	Model	4.945	7	0.706	1.434	0.263
	Intercept	0.140	1	0.140	0.283	0.602
	Treat	0.020	1	0.020	0.041	0.842
	Time	0.024	1	0.024	0.049	0.828
	Mas	0.001	1	0.001	0.002	0.969
	Treat*Time	0.001	1	0.001	0.001	0.974
	Treat*Mas	0.181	1	0.181	0.366	0.554
	Time*Mas	0.090	1	0.090	0.183	0.675
	Treat*Time*Mas	0.097	1	0.097	0.197	0.663
	Error	7.392	15	0.493		
	Total	14.633	23			
IL6	Model	1.464	7	0.209	1.014	0.462
	Intercept	0.080	1	0.080	0.386	0.544
	Treat	0.005	1	0.005	0.024	0.878
	Time	0.148	1	0.148	0.716	0.412
	Mas	0.104	1	0.104	0.504	0.489
	Treat*Time	0.336	1	0.336	1.628	0.223
	Treat*Mas	0.004	1	0.004	0.018	0.896
	Time*Mas	0.020	1	0.020	0.098	0.758
	Treat*Time*Mas	0.120	1	0.120	0.584	0.457
	Error	2.887	14	0.206		
	Total	4.354	22			
IL10	Model	4.792	7	0.685	1.573	0.241
	Intercept	0.446	1	0.446	1.026	0.333
	Treat	0.147	1	0.147	0.337	0.573
	Time	0.014	1	0.014	0.032	0.861
	Mas	1.360	1	1.360	3.125	0.105
	Treat*Time	0.009	1	0.009	0.022	0.885
	Treat*Mas	0.731	1	0.731	1.680	0.221
	Time*Mas	0.103	1	0.103	0.237	0.636
	Treat*Time*Mas	0.093	1	0.093	0.213	0.653
	Error	4.787	11	0.435		
	Total	11.096	19			
IFNγ	Model	1.138	7	0.163	0.196	0.982
	Intercept	0.104	1	0.104	0.126	0.728
	Treat	0.015	1	0.015	0.018	0.896
	Time	0.079	1	0.079	0.095	0.762
	Mas	0.260	1	0.260	0.313	0.584
	Treat*Time	0.418	1	0.418	0.504	0.489
	Treat*Mas	0.043	1	0.043	0.052	0.823
	Time*Mas	0.041	1	0.041	0.050	0.826
	Treat*Time*Mas	0.254	1	0.254	0.307	0.588
	Error	12.449	15	0.830		
	Total	13.941	23			
C1S	Model	0.799	7	0.114	0.280	0.952
	Intercept	0.151	1	0.151	0.370	0.552
	Treat	0.080	1	0.080	0.195	0.665
	Time	0.062	1	0.062	0.153	0.701
	Mas	0.014	1	0.014	0.034	0.856
	Treat*Time	0.017	1	0.017	0.042	0.841
	Treat*Mas	0.019	1	0.019	0.046	0.833
	Time*Mas	0.186	1	0.186	0.456	0.510
	Treat*Time*Mas	0.021	1	0.021	0.051	0.824
	Error	6.120	15	0.408		
	Total	7.958	23			

Note. **Type III SS** = Type III Sum of Squares; **DF** = Degrees of Freedom; **MS** = Mean Square. **Treat** = Treatment. **Mas =** Body Mass. **Asterisk (*)** = $p \leq 0.05$.

