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SYMPOSIUM

Time Course of Splenic Cytokine mRNA and Hormones during a Lipopolysaccharide-Induced Inflammation in Toads

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Synopsis Inflammation comprises alterations in glucocorticoids (in amphibians, corticosterone—CORT) and melatonin (MEL) levels, two hormones with immunomodulatory effects on cytokine production in several vertebrates. Cytokines mediate inflammation progress differently depending on their function. While some are secreted during the acute phase of the immune response, others prevail during the resolution phase. Major efforts have been made to understand the interaction of endocrine mediators and cytokine production in endotherms, but little is known for ectotherms so far. Characterizing the stages of inflammation and their interplay with endocrine mediators is crucial for an assertive and integrative approach to amphibian physiology and ecoimmunology. Herein, we investigated CORT and MEL plasma levels as well as splenic cytokine (IL-1β, IL-6, and IL-10) mRNA levels during the progression of the inflammatory response in toads (*Rhinella diptycha*) in four time-points (1, 3, 6, and 18 h) after an immune challenge with lipopolysaccharide (LPS) using independent samples. Toads were responsive to LPS, with all hormones and cytokines affected by LPS. IL-1β and IL-6 were up-regulated after 1 h, but IL-1β decreased right after 3 h, while IL-6 sustained up-regulation throughout all time-points. IL-10 had not been detected until 6 h post-LPS-stimulation, when it showed up-regulation, along with a CORT increase at the same time-point. After 18 h, CORT levels were still high, and IL-1β was up-regulated again, along with up-regulated IL-6 and an IL-10 decrease. We also found positive correlations between IL-1β with IL-6 for LPS and saline groups. LPS-treated individuals showed an overall decrease in MEL plasma levels compared to saline counterparts. Our results showcase the early endocrine and molecular events of the amphibian immune response. We also report activation of the hypothalamus–pituitary–interrenal (HPI) axis during inflammation and increasing evidence for an immune–pineal axis to be described in amphibians.

Introduction

The innate immune response constitutes the first line of defense against damage/pathogen-associated molecular patterns (DAMPs and PAMPs, respectively) and other non-self particles (Abbas et al. 2018). Its mechanisms have been evolutionarily conserved, making the innate immunity of all vertebrates essentially the same (Robert and Ohta 2009). For example, recognition of PAMPs (such as bacterial lipopolysaccharide—LPS) by cellular membrane receptors leads to intracellular NF-κB activation, starting the inflammatory process by increasing the transcription of pro-inflammatory cytokines genes in the immune cell nucleus (Verstrepen et al. 2008). In this way, circulating pro-inflammatory cytokines activate specific receptors on the membrane of immune cells, triggering the immune response propagation (Verstrepen et al. 2008).

Cytokines are secreted proteins that modulate inflammation by mediating the communication among leukocytes (Turnbull and Rivier 1999; Akdis et al. 2016). Pro-inflammatory cytokines (e.g., IL-1β and IL-6) are primarily secreted during the initial hours of the inflammatory process (Givalois et al. 1994; Layé et al. 1994; Webel et al. 1997; Kim et al. 2007) to boost and propagate the response. IL-1β is essential for neutrophil and macrophage activation and recruitment

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(Miller et al. 2007; Rider et al. 2011), while IL-6 plays an important role in leukocyte trafficking, and activation and the production of acute-phase proteins in the liver (Akdis et al. 2016). IL-1β and IL-6 can also activate the hypothalamus–pituitary–adrenal/interrenal (HPA/I) axis (Dunn 2000; Cain and Cidlowski 2017), leading to glucocorticoids (GCs) secretion. Conversely, anti-inflammatory cytokines (e.g., IL-10) are secreted in the latter resolution phase of the inflammatory process. IL-10 production inhibits the synthesis of IL-1β and IL-6, and other pro-inflammatory cytokines to moderate the response intensity and prevent autoimmune damage (de Waal Malefyt et al. 1991; Hopkins 2003; Akdis et al. 2016).

Under an inflammatory context, increased circulating GCs (e.g., cortisol and corticosterone) can inhibit several acute-phase inflammatory stimuli, such as down-regulating pro-inflammatory cytokine expression and altering the circulation of leukocytes (Barnes 1998; Coutinho and Chapman 2011; Cain and Cidlowski 2017). GCs also stimulate the production of IL-10 (Gayo et al. 1998; Elenkov and Chrousos 2002), emphasizing the anti-inflammatory nature of GCs during the inflammatory process and protection against autoimmune damage (Sapolsky et al. 2000; Elenkov and Chrousos 2002). Nevertheless, GCs are naturally secreted under non-pathogenic conditions, marking the individual's activity period in a circadian rhythm (Dumbell et al. 2016). In nocturnal vertebrates (such as amphibians), peaking corticosterone (CORT) levels at night mark the phase of daily activities in the field (Jessop et al. 2014) and under captivity (Titon et al. 2021).

Another hormone that plays an important role as a mediator of the immune parameters is melatonin (MEL). Central MEL production occurs in the pineal gland in response to darkness (Tan et al. 2010). Under homeostatic conditions, the nocturnal production of MEL by the pineal gland impairs the leukocyte migration from the blood vessels to peripheral tissues (Lotufo et al. 2001; Marçola et al. 2013). However, pinealocytes are able to recognize circulating PAMPs and cease central (i.e., pineal) production and systemic circulation of MEL in the context of a pathogen invasion (Cruz-Machado et al. 2010). Subsequently, leukocyte migration through the blood vessels is restored, and MEL production occurs locally in the inflammatory site by activated immunocompetent cells (Markus et al. 2007, 2018). This shift from central MEL production by the pinealocytes to local MEL production by immune cells is known as the immune–pineal axis, which has already been described for mammals (Markus et al. 2007, 2018). Likewise, recent data from our lab raised evidence on the existence of the immune–pineal axis in amphibians, since the immune challenge with LPS decreased MEL plasma levels in bullfrogs (*Lithobates catesbeianus*) 24 h post-injection (Figueiredo et al. 2021), and in Cururu toads (*Rhinella icterica* and *Rhinella diptycha*) 2 and 6 h post-injection, respectively (Bastos et al. 2021; Ferreira et al. 2021).

So far, the majority of studies involving molecular approaches to investigate dynamics of endocrine immunomodulators and/or cytokine gene expression following an immune challenge use endothermic models (Givalois et al. 1994; Layé et al. 1994; Meltzer et al. 2004; Kim et al. 2007; Williams et al. 2009; Shini et al. 2010). Nevertheless, Gardner et al. (2018) showed up-regulated gene expression of several cytokines, chemokines, and other immune-related genes in cane toads (*Rhinella marina*) 2 h after an LPS challenge. Recently, Ferreira et al. (2021) demonstrated up-regulation of pro-inflammatory cytokines gene expression in *R. diptycha* toads 6 h after LPS stimulation. Still, most studies using reptile or amphibian models sampled a single time-point after the immune challenge (Zou et al. 2000; Gardner et al. 2018; Rayl et al. 2019; Ferreira et al. 2021), or sampled over days after the immune challenge (Andino et al. 2012). Thus, the time course of the inflammatory response remains unexplored in amphibians.

We aimed to investigate the temporal dynamics of endocrine (CORT and MEL) and molecular (cytokines mRNA) mediators during the initial hours of the inflammation. We submitted toads (*R. diptycha*) to an immune challenge with LPS and collected blood and tissue (spleen) at four different time-points: 1, 3, 6, and 18 h post-stimulation. The amphibian spleen represents the main peripheral lymphoid organ since these animals lack a mammalian-equivalent lymph node (Robert and Ohta 2009; Grogan et al. 2018). Moreover, the spleen coordinates nervous and endocrine stimuli with immune cells during an inflammatory response (Straub 2004). We hypothesized that the LPS challenge would trigger the inflammatory response and alter plasma hormone levels and splenic cytokine mRNA distinctively throughout the 18-h course. In general, we predicted that LPS stimulation would increase all cytokines mRNA levels and CORT levels while decreasing MEL levels compared to saline injection. More specifically, we predicted that (1) LPS stimulation would increase pro-inflammatory cytokines (IL-1β and IL-6) mRNAs, peaking at initial time-points (1–6 h), while (2) the anti-inflammatory cytokine (IL-10) should peak in the last time-point (18 h). Moreover, (3) CORT levels should rise in both groups within 3–6 h (circadian variation), but this increase would be more pronounced in the LPS-stimulated toads. Meanwhile, (4) MEL levels should rise after 1–3 h in saline-treated toads (circadian variation),

but decrease in LPS-stimulated individuals (immune–pineal axis activation).

Materials and methods

Species and collection site

Rhinella diptycha—formerly *Rhinella schneideri* (Lavilla and Brusquetti 2018)—is a nocturnal generalist toad species widely distributed across South America (Maciel et al. 2010). Adult males were collected in the municipality of Botucatu (22°46"59.9"S, 48°28"28.1"O), Sao Paulo/Brazil, in November 2017 ($N = 32$) and November 2018 ($N = 32$). Toads were located by visual inspection and manually placed in plastic bins with perforated lids (eight animals each). Animals were transported to the laboratory, transferred to a controlled room in the Department of Physiology (Institute of Biosciences, University of Sao Paulo), and allocated individually inside 20 L plastic bins with perforated lids for air circulation and water *ad libitum*. The temperature set was 21 ± 2°C, and the photoperiod was 13 h of light and 11 h of darkness (light/dark 13:11). Body mass (BM, 0.01 g) and snout-vent length (SVL; 0.01 mm) were measured after 7 days in captivity. The body index (BI) was calculated (unstandardized residuals of a linear regression of body mass as a function of SVL) and used as a proxy of body condition. Considering these morphometric parameters, toads were equally distributed in the treatment groups, as described in the next section.