Table A5. Hormonal and immune variables for individuals of *R. icterica* analyzed by a set of ANCOVAs, with plasma corticosterone, testosterone and melatonin levels, and bacterial killing ability as dependent variables, environmental component as co-variable and treatment (saline and LPS) and time (early or late night) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
CORT	Model	2712.241	7	387.463	0.992	0.468
	Intercept	13318.978	1	13318.978	34.107	0.000
	Treat	1553.100	1	1553.100	3.977	0.061
	Time	2.206	1	2.206	0.006	0.941
	Env	282.169	1	282.169	0.723	0.406
	Treat*Time	7.573	1	7.573	0.019	0.891
	Treat*Env	829.891	1	829.891	2.125	0.162
	Time*Env	110.663	1	110.663	0.283	0.601
	Treat*Time*Env	242.074	1	242.074	0.620	0.441
	Error	7029.187	18	390.510		
	Total	23906.941	26			
T	Model	6505.279	7	929.326	0.780	0.612
	Intercept	40570.654	1	40570.654	34.049	0.000
	Treat	225.776	1	225.776	0.189	0.669
	Time	415.967	1	415.967	0.349	0.562
	Env	2370.084	1	2370.084	1.989	0.175
	Treat*Time	751.402	1	751.402	0.631	0.437
	Treat*Env	999.628	1	999.628	0.839	0.372
	Time*Env	1910.353	1	1910.353	1.603	0.222
	Treat*Time*Env	44.668	1	44.668	0.037	0.849
	Error	21447.497	18	1191.528		
	Total	77626.569	26			
MEL	Model	7847.304	7	1121.043	2.597	0.051
	Intercept	18600.702	1	18600.702	43.095	0.000
	Treat	187.783	1	187.783	0.435	0.518
	Time	0.064	1	0.064	0.000	0.990
	Env	170.954	1	170.954	0.396	0.537
	Treat*Time	0.062	1	0.062	0.000	0.991
	Treat*Env	328.363	1	328.363	0.761	0.395
	Time*Env	6337.857	1	6337.857	14.684	**0.001***
	Treat*Time*Env	77.695	1	77.695	0.180	0.677
	Error	7337.622	17	431.625		
	Total	29629.601	25			
BKA	Model	234.728	7	33.533	0.901	0.528
	Intercept	157769.520	1	157769.520	4240.865	0.000
	Treat	10.308	1	10.308	0.277	0.605
	Time	40.547	1	40.547	1.090	0.311
	Env	.032	1	0.032	0.001	0.977
	Treat*Time	25.286	1	25.286	0.680	0.421
	Treat*Env	77.386	1	77.386	2.080	0.167
	Time*Env	64.790	1	64.790	1.742	0.204
	Treat*Time*Env	35.041	1	35.041	0.942	0.345
	Error	632.437	17	37.202		
	Total	210933.554	25			

Note. **Type III SS** = Type III Sum of Squares; **DF** = Degrees of Freedom; **MS** = Mean Square. **CORT** = corticosterone; **T** = testosterone; **MEL** = melatonin; **BKA** = bacterial killing ability. **Treat** = Treatment. **Env =** Environmental Component. **Asterisk (*)** = $p \leq 0.05$.

Table A6. Cytokines and complement protein expression for individuals of *R. icterica* analyzed by a set of ANCOVAs, with IL1β, IL6, IL10, IFNγ and C1S expression as dependent variables, environmental component as co-variable and treatment (saline and LPS) and time (early or late night) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
IL1β	Model	6.559	7	0.937	2.432	.070
	Intercept	1.383	1	1.383	3.590	0.078
	Treat	1.863	1	1.863	4.836	**0.044***
	Time	0.126	1	0.126	0.328	0.575
	Env	0.033	1	0.033	0.085	0.774
	Treat*Time	1.786	1	1.786	4.635	**0.048***
	Treat*Env	1.452	1	1.452	3.769	0.071
	Time*Env	0.144	1	0.144	0.373	0.551
	Treat*Time*Env	0.894	1	0.894	2.320	0.149
	Error	5.778	15	0.385		
	Total	14.633	23			
IL6	Model	1.504	7	0.215	1.056	0.438
	Intercept	0.000	1	0.000	0.002	0.965
	Treat	0.014	1	0.014	0.068	0.799
	Time	0.430	1	0.430	2.116	0.168
	Env	0.177	1	0.177	0.868	0.367
	Treat*Time	0.656	1	0.656	3.224	0.094
	Treat*Env	0.000	1	0.000	0.001	0.976
	Time*Env	0.007	1	0.007	0.036	0.853
	Treat*Time*Env	0.079	1	0.079	0.390	0.542
	Error	2.847	14	0.203		
	Total	4.354	22			
IL10	Model	4.759	7	0.680	1.552	0.247
	Intercept	1.944	1	1.944	4.436	0.059
	Treat	2.033	1	2.033	4.640	0.054
	Time	0.142	1	0.142	0.324	0.581
	Env	1.213	1	1.213	2.769	0.124
	Treat*Time	0.178	1	0.178	0.406	0.537
	Treat*Env	0.855	1	0.855	1.950	0.190
	Time*Env	0.162	1	0.162	0.370	0.555
	Treat*Time*Env	0.151	1	0.151	0.346	0.569
	Error	4.819	11	0.438		
	Total	11.096	19			
IFNγ	Model	2.041	7	0.292	0.379	0.901
	Intercept	0.190	1	0.190	0.247	0.626
	Treat	0.020	1	0.020	0.026	0.875
	Time	0.001	1	0.001	0.001	0.974
	Env	0.563	1	0.563	0.732	0.406
	Treat*Time	0.027	1	0.027	0.035	0.853
	Treat*Env	0.824	1	0.824	1.070	0.317
	Time*Env	0.123	1	0.123	0.160	0.695
	Treat*Time*Env	0.198	1	0.198	0.257	0.620
	Error	11.546	15	0.770		
	Total	13.941	23			
C1S	Model	1.598	7	0.228	0.643	0.714
	Intercept	1.173	1	1.173	3.306	0.089
	Treat	0.394	1	0.394	1.111	0.309
	Time	0.508	1	0.508	1.433	0.250
	Env	0.301	1	0.301	0.850	0.371
	Treat*Time	0.065	1	0.065	0.184	0.674
	Treat*Env	0.524	1	0.524	1.478	0.243
	Time*Env	0.130	1	0.130	0.365	0.555
	Treat*Time*Env	0.297	1	0.297	0.836	0.375
	Error	5.321	15	0.355		
	Total	7.958	23			