Animals were collected under license from Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, process number 29, 896–1). All procedures performed were approved by the Ethics Committee from the Institute of Biosciences, University of Sao Paulo (CEUA: nº 242/2016).

Immune challenge with lipopolysaccharide (LPS)

Animals were equally divided into two groups: (1) LPS: received an intraperitoneal injection of LPS (*Escherichia coli* 0127: B8, Sigma-Aldrich, L3129) in the concentration of 2 mg/kg diluted in amphibian phosphate buffer solution (APBS) (NaCl: 8 g; KCl 0.2 g; Na_2PO_4: 1.44 g; KH_2PO_4: 0.24 g, diluted in 1.3 L of distilled water); and (2) saline: received an equivalent volume of APBS (1 µL of APBS/g of BM). BM ($F = 0.607$, $P = 0.614$), SVL ($F = 0.606$, $P = 0.614$), and BI ($F = 0.351$, $P = 0.788$) did not differ between groups. Animals started receiving the injections randomly 20 min after lights off (7 PM) intercalating groups. We chose to perform the immune challenge at night because toads are nocturnal animals (Jessop et al. 2014). Thus, exposure to foraging and mating activities might increase the likelihood of encountering pathogens at night.

Blood and tissue collection

Toads were bled 1 h (8 PM), 3 h (10 PM), 6 h (1 AM), and 18 h (1 PM) post-injection (independent samples, $N = 8$ in each group and time) via cardiac puncture (800 µL of blood) with 1 mL syringes and 26 G x 1/2" needles previously heparinized. Blood samples were kept on ice (<2 h) and centrifuged (604 x g, 4 min, 23°C) to separate the plasma. Plasma samples were stored at −80°C, and further used to measure CORT and MEL levels. Immediately after blood sampling, toads were euthanized by immersion in a lethal solution of benzocaine (0.2%). Spleens were collected, immediately frozen in liquid nitrogen, and then transferred to a −80°C freezer for cytokine gene expression evaluation.

Molecular data

Cytokines selection and primers testing

Based on previous results obtained for *R. diptycha* toads 6 h post-injection (Ferreira et al. 2021), we selected three cytokines that could potentially mark the pace of the inflammatory response: the pro-inflammatory IL-1β and IL-6, both with primers designed by Gardner et al. (2018); and the anti-inflammatory IL-10, primer designed by Ferreira et al. (2021). The primer for the housekeeping gene β-actin was designed by Halliday et al. (2008). All primers were purchased from Thermo Fisher Scientific (Waltham/MA, USA) and tested by Ferreira et al. (2021).

RNA extraction and conversion to complementary DNA (cDNA)

RNA extraction and reverse transcription were performed following the methods in Ferreira et al. (2021). In brief, spleens (~50 mg) were homogenized in 1.5-mL sterile microtubes with 750 µL cold TRIzol® reagent (Thermo Fisher Scientific, 15596018, Waltham/MA, USA). RNA concentration and quality were measured in a spectrophotometer at A260/A280 (Nanodrop ND1000, Thermo Scientific, Waltham/MA, USA). Next, the remaining gDNA was eliminated using 1 µL DNase I solution (Thermo Scientific, 89836, Waltham/MA, USA) per 1 µg of RNA, followed by incubation (37°C, 60 min). Then, 2 µL EDTA (25 mM) was added for enzyme inactivation (65°C, 10 min) in a thermocycler (Nexus Mastercycler® Eppendorf®, Hamburg, Germany).

The reverse transcription was performed using a solution of 2 µg RNA sample, 2 µL Random Hexamer Primer (Thermo Scientific, SO142, Waltham/MA), and RNAse-free water to obtain a total of 28 µL. First,

the reactions were incubated at 25°C for 10 min in a thermocycler (Nexus Mastercycler® Eppendorf®, Hamburg, Germany). Then, 2 μL dNTP Mix (10 mM, Thermo Scientific, R0192), 2 μL RevertAid H Minus Reverse Transcriptase, and 8 μL 5X Buffer (Thermo Scientific, EP0452, Waltham/MA) were added to each tube, following incubation (42°C for 60 min, and 70°C for 10 min), in a thermocycler.

RT-qPCR

Prior to plate assemblage, a reaction mix was prepared with 10 μL 2X SYBR Green (Thermo Scientific, K0223, Waltham/MA), 0.1 μL target primer (10 μM, forward + reverse mix), 5 μL of cDNA (RNA 50 ng), and 4.9 μL RNA-free water. Samples and non-template controls (NTC) were pipetted in duplicates into 96-well PCR plates (Applied Biosystems™, N8010560, Waltham/MA) and submitted to the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min, in a thermocycler (Applied Biosystems™ StepOnePlus™ Real-Time PCR System, Waltham/MA). Results were obtained using StepOne™ Software v2.3. The fold change was calculated by relative quantification using the $\Delta\Delta Ct$ method (Livak and Schmittgen 2001). The data were normalized by β-actin housekeeping gene expression, and fold change of the target gene expression in the LPS-stimulated group samples was calculated relative to the average values of saline group samples from each time-point.

Hormone assays

CORT assays were run following the methods in Assis et al. (2017). Briefly, 10 μL of each plasma sample was transferred to a glass test tube. Then, 3 mL of ether was added to each tube. Tubes were agitated for 30 s and centrifuged (583 x g, 9 min, 4°C). Samples were kept in a −80°C freezer for 7 min. After that, the sample was allowed to thaw and the liquid content was transferred to a smaller test tube. The tubes were covered with a paper towel and kept at room temperature (23 ± 2°C) until complete ether evaporation (~24 h). According to the manufacturer's instructions, samples were resuspended in ELISA buffer to perform the ELISA corticosterone kit (CORT number 5,01,320; Cayman Chemical, MI, USA). Intra-assay (between sample replicates) and inter-assay (between multiple points on the standard curve replicates) coefficient of variations were 4.10 and 4.55%, respectively. The sensitivity of the assays was 31.62 pg/mL. Assay sensitivity was calculated according to the manufacturer's instructions and represented 80% B/B_0 (bound/maximum bound) of the standard curve.

For MEL, 250 μL of plasma was extracted using silica columns (Waters Sep-Pak® Vac, supplied in the IBL kit) and methanol. Briefly, columns were prepared with pure methanol and ultrapure water. Then, the eluate was passed through the column and subsequentially washed with ultrapure water and methanol (10%). Retained MEL in the column was recovered with pure methanol. Samples were allowed to evaporate, and pellets were resuspended in ultrapure water for the hormone assay. ELISA MEL kits were used to determine concentrations (IBL, RE54021, Switzerland) according to the manufacturer's instructions and methods in Titon Jr et al. (2021) . The intra- and inter-assay coefficients of variation were 3.2 and 2.25%, respectively. The lower sensitivity of the MEL assays was 1.3 pg/ml.

Statistical analysis

Hormonal (CORT and MEL) and molecular (IL-1β, IL- 6, and IL-10) data were initially submitted to the Shapiro-Wilk ($n \leq 50$) test of normality and Levene´s test for homogeneity of variance. Since all variables showed an absence of normality or homogeneity of variance, CORT was transformed into square root, and the other variables into Log_{10} (MEL, IL-1β, IL-6, and IL-10) to fit the assumptions of parametric tests. However, CORT and IL-10 still showed heterogeneous variance. Despite this, we used ANOVAs since they are robust when group sizes are equal and of reasonable size (Kohr and Games 1974; Field 2013), and non-parametric test of Mann–Whitney for independent samples does not allow us to analyze the interactions between time and treatment. Effects of the treatment (LPS or saline injection) and time post-injection (1, 3, 6, and 18 h) were analyzed through a general linear model (two-way ANOVA) followed by *post-hoc* multiple pairwise comparison tests (Bonferroni's adjustment). CORT, MEL, and the cytokines mRNA were used as dependent variables, while treatment and time were used as factors. Non-parametric Spearman correlations were used to investigate possible relations amongst the variables in each group. Missing values are due to samples that were not quantified and had no spare for a second measure (see Supplementary Table S1 for the *N* in each group and time). All data were analyzed using the SPSS 26 for Windows.

Results

Descriptive statistics for endocrine and molecular variables throughout the time course post-LPS or saline injection in *R. diptycha* toads are shown in Supplementary Table S1.

Gene expression

Treatment with LPS affected the splenic mRNA of all investigated cytokines (Table 1). LPS-stimulated toads

Table 1 LPS stimulation effects on endocrine and molecular variables in *Rhinella diptycha* toads throughout time

Variable	Source	Type III SS	df	Mean square	*F*	*P*
CORT (ng/mL)	Intercept	318.175	1	318.175	339.169	**<0.001**
	Treatment	32.062	1	32.062	34.178	**<0.001**
	Time	28.630	3	9.543	10.173	**<0.001**
	Treatment ∗ Time	15.438	3	5.146	5.486	0.002
	Error	49.719	53	0.938		
MEL (pg/mL)	Intercept	4.024	1	4.024	39.203	**<0.001**
	Treatment	0.866	1	0.866	8.433	0.006
	Time	1.246	3	0.415	4.047	0.012
	Treatment ∗ Time	0.062	3	0.021	0.202	0.895
	Error	4.722	46	0.103		
IL-1β (FC)	Intercept	9.843	1	9.843	19.582	**<0.001**
	Treatment	7.368	1	7.368	14.658	**<0.001**
	Time	3.742	3	1.247	2.481	0.071
	Treatment ∗ Time	2.551	3	0.850	1.691	0.180
	Error	27.647	55	0.503		
IL-6 (FC)	Intercept	32.405	1	32.405	56.767	**<0.001**
	Treatment	26.485	1	26.485	46.396	**<0.001**
	Time	1.578	3	0.526	0.921	0.437
	Treatment ∗ Time	1.806	3	0.602	1.054	0.376
	Error	30.254	53	0.571		
IL-10 (FC)	Intercept	12.111	1	12.111	23.979	**<0.001**
	Treatment	12.089	1	12.089	23.936	**<0.001**
	Time	0.003	1	0.003	0.005	0.943
	Treatment ∗ Time	0.003	1	0.003	0.005	0.942
	Error	10.606	21	0.505		