Note. **Type III SS** = Type III Sum of Squares; **DF** = Degrees of Freedom; **MS** = Mean Square. **Treat** = Treatment. **Env =** Environmental Component. **Asterisk (*)** = $p \leq 0.05$.

Table A7. Hormonal and immune variables for individuals of *R. icterica* analyzed by a set of ANOVAs, with plasma corticosterone, testosterone and melatonin levels, and bacterial killing ability as dependent variables, and treatment (saline and LPS) and time (early or late night) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
CORT	Model	1466.771	3	488.924	1.300	0.299
	Intercept	14199.591	1	14199.591	37.753	0.000
	Treat	1416.793	1	1416.793	3.767	0.065
	Time	41.531	1	41.531	0.110	0.743
	Treat*Time	33.562	1	33.562	0.089	0.768
	Error	8274.657	22	376.121		
	Total	23906.941	26			
T	Model	1772.993	3	590.998	0.497	0.688
	Intercept	49585.643	1	49585.643	41.669	0.000
	Treat	409.511	1	409.511	0.344	0.563
	Time	36.155	1	36.155	0.030	0.863
	Treat*Time	1434.985	1	1434.985	1.206	0.284
	Error	26179.783	22	1189.990		
	Total	77626.569	26			
MEL	Model	452.418	3	150.806	0.215	0.885
	Intercept	13902.426	1	13902.426	19.817	0.000
	Treat	450.890	1	450.890	0.643	0.432
	Time	3.227	1	3.227	0.005	0.947
	Treat*Time	2.774	1	2.774	0.004	0.950
	Error	14732.508	21	701.548		
	Total	29629.601	25			
BKA	Model	77.767	3	25.922	0.690	0.569
	Intercept	208777.980	1	208777.980	5554.024	0.000
	Treat	0.094	1	0.094	0.003	0.961
	Time	76.357	1	76.357	2.031	0.169
	Treat*Time	0.431	1	0.431	0.011	0.916
	Error	789.398	21	37.590		
	Total	210933.554	25			