A set of two-way ANOVAs, with CORT, MEL, IL-1β, IL-6, and IL-10 as dependent variables, treatment (LPS or saline), and time (1, 3, 6, and 18 h) as factors. Abbreviations: Type III SS = type III sum of squares; DF = degrees of freedom; CORT = corticosterone plasma levels; MEL = melatonin plasma levels; IL-1β = IL-1β gene expression; IL-6 = IL-6 gene expression; IL-10 = IL-10 gene expression; and FC = fold change. Variables with *P*-value significance at 0.05 are highlighted in bold.

showed an overall 15 times higher levels of IL-1β than saline toads. IL-1β was up-regulated in LPS toads in 1 h (18-fold) and 18 h (16-fold) post-injection compared to saline. Within the LPS-stimulated toads, IL-1β mRNA was higher in 18 h (six-fold) than in 6 h post-injection (Fig. 1A).

In general, LPS-stimulated toads expressed 27 times more IL-6 transcripts than saline toads. IL-6 was up-regulated in all time-points post-injection: 14-fold at 1 h, 36-fold at 3 h, 57-fold at 6 h, and 80-fold at 18 h post-injection compared to saline toads (Fig. 1B). We also found a positive correlation between IL-6 and IL-1β for both LPS (Fig. 2A) and saline-injected toads (Fig. 2B).

LPS-stimulated toads showed 98 times higher levels of IL-10 than saline toads in general, being 159 times higher than saline-injected in 6 h and 44 times higher in 18 h. We could not detect IL-10 gene expression either in 1 or 3 h post-injection (Fig. 1C).

Hormonal data

The stimulation with LPS affected CORT plasma levels (Fig. 3A, Table 1). LPS-stimulated toads showed 4 times higher CORT levels than saline toads in general. CORT levels were higher in LPS-stimulated toads from 6 h in (Fig. 3A), with LPS toads exhibiting 6 times higher CORT in 6 h and 9 times higher CORT in 18 h compared to the respective saline groups. Similarly, LPS-stimulated toads in 6 h showed CORT levels 3 and 8 times higher than LPS toads in the 1 and 3 h time-points, respectively. In contrast, LPS toads showed a 46% reduction in CORT levels from 6 to 18 h. LPS stimulation and time affected MEL plasma levels in toads independently (Fig. 3B, Table 1), with LPS toads showing a 39% reduction in MEL levels compared to saline toads in general. We did not find *post-hoc* differences within or between groups for MEL plasma levels.

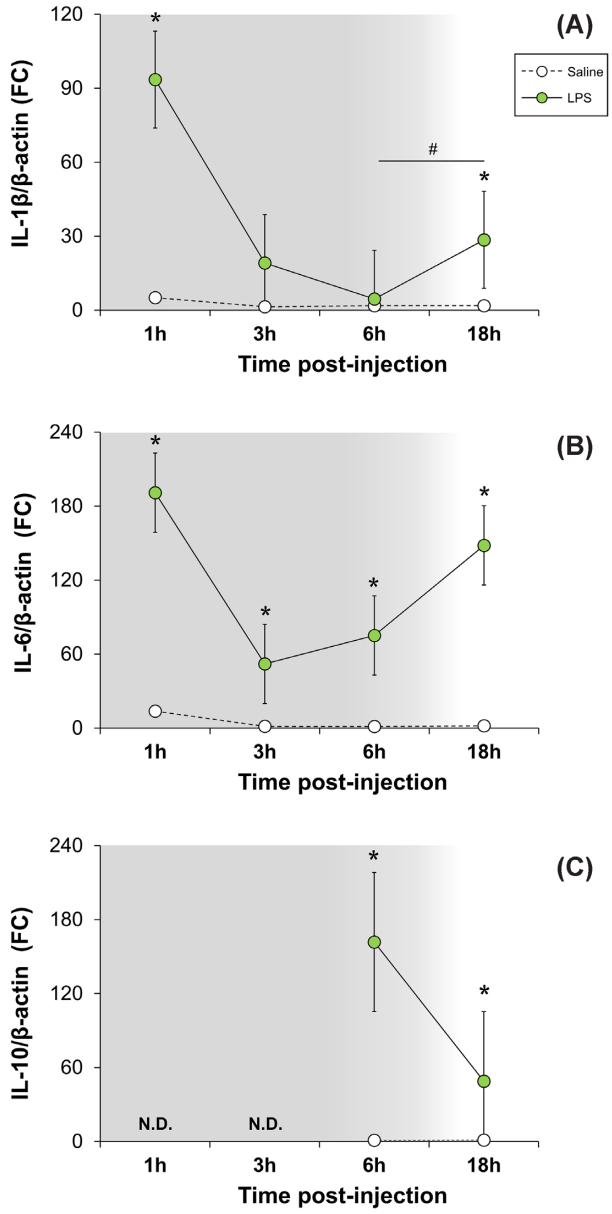

Fig. 1 Means of *R. diptycha* splenic cytokine gene expression following an LPS immune stimulation. **(A)** IL-1β, **(B)** IL-6, and **(C)** IL-10. FC = fold change. Asterisks (*) indicate statistical differences ($P < 0.05$) between treatment groups while hashtags (#) indicate statistical differences ($P < 0.05$) within an LPS-stimulated group. The gray area represents the dark phase of the circadian rhythm, and the bars represent the standard error.

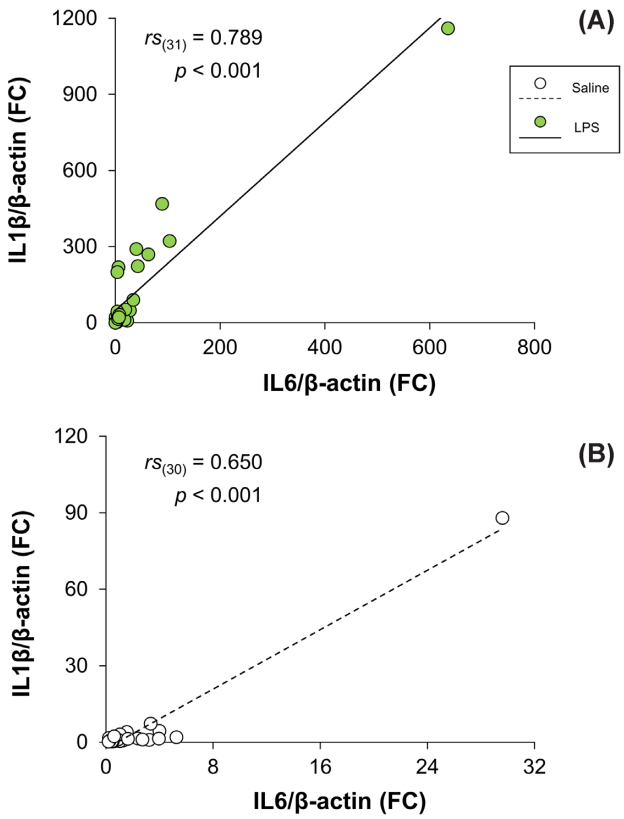

Fig. 2 Non-parametric correlations (Spearman) between splenic cytokines and endocrine mediators following an LPS immune stimulation in *R. diptycha* toads. **(A)** IL-6 and IL-1β for LPS-stimulated toads; and **(B)** IL-6 and IL-1β for saline-injected toads. Dashed lines represent best-fitting lines, *rs* represents correlation value, and numbers between parentheses represent the number of individuals used in the analysis.

Discussion

Supporting our hypothesis, we found that the LPS stimulation differently modulated cytokines gene expression during the inflammatory response compared to the saline injection. IL-1β and IL-6 mRNAs levels rose from 1 h onward after the LPS injection, in agreement with previous studies. Layé et al. (1994) found maximum IL-1β and IL-6 mRNAs levels after 1 and 2 h in the spleen of mice injected with LPS, respectively. Similarly, Givalois et al. (1994) demonstrated IL-1β and IL-6 plasma levels both peaked 2 h post-injection with LPS in rats. Upon recognition of bacterial LPS by toll-like receptor 4 (TLR4), pro-inflammatory cytokines such as IL-1β and IL-6 are the primary mRNA transcripts from the activation of the NF-κB pathway (Baker et al. 2011; Abbas et al. 2018). It is broadly understood that IL-1β and IL-6 secretions have a temporal, causal, and complex relationship (greatly reviewed in Chrousos 1995; Turnbull and Rivier 1999; Dunn 2000), illustrated in our results by the correlations between these two cytokines. Such correlations between IL-1β and IL-6 are consistent with the corresponding early expression of these cytokines in our results since IL-1β stimulates the production of IL-6 (Tosato and Jones 1989; Libert et al. 1990; Chrousos 1995).

IL-6 mRNA sustained the up-regulation in 3 and 6 h post-injection while IL-1β did not, which could be due to IL-6 inhibiting the production of IL-1β (Chrousos 1995). Kakizaki et al. (1999) found a similar pattern for LPS-challenged rats, showing that IL-1β plasma