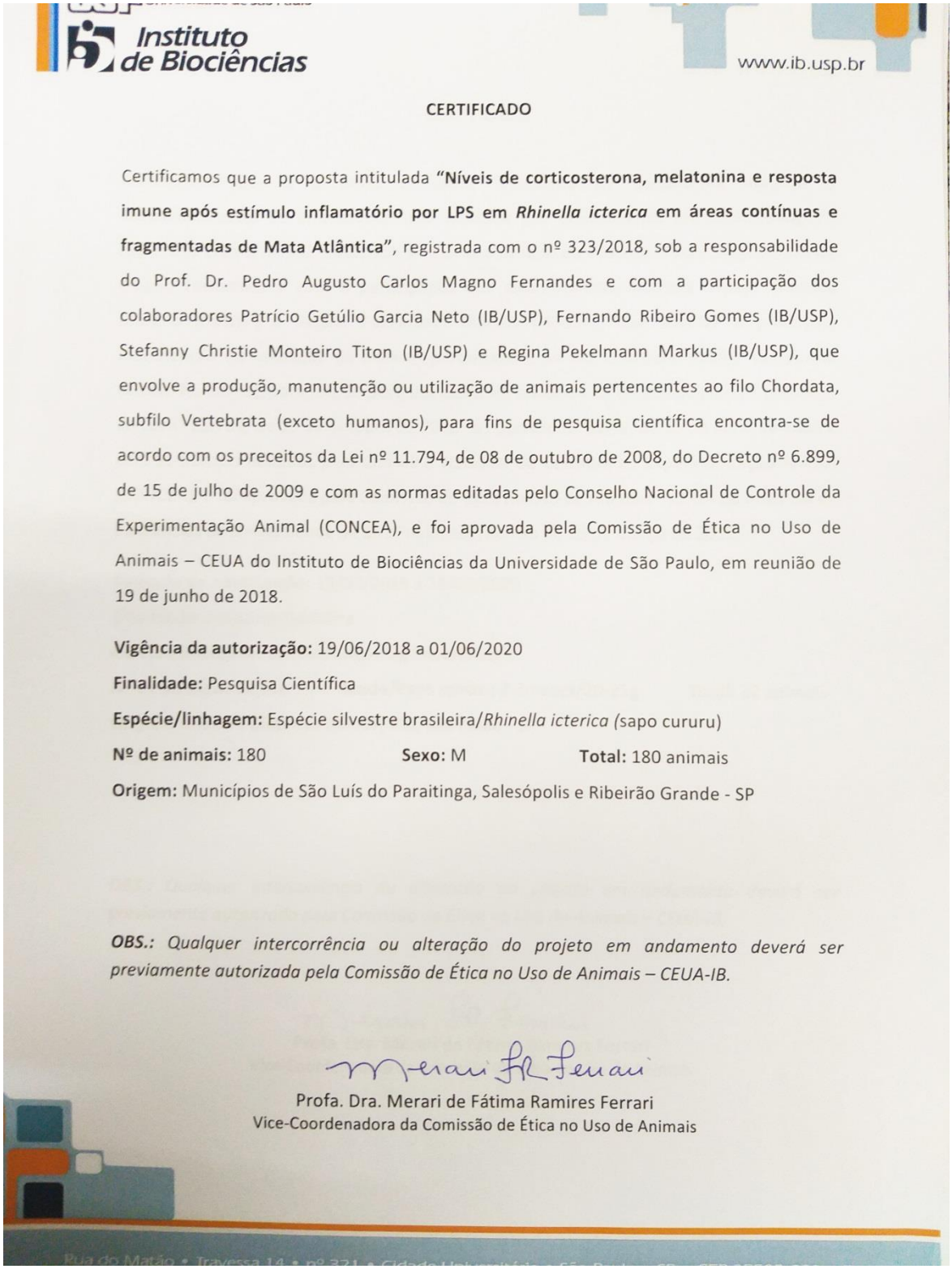
Note. **Type III SS** = Type III Sum of Squares; **DF** = Degrees of Freedom; **MS** = Mean Square. **CORT** = corticosterone; **T** = testosterone; **MEL** = melatonin; **BKA** = bacterial killing ability. **Treat** = treatment. **Asterisk (*)** = $p \leq 0.05$.

Table A8. Cytokines and complement protein expression for individuals of *R. icterica* analyzed by a set of ANOVAs, with IL1β, IL6, IL10, IFNγ and C1S expression as dependent variables, and treatment (saline and LPS) and time (early or late night) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
IL1β	Model	4.550	3	1.517	3.700	0.030
	Intercept	1.993	1	1.993	4.863	0.040
	Treat	3.386	1	3.386	8.262	**0.010***
	Time	0.271	1	0.271	0.660	0.427
	Treat*Time	0.900	1	0.900	2.195	0.155
	Error	7.788	19	0.410		
	Total	14.633	23			
IL6	Model	1.172	3	0.391	2.212	0.122
	Intercept	0.003	1	0.003	0.018	0.896
	Treat	0.003	1	0.003	0.018	0.896
	Time	0.638	1	0.638	3.610	0.074
	Treat*Time	0.638	1	0.638	3.610	0.074
	Error	3.179	18	0.177		
	Total	4.354	22			
IL10	Model	2.698	3	0.899	1.961	0.163
	Intercept	2.080	1	2.080	4.535	0.050
	Treat	2.080	1	2.080	4.535	**0.050***
	Time	0.353	1	0.353	0.769	0.394
	Treat*Time	0.353	1	0.353	0.769	0.394
	Error	6.880	15	0.459		
	Total	11.096	19			
IFNγ	Model	0.456	3	0.152	0.220	0.881
	Intercept	0.350	1	0.350	0.507	0.485
	Treat	0.096	1	0.096	0.139	0.713
	Time	0.060	1	0.060	0.087	0.771
	Treat*Time	0.278	1	0.278	0.402	0.534
	Error	13.131	19	0.691		
	Total	13.941	23			
C1S	Model	0.550	3	0.183	0.547	0.656
	Intercept	1.039	1	1.039	3.100	0.094
	Treat	0.222	1	0.222	0.661	0.426
	Time	0.303	1	0.303	0.903	0.354
	Treat*Time	3.026E-6	1	3.026E-6	0.000	0.998
	Error	6.369	19	0.335		
	Total	7.958	23			

Note. **Type III SS** = Type III Sum of Squares; **DF** = Degrees of Freedom; **MS** = Mean Square. **Treat** = Treatment. **Asterisk (*)** = $p \leq 0.05$.