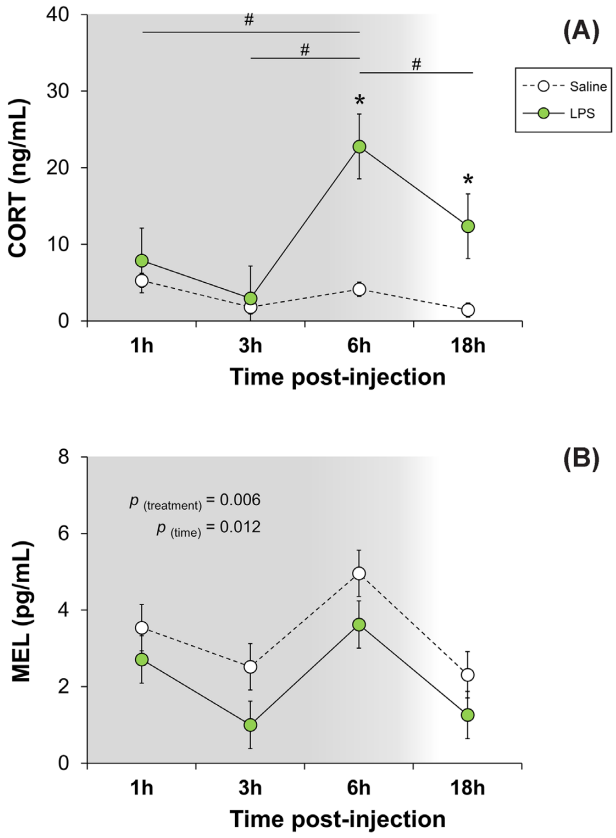

Fig. 3 Means of CORT **(A)** and MEL **(B)** of *R. diptycha* toads following an LPS immune stimulation. Asterisks (∗) indicate statistical differences ($P < 0.05$) between treatment groups while hashtags (#) indicate statistical differences ($P < 0.05$) within LPS-stimulated group. The gray area represents the dark phase of the circadian rhythm, and the bars represent the standard error.

levels were not responsive to LPS within 4.5 h post-injection, while IL-6 plasma levels rose significantly 1.5 h post-injection and sustained high levels throughout the entire timeline of 4.5 h. In fact, contrary to our prediction, IL-6 was up-regulated at all time-points in our study. IL-6 is a pleiotropic regulatory cytokine with a vast range of essential systemic effects during inflammation (Hopkins 2003; Akdis et al. 2016), such as the shift of neutrophil to monocyte recruitment during the acute phase in mammals (reviewed in Kaplanski et al. 2003; Jones 2005). Moreover, IL-6 is produced by a huge variety of cells throughout the body, in contrast to local production of IL-1β restricted to macrophages and dendritic cells (Hopkins 2003). In a review, Turnbull and Rivier (1999) suggest that IL-6 levels can be elevated for greater periods than IL-1β during local inflammation. A solid line of evidence also indicates IL-6 as a crucial cytokine for the transition from innate to acquired immunity (Jones 2005; Scheller et al. 2011; Kaur et al. 2020). Altogether, the aforementioned may explain why IL-6 mRNA up-regulation was sustained until the last time-point, while IL-1β did not show sustained expression in 3 and 6 h post-LPS injection.

Nevertheless, contrary to our predictions, IL-1β showed a late up-regulation in 18 h post-injection. During inflammation in mammals, IL-1β is secreted by the canonical activation of the NLRP3 inflammasome in a cascade response to TLRs recognizing pathogens (Kelley et al. 2019). However, cytoplasmic Gram-negative bacteria (i.e., LPS) trigger a non-canonical caspase-11-dependent pathway of the NLRP3 inflammasome for IL-1β secretion (Pellegrini et al. 2017; Yi 2017; Kelley et al. 2019; Downs et al. 2020). Both pathways can occur independently, and non-canonical activation enhances IL-1β maturation and secretion by inducing cell death through pyroptosis (Kayagaki et al. 2015; Downs et al. 2020). Moreover, Kayagaki et al. (2015) suggest that delayed pyroptosis (thus, IL-1β secretion) might be mediated by different caspase-1 orthologues in non-mammalian vertebrates, considering caspase-4/5/11 and gasdermin are exclusive to mammals. However, literature investigating the temporal dynamics of IL-1β secretion by the different NLRP3 inflammasome activation pathways is still scarce (especially in non-mammalian models) and exploring biomarkers for pyroptosis was beyond the scope of this study.

The IL-10 mRNA expression was observed only 6 h post-injection but did not peak after 18 h as predicted. IL-10 is an anti-inflammatory cytokine, with several downregulating and inhibitory effects on pro-inflammatory stimuli (Elenkov and Chrousos 2002; Amoroso et al. 2012; Akdis et al. 2016) including the inhibition of pro-inflammatory cytokines (de Waal Malefyt et al. 1991), thus leading to an inverted dynamic between IL-10 and IL-1β in our study. Consequently, our results suggest that the decrease in IL-10 mRNA levels 18 h after the stimulus might have a permissive role for the synchronous IL-1β surge. Interestingly, Qi et al. (2015) found a 15-fold increase in IL-10 gene expression after 24 h of an LPS injection in frogs, indicating that this cytokine is kept up-regulated for later than 18 h. In agreement with our findings, other studies using LPS stimulation in mammalian models reported production of several pro- and anti-inflammatory cytokines simultaneously within the first hours after stimulation (Kemna et al. 2005; Erickson and Banks 2011; Li et al. 2014). Nonetheless, although our study design sets a solid timeframe for the initial stages of the inflammatory response, the dynamics between pro- and anti-inflammatory cytokines in further time-points (i.e., days post-injection) remain to be explored.

Although supporting our hypothesis that the LPS injection would modulate CORT levels, significant differences from the saline group were only reached later than we predicted, in 6 and 18 h post-injection. Both IL-1β and IL-6 can activate the HPA/I axis (Chrousos 1995; Dunn 2000), leading to CORT secretion, but stimulation with IL-1β offers a more responsive and prolonged GC release (Turnbull and Rivier 1999; Dunn 2000). Moreover, intraperitoneal cytokine stimulation shows a slower GC response when compared to intravenous stimulation (reviewed in Dunn 2000). Considering the aforementioned, pro-inflammatory cytokine secretion following the short-lived first IL-1β mRNA surge and the early onset of IL-6 mRNA expression might have contributed to the observed CORT elevation in 6 h after the intraperitoneal LPS stimulus.

LPS-stimulated toads showed lower MEL levels overall, thus sustaining our prediction. In mammals, a decrease in central MEL production is expected to follow an immune challenge upon activation of the immune–pineal axis (Markus et al. 2007, 2018). Similarly, recent studies with amphibians showed a decrease in MEL following LPS injection. Bastos et al. (2021) documented lower plasma MEL levels in toads (*R. ictterica*) 2 h after injection with LPS. Figueiredo et al. (2021) also observed effects of LPS stimulation on MEL levels in *L. catesbeianus* frogs, with animals sampled 24 h post-injection having lower plasma levels of this hormone. Finally, Titon Jr et al. (2021) and Ferreira et al. (2021) showed LPS-injected toads (*R. diptycha*) decreased plasma MEL levels 6 h post-injection. Although our results integrate a number of clues towards the confirmation of the immune–pineal axis in amphibians, local measurements of MEL levels produced by immune cells are still necessary to fully describe this phenomenon.

When considering similar studies using endothermic models (briefly reviewed in Supplementary Table S2), toads seem to have a concomitant initial surge of pro-inflammatory interleukins (around 1–2 h post-stimulus), but the peak gene expression appeared later in our model compared to endotherms. Alternatively, anti-inflammatory stimuli (IL-10 and CORT) exhibit delayed initial surges and peaks in our study compared to humans, rats, and pigs (Supplementary Table S2). Such patterns might indicate that the inflammatory response in toads can be triggered as early as endothermic vertebrates, but may take longer to fully assemble. Still, it is important to state that we restricted our comparations to splenic tissue sampled over a timeframe after an LPS stimulus. Even so, the compared studies varied in a plethora of variables, such as the quantification method (plasma concentration *vs.* mRNA levels) and LPS strain and dose (Supplementary Table S2). In this context, we highlight the importance of standardization and developing novel techniques to facilitate the quantification of inflammatory markers for fish, amphibians, and non-avian reptiles to expand the knowledge around inflammation in ectotherms.

Conclusions

Our study provides an overview of the first hours of the inflammatory response assemblage in an amphibian species, integrating hormonal and molecular data. Toads were responsive to the peritoneal LPS injection by differently modulating plasma hormone levels and cytokine expression within an 18-hour timeframe post-injection. IL-1β and IL-6 mRNA transcripts were rapidly up-regulated at 1 h, but only IL-6 was sustained through 3 and 6 h post-LPS injection. IL-10 was detected after 6 h, followed by a decrease of its mRNA levels at 18 h post-injection. Regarding hormone levels, CORT levels increased after 6 h, while MEL levels were overall lower in the LPS-treated animals. These data are consistent with the activation of the HPI axis leading to CORT secretion and add evidence for an amphibian immune–pineal axis to be investigated. By establishing the dynamics of splenic pro- and anti-inflammatory cytokines, and their interplay with immunomodulatory hormones, our results offer an integrative framework for future studies, contributing to the establishment of more accurate experimental designs in terms of matching variables and temporal sampling choices for amphibians. That being so, exploring further time-points and/or gene expression in different tissues can be future perspectives to help build more information around the molecular features of the amphibian immune response.

Future studies might also investigate whether environmental stressors disrupt the temporal dynamics of the described inflammatory process.

Author contributions

F.R.F.: Conceptualization, methodology, investigation, writing—original draft, visualization, project administration, and aunding acquisition; B.T.: Methodology, formal analysis, writing—review and editing, and funding acquisition; S.C.M.T.: Methodology, investigation, writing—review and editing; S.M.M.: Conceptualization, formal analysis, and writing—review and editing; F.R.G.: Resources, writing—review and editing, and funding acquisition; V.R.A.: Conceptualization, methodology, writing—review and editing, supervision, project administration, and funding acquisition.

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Supplementary data

Supplementary data available at *ICB* online.

Data availability

The raw data underlying this article are available in Mendeley Data (https://data.mendeley.com/datasets/9x65dr6mzj/1).

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