9. Attachment



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CERTIFICADO

Certificamos que a proposta intitulada **"Níveis de corticosterona, melatonina e resposta imune após estímulo inflamatório por LPS em *Rhinella icterica* em áreas contínuas e fragmentadas de Mata Atlântica"**, registrada com o nº 323/2018, sob a responsabilidade do Prof. Dr. Pedro Augusto Carlos Magno Fernandes e com a participação dos colaboradores Patrício Getúlio Garcia Neto (IB/USP), Fernando Ribeiro Gomes (IB/USP), Stefanny Christie Monteiro Titon (IB/USP) e Regina Pekelmann Markus (IB/USP), que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais – CEUA do Instituto de Biociências da Universidade de São Paulo, em reunião de 19 de junho de 2018.

Vigência da autorização: 19/06/2018 a 01/06/2020

Finalidade: Pesquisa Científica

Espécie/linhagem: Espécie silvestre brasileira/*Rhinella icterica* (sapo cururu)

Nº de animais: 180 **Sexo:** M **Total:** 180 animais

Origem: Municípios de São Luís do Paraitinga, Salesópolis e Ribeirão Grande - SP

OBS.: *Qualquer intercorrência ou alteração do projeto em andamento deverá ser previamente autorizada pela Comissão de Ética no Uso de Animais – CEUA-IB.*

Profa. Dr. Merari de Fátima Ramires Ferrari
Vice-Coordenadora da Comissão de Ética no Uso de Animais

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Curriculum Vitae

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Voluntariado – Organização Não-Governamental Orla Viva - Maranhão
Maio 2012 – Maio 2014
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Agosto 2012 – Agosto 2013
Aluno Bolsista – Projeto Herpetofauna: Conhecer para Conservar
Laboratório de Herpetologia aplicada à Conservação – Universidade Federal do Maranhão (UFMA)
Agosto 2013 – Julho 2014
Estágio – Monitoramento de Serpentes Exóticas
Todd Lab – University of California, Davis (UCDAVIS)
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Aluno Bolsista – Projeto Girinos de áreas ecotonais no nordeste brasileiro: caracterização morfológica, distribuição espacial e padrões de diversidade.
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Instituto de Biociências/Universidade de São Paulo
01 – 19 Julho 2019

Outros Cursos

Serpentes e Ofidismo – 08h
Mastofauna Brasileira – 08h
VIII Curso "Bioestatística na Prática" – 24h
Ferramentas Biomoleculares e Biodiversidade – 06h
Princípios Básicos em Genética da Conservação – 22h
Diversidade de Peixes Recifais – 10h
Introdução à Linguagem R – 8h

Publicações

GARCIA NETO, P. G.; NOWAKOWSKI, A. J. ; SILVA, A. F. C.; OLIVEIRA, O. C. C.; GUERRA, R. N. M.; ANDRADE, G. V. Leukocyte profiles of two neotropical anuran species affected by anthropogenic habitat alteration. Animal Conservation, 2020. http://dx.doi.org/10.1111/acv.12564

FIGUEIREDO, A. Q. S.; MENDES, M. B. P.; NASCIMENTO, A. D.; VALE, A. A. M.; PAIVA, B. H. I.; SARAIVA JUNIOR, C. C. F.; PEREIRA, D. M.; ARAUJO JUNIOR, E. C.; LIMA, G. P.; DUTRA, I. L.; SILVA, J. U. ; FEITOSA, L. M.; TROVAO, L. O.; MARTINS, L. P.; LIMA, L. S.; FERREIRA, M. A. M.; BELFORT, M. R. C.; OLIVEIRA, O. C. C.; **GARCIA NETO, P. G.**; BRANDAO, R. A.; LIMA, R. R.; AZEVEDO, G. G.. O perfil de sensibilização acerca do descarte e reutilização de resíduos sólidos na cidade universitária, Universidade Federal do Maranhão. Revista Eletrônica em Gestão, Educação e Tecnologia Ambiental, v. 19, p. 152-159, 2